On-Line Radical Scavenging Detection and Characterization of Antioxidants from *Artemisia herba-alba*

by **Nacéra Dahmani-Hamzaoui**[a]), **Sofía Salido**[b]), **Pablo J. Linares-Palomino**[b]), **Aoumeur Baaliouamer**[c]), and **Joaquín Altarejos***[b])

[a]) Département de Chimie, Faculté des Sciences, Université Mouloud Mammeri, Tizi Ouzou, Algeria
[b]) Departamento de Química Inorgánica y Orgánica, Facultad de Ciencias Experimentales, Universidad de Jaén, ES-23071 Jaén (phone: +34-953-212743; fax: +34-953-211876; e-mail: jaltare@ujaen.es)
[c]) Département de Chimie Organique, Faculté de Chimie, Université des Sciences et de la Technologie Houari Boumediene, B.P. 32, El-Alia, Bab-Ezzouar, Alger 16111, Algeria

Four caffeoylquinic acid (CQA) derivatives, 5-*O*-caffeoylquinic acid (**1**), 3,5-di-*O*-caffeoylquinic acid (**3**), 4,5-di-*O*-caffeoylquinic acid (**4**), and 3,4,5-tri-*O*-caffeoylquinic acid (**5**), have been isolated from *Artemisia herba-alba* growing wild in Algeria, using the on-line HPLC–DAD–DPPH radical-scavenging detection technique as guidance. In the course of the purification work, the non-frequent (*E*)-2-(*β*-D-glucopyranosyloxy)-4-methoxycinnamic acid (**2**) has also been isolated. The CQAs showed fair-to-good antioxidant activities determined by the DPPH· scavenging assay. The structures of the five isolated compounds were determined by spectroscopic methods. The on-line HPLC–DAD–DPPH technique allowed for a rapid pinpointing of antioxidants in the studied plant, accomplishing the facile guided isolation of the target molecules. Algerian *A. herba-alba* could be an interesting source of natural antioxidants that deserve further work.

Introduction. – A growing body of evidence indicates that many pathologies, such as diabetes, cancer, or neurodegenerative disorders, among others, are associated with the damaging effects of uncontrolled free radicals in living organisms [1]. Natural antioxidants, mainly contained in fruits and vegetables, should be an important part in human diets, since they have the ability to scavenge free radicals and, therefore, keeping them at a physiological non-dangerous level in the organism [1]. Some synthetic antioxidants used to protect food from oxidation processes are suspected to be toxic, and consequently both consumers and producers often prefer natural antioxidants more than synthetic ones as food additives [2]. These are the reasons, at least in part, why a great number of studies searching for sources of potentially safe, effective, and cheap antioxidants have been reported over the past years [3].

The recent development of on-line activity screening (also called high-resolution screening) of very complex mixtures such as plant extracts, to achieve a rapid analysis of their bioactive components, represents a great progress in phytochemistry and natural product-based drug discovery [4]. This method combines an efficient separation technique like HPLC with a fast post-column (bio)chemical assay. Thus, HPLC separates the complex mixture, and the post-column assay determines the activity of each individual component of the mixture, avoiding time-consuming, labor-intensive, and expensive strategies. Among all these screening techniques, the HPLC

on-line assays for antioxidants have now become a very useful tool for the rapid identification of antioxidants in plant extracts, foods, and beverages [5]. *van Beek* and co-workers developed in 2000 [6a] a simple and rapid on-line method for detection of antioxidants in crude plant extracts using the stable free radical DPPH (=2,2-diphenyl-1-picrylhydrazyl radical), and, since then, several applications of the technique have been reported [4a][7] apart from ours [8]. In this method, the radical scavengers present in the mixture react with DPPH$^{\bullet}$ post-column, and the reduction is detected as a negative peak by an absorbance detector at 515 nm. In this way, it is easy to rapidly pinpoint antioxidants in a mixture and, therefore, to focus subsequent efforts in the preparative separation and identification of just the active constituents.

The present study deals with the detection, and subsequent isolation and characterization of the antioxidants present in *Artemisia herba-alba* Asso (Asteraceae) wild-growing in Algeria, using the on-line HPLC–DAD[1]) radical-scavenging detection technique [6]. The structures of these compounds have been elucidated by standard spectroscopic methods (NMR, UV, IR, and MS), and the antioxidant activity of extract, fractions and pure compounds have been evaluated by determining their trapping capacities of the DPPH radical.

A. herba-alba is a medicinal and aromatic dwarf shrub that grows wild in the steppes and deserts of the Middle-East, North Africa, and the arid areas of Spain, extending into north western Himalayas. In Algeria, this greenish-silver perennial plant abounds over large areas of steppes and Sahara desert [9]. The plant is used for flavoring coffee, and mainly for the treatment of gastric disturbances and as an antidiabetic agent in folk medicine [10]. The chemical composition and biological activity of the plant have been reviewed recently [11], and the essential oil composition has received ample attention [12]. Indeed, our groups have studied several times the volatile components of *A. herba-alba* collected in Spain [13] and Algeria [14]. Sesquiterpene lactones, flavonoids, some phenolic compounds, and waxes have also been found in the plant [11]. The antioxidant activity of *A. herba-alba* has been evaluated several times [11], although the compounds responsible for such activity are still unknown.

Results and Discussion. – *Activity-Guided Chromatographic Isolation of Antioxidants.* A sample of *A. herba-alba* in bloom, collected in an arid region of Algeria, was macerated with EtOH at room temperature to yield crude extract **N0** (6.0% yield with respect to dry plant). This EtOH extract was liquid–liquid partitioned with solvents of increasing polarity according to the scheme depicted in *Fig. 1*. The extraction with CH_2Cl_2 (**N1**; 59.6% yield with respect to crude EtOH extract **N0**) allowed removal of the non-phenolic (inactive) compounds from **N0**. The other five extracts (**N0**, **N2**, and **N3**–**N5**) were assessed for their abilities to scavenge the free radical DPPH. The radical scavenging activity of these extracts was expressed as the amount (in μg/ml) of antioxidant necessary to decrease the initial DPPH$^{\bullet}$ concentration by 50% after 15 min (efficient concentration, EC_{50}). Thus, extracts **N0** and **N5** exhibited similar intermediate antioxidant activities, while the more active extracts **N3**, **N2**, and **N4** displayed EC_{50} values of 12.3, 16.9, and 44.0 μg/ml, respectively (*Table 1*). The five main extracts, **N0** and **N2**–**N5**, were further monitored by the on-line HPLC–DAD–DPPH method [5],

[1]) DAD: Diode array detector.

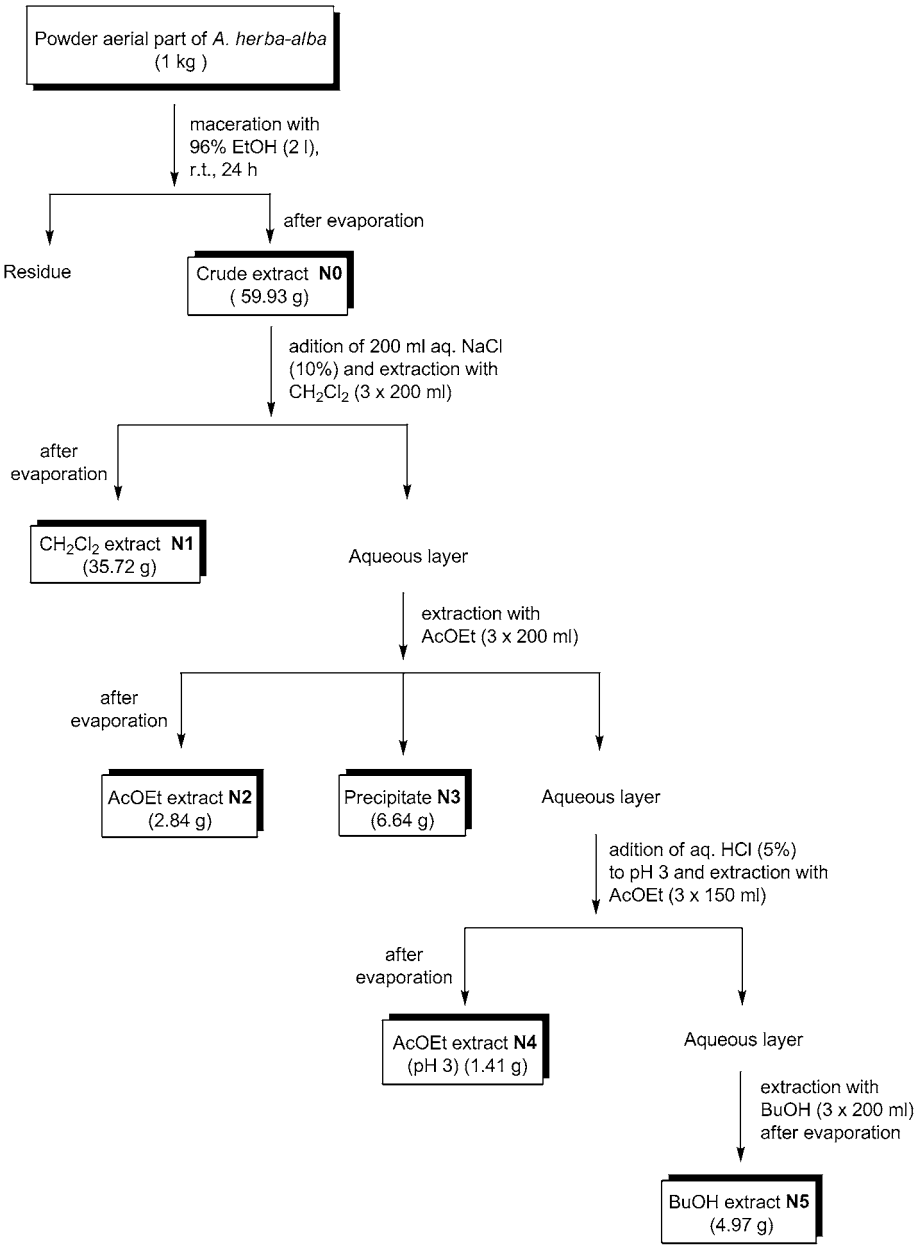


Fig. 1. *Extraction scheme for the preparation of extracts from* Artemisia herba-alba

and the active components present in each one were easily detected. This on-line DPPH$^{\bullet}$ bleaching assay is based on the decrease of DPPH$^{\bullet}$ absorbance at 515 nm, *i.e.*, reduction of that relatively stable radical by the antioxidants which elute from a HPLC

Table 1. *Yields and DPPH Radical-Scavenging Activity* (expressed as efficient concentration, EC_{50}) *of the Crude EtOH Extract of* Artemisia herba-alba *and Its Liquid–Liquid Partitioning Extracts*

Extract[a])	Yield [%]	EC_{50} [μg/ml][b])
N0	6.0[c])	98.7
N1	59.6[d])	–
N2	4.7[d])	16.9
N3	11.1[d])	12.3
N4	2.4[d])	44.0
N5	8.3[d])	99.4

[a]) For abbreviations, see *Fig. 1*. [b]) EC_{50} Values are means of three replicates and RSD is less than 1%. [c]) Yield with respect to dry plant. [d]) Yield with respect to crude EtOH extract (**N0**).

column [6]. The equipment used for the on-line detection of radical-scavenging compounds is shown in *Fig. 2*, and explained in detail in the *Exper. Part*. As illustrated in *Fig. 3* for the on-line assay applied to extract **N2**, two HPLC profiles were obtained with the technique: the 'normal' one, with positive peaks, and the 'negative' one, where inverted peaks indicate disappearance of DPPH· and, thus, the presence of radical scavengers. Among the extracts studied by this method, **N2** – **N4** seemed to be the most suitable extracts to achieve purifications of active compounds. Thus, extracts **N2** (4.7% yield) and **N3** (11.1% yield) were fractionated by open column chromatography on silica gel with CH_2Cl_2/EtOH mixtures of increasing polarity, while **N4** extract (2.4% yield) was subjected to column chromatography, with CH_2Cl_2/EtOH/HCOOH mixtures of increasing polarity. As a result of these chromatographic fractionations

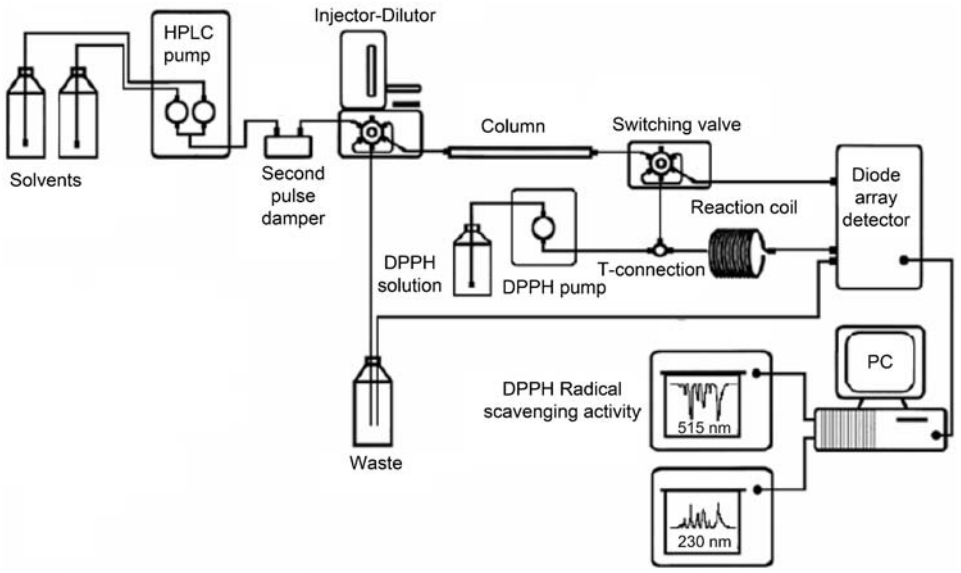


Fig. 2. *Instrumental set-up for the on-line detection of radical-scavenging compounds* (HPLC–DAD–DPPH assay)

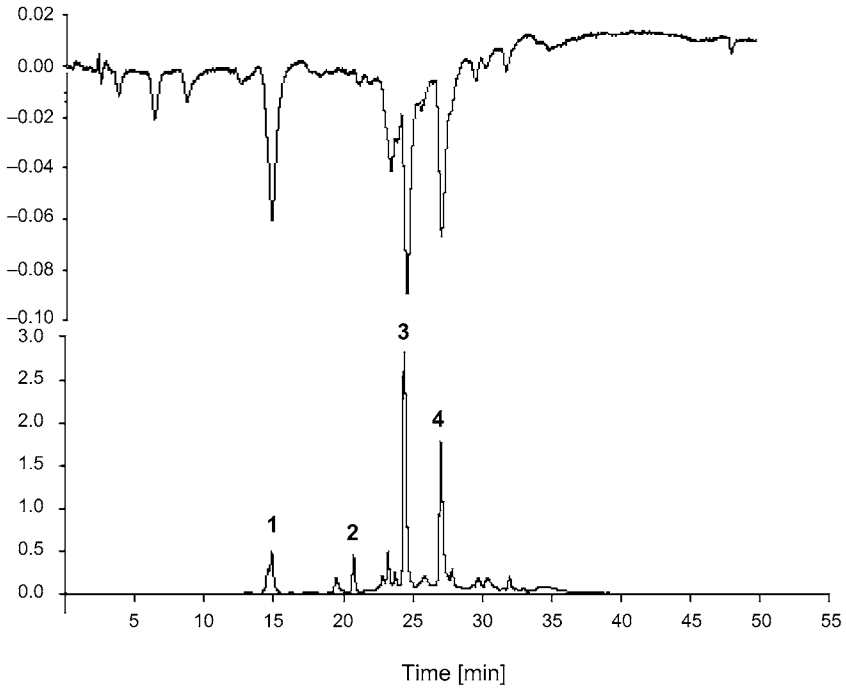
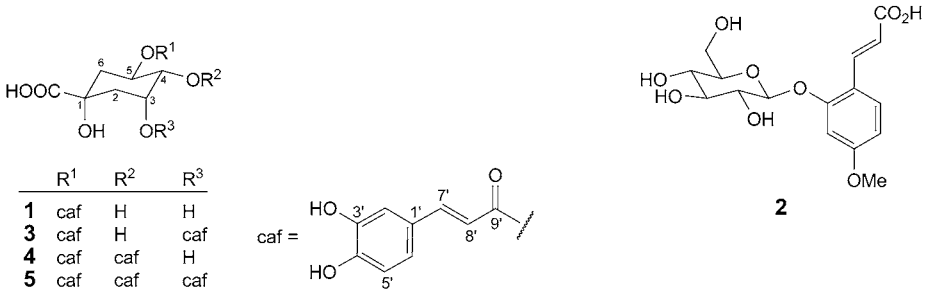


Fig. 3. *On-line HPLC–DAD–DPPH analysis of AcOEt extract* **N2** *from* Artemisia herba-alba*. Upper profile:* DPPH$^{\bullet}$ reduction signal at 515 nm. *Lower profile:* UV signal at 230 nm. Active compounds are those that show negative peaks in the DPPH upper profile.

and subsequent preparative reversed-phase (RP)-HPLC purifications, the following four quinic acid derivatives were isolated in pure form: 5-*O*-caffeoylquinic acid (**1**), 3,5-di-*O*-caffeoylquinic acid (**3**), 4,5-di-*O*-caffeoylquinic acid (**4**), and 3,4,5-tri-*O*-caffeoylquinic acid (**5**) (*Fig. 4*). From the chromatographic fractionation of **N3**, the non-active (*E*)-2-(*β*-D-glucopyranosyloxy)-4-methoxycinnamic acid (**2**) was easily isolated as well. Compounds **1** and **4** were already isolated from the leaves of *A. herba-alba* [11], compounds **3** and **5** have been identified for the first time in this species, and compound



	R^1	R^2	R^3
1	caf	H	H
3	caf	H	caf
4	caf	caf	H
5	caf	caf	caf

Fig. 4. *Structures of the isolated compounds from* Artemisia herba-alba

2, which was only detected in extracts of *Matricaria recutita* [15], is also found, for the first time in genus *Artemisia*.

Structure Elucidation of the Isolated Antioxidants. Compounds **1**–**5** were characterized and identified by UV, IR, MS, HR-MS, ^{1}H- and ^{13}C-NMR, 2D-NMR, and specific optical-rotation data. The spectral and physical data of compounds **1**, **3**, **4**, and **5** are in agreement with those published earlier: 5-*O*-caffeoylquinic acid (5-CQA; **1**) [16], 3,5-di-*O*-caffeoylquinic acid (**3**), 4,5-di-*O*-caffeoylquinic acid (**4**) [16b][17], and 3,4,5-tri-*O*-caffeoylquinic acid (**5**) [18]. These caffeoylquinic acid derivatives comprise a category of prominent natural products, as 5-CQA (**1**) and its isomers, as well as related di- and triesters of quinic acid with cinnamic acid derivatives. The chemical structure of **2** was unambiguously established by analysis and comparison of the NMR data with those already reported for the same compound although recorded in other solvent [15]. In this article, the *IUPAC* nomenclature recommendations for cyclitols [19] have been followed for ring numbering of quinic acid, and the conclusions of *Pauli et al.* [16] regarding the NMR spectroscopy of these compounds have been applied.

Compound **1** was assigned a molecular formula of $C_{16}H_{18}O_9$ by HR-ESI-MS (pseudo-molecular-ion peak at *m*/*z* 353 ($[M-H]^-$)). The ^{1}H- and ^{13}C-NMR spectra revealed the presence of quinic acid and one caffeoyl group (*Table 2*). The UV spectrum showed the typical absorbances (203 and 327 nm) for a caffeic acid moiety. The caffeoyl group can be attached to C(1), C(3), C(4), or C(5) of quinic acid. However, these four compounds can be distinguished by their individual NMR data [16][20][21]. The detailed analysis and comparison of NMR data (*Table 2*) allowed the identification of compound **1** as 5-*O*-caffeoylquinic acid (5-CQA), also called chlorogenic acid.

Compound **2** was assigned a molecular formula of $C_{16}H_{20}O_9$ by HR-ESI-MS (pseudo-molecular-ion peak at *m*/*z* 355 ($[M-H]^-$)). Sixteen C-atom signals were observed in the ^{13}C-NMR spectrum, where six of them (corresponding to five non-aromatic CH and one CH_2 group) were easily assignable to a glucopyranose moiety (δ(C) 102.55, 78.44, 78.14, 74.86, 71.44, and 62.60) and the rest to a cinnamic acid derivative (*Table 3*). In the ^{1}H-NMR spectrum a large coupling constant of a typical anomeric H-atom was observed (δ(H) 4.95 (*d*, $J=7.8$)), being consistent with the β-configuration of a glucopyranose. Three aromatic H-atom signals (δ(H) 6.64 (*dd*, $J=$ 8.7, 2.5); 6.84 (*d*, $J=2.5$); 7.55 (*d*, $J=8.7$)) were in accordance with a 1,2,4-trisubstitution pattern of the phenyl ring. Besides, the olefinic *AB* system (δ(H) 6.40 (*d*, $J=16.2$) and 8.02 (*d*, $J=16.2$)) suggested that compound **2** could be a dioxygenated derivative of the cinnamic acid. At this point, three patterns of trisubstitution can be proposed (*Fig. 5*): 3,4-dioxygenated derivative (*i.e.*, **A** and **B**), 2,4-dioxygenated derivative (*i.e.*, **C** and **D**), and 2,5-dioxygenated derivative (*i.e.*, **E** and **F**). The HMBC spectrum exhibited cross-peaks between the olefinic H-atom H–C(7) (δ(H) 8.02) and three aromatic C-atoms: *i.e.*, the quaternary oxygenated C-atom (δ(C) 159.04; it must be C(2)), the quaternary non-oxygenated C-atom (δ(C) 118.08; it must be C(1)), and the quaternary non-oxygenated CH C-atom (δ(C) 129.98; it must be C(6)) (*Fig. 5*). These long-range H,C correlations exclude models **A** and **B**. In addition, the HMBC cross-peak between the anomeric H-atom (δ(H) 4.95 (H–C(1′)) and the aromatic C-atom C(2) (δ(C) 159.04) suggested the presence of the glucosyl group at C(2) instead of the MeO group (*Fig. 6*). This excludes models **C** and **E**. According to the HSQC

Table 2. *^1H- (400 MHz) and ^{13}C-NMR (100 MHz) Data of Compounds* **1**, **3**, **4**, *and* **5**[a]). δ in ppm, J in Hz.

	1		**3**		**4**		**5**	
	δ(H)	δ(C)	δ(H)	δ(C)[b])	δ(H)	δ(C)[b])	δ(H)	δ(C)[b])
H–C(1)	–	74.16	–	75.03	–	75.87	–	74.87
CH_2(2)	2.02–2.07 (*m*, H_{eq}), 2.15–2.19 (*m*, H_{ax})	38.22	2.10–2.18 (*m*, H_{eq}), 2.32 (br. *d*, *J* = 12.0, H_{ax})	36.17	2.07–2.14 (*dd*, *J* = 14.2, 3.5, H_{eq}), 2.24–2.32 (br. *d*, *J* = 14.2, H_{ax})	38.41	2.18 (*dd*, *J* = 13.3, 4.3, H_{eq}), 2.46 (br. *d*, *J* = 13.3, H_{ax})	36.71
H–C(3)	4.21 (*m*)	72.48	5.42 (*m*)	72.75	4.37 (*m*)	69.49	5.67 (*m*)	70.07
H–C(4)	3.73 (br. *d*, *J* = 7,0)	74.16	3.96 (*dd*, *J* = 7.6, 2.9)	70.94	5.11 (*dd*, *J* = 9.1, 2.9)	75.87	5.32 (*dd*, *J* = 8.5, 3.3)	72.81
H–C(5)	5.35 (*m*)	72.08	5.39 (br. *t*, *J* = 6.5)	72.09	5.62 (*ddd*, *J* = 13.8, 9.3, 5.0)	69.02	5.67 (*m*)	69.15
CH_2(6)	2.20–2.25 (*m*, H_{eq}), 2.04–2.10 (*m*, H_{ax})	39.78	2.19 (*dd*, *J* = 16.0, 6.0, H_{eq}), 2.16–2.21 (*m*, H_{ax})	37.95	2.24–2.32 (*m*, H_{eq}), 2.18–2.27 (*m*, H_{ax})	39.48	2.25–2.40 (*m*, H_{eq}), 2.25–2.40 (*m*, H_{ax})	38.73
H–C(7)	–	–[c])	–	–[c])	–	–[c])	–	–[c])
C(1′)	–	127.80	–	127.81	–	127.66	–	127.78
H–C(2′)	7.03 (*d*, *J* = 1,4)	115.19	7.06 (br. *s*)	115.16	6.99 (*d*, *J* = 1.9)	115.17	7.06 (*d*, *J* = 1.6)	115.15
C(3′)	–	146.79	–	146.76	–	146.74	–	146.71
C(4′)	–	149.55	–	149.47	–	149.66	–	149.61
H–C(5′)	6.76 (*d*, *J* = 8,3)	116.50	6.77 (*d*, *J* = 8.1)	116.48	6.73 (*d*, *J* = 8.0)	116.47	6.77 (*d*, *J* = 8.3)	116.50
H–C(6′)	6.92 (br. *d*, *J* = 8,3)	122.96	6.96 (br. *d*, *J* = 8.1)	123.05	6.89 (*dd*, *J* = 8.0, 1.9)	123.12	6.93 (*dd*, *J* = 8.3, 1.6)	123.22
H–C(7′)	7.55 (*d*, *J* = 15,9)	147.03	7.57 (*d*, *J* = 15.9)	147.24	7.50 (*d*, *J* = 15.8)	147.57	7.60 (*d*, *J* = 15.8)	147.57
H–C(8′)	6.26 (*d*, *J* = 15,9)	115.37	6.25 (*d*, *J* = 15.9)	115.26	6.18 (*d*, *J* = 15.8)	114.71	6.32 (*d*, *J* = 15.8)	115.16
C(9′)	–	168.95	–	168.45		168.27	–	168.51
C(1″)			–	127.94		127.72	–	127.54
H–C(2″)			7.06 (br. *s*)	115.16	7.01 (*d*, *J* = 1.9)	115.19	7.01 (*d*, *J* = 2.1)	115.24
H–C(3″)			–	146.76	–	146.76	–	146.71
C(4″)			–	149.56	–	149.66	–	149.66
H–C(5″)			6.77 (*d*, *J* = 8.1)	116.48	6.74 (*d*, *J* = 8.0)	116.47	6.74 (*d*, *J* = 8.3)	116.54
H–C(6″)			6.96 (br. *d*, *J* = 8.1)	122.99	6.91 (*dd*, *J* = 8.0, 1.9)	123.12	6.91 (*dd*, *J* = 8.6, 1.8)	123.18
H–C(7″)			7.61 (*d*, *J* = 15.9)	147.04	7.59 (*d*, *J* = 15.8)	147.70	7.53 (*d*, *J* = 15.9)	147.71
H–C(8″)			6.35 (*d*, *J* = 15.9)	115.62	6.26 (*d*, *J* = 15.8)	114.75	6.21 (*d*, *J* = 15.9)	114.61
C(9″)			–	168.90	–	168.57	–	168.09
C(1‴)							–	127.65
H–C(2‴)							6.98 (*d*, *J* = 1.9)	115.02
C(3‴)							–	146.76
C(4‴)							–	149.71
H–C(5‴)							6.70 (*d*, *J* = 8.3)	116.54
H–C(6‴)							6.83 (*dd*, *J* = 8.3, 1.9)	123.41
H–C(7‴)							7.51 (*d*, *J* = 15.9)	147.91
H–C(8‴)							6.20 (*d*, *J* = 15.9)	114.34
C(9‴)							–	168.12

[a]) Spectra recorded in CD_3OD. [b]) Some ^{13}C signals of the different caffeoyl groups may be interchangeable. [c]) Quaternary C-atom signals were not observed because of insufficient signal-to-noise ratio.

Table 3. *^1H-* (400 MHz) *and ^{13}C-NMR* (100 MHz) *Data of Compound* **2**[a])

	δ(H)	δ(C)
C(1)	–	118.08
C(2)	–	159.04
H–C(3)	6.84 (*d*, *J* = 2.5)	102.69
C(4)	–	164.36
H–C(5)	6.64 (*dd*, *J* = 8.7, 2.5)	109.91
H–C(6)	7.55 (*d*, *J* = 8.7)	129.98
H–C(7)	8.02 (*d*, *J* = 16.2)	141.25
H–C(8)	6.40 (*d*, *J* = 16.2)	116.84
C(9)	–	171.80
MeO	3.82 (*s*)	56.04
H–C(1′)	4.95 (*d*, *J* = 7.8)	102.55
H–C(2′)	3.56 (*dd*, *J* = 9.1, 7.8)	74.86
H–C(3′)	3.48 (*t*, *J* = 9.1)	78.14
H–C(4′)	3.39 (*t*, *J* = 9.1)	71.44
H–C(5′)	3.48 (*ddd*, *J* = 9.1, 6.0, 2.2)	78.44
CH_2(6′)	3.90 (*dd*, *J* = 12.2, 2.2), 3.68 (*dd*, *J* = 12.2, 6.0)	62.60

[a]) Spectra recorded in CD_3OD; δ values in ppm with reference to Me_4Si and *J* in Hz.

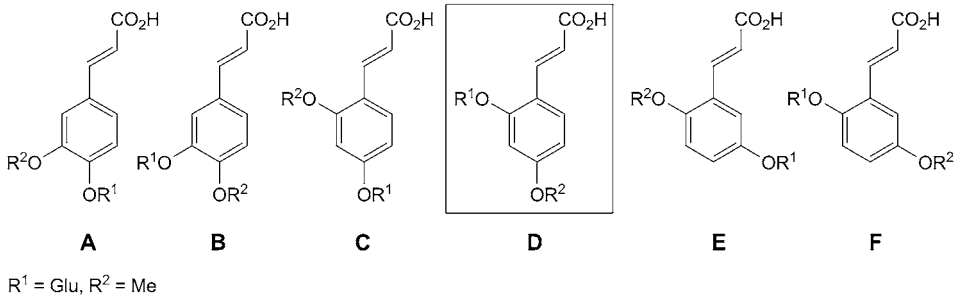


Fig. 5. *Possible trisubstitution patterns for dioxygenated derivatives of cinnamic acid*

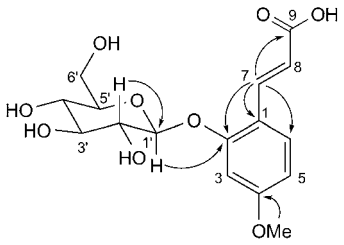


Fig. 6. *Significant HMBCs of* (E)*-2-(β-*D*-glucopyranosyloxy)-4-methoxycinnamic acid* (**2**)

spectrum, the ^{13}C peak at δ(C) 129.98 (C(6)) correlates with the ^1H *doublet* at δ(H) 7.55 (*J* = 8.7, H–C(6)), which was clearly the signal of a H-atom with another H-atom in

ortho position. It finally excludes model **F**, allowing the identification of compound **2** as (*E*)-2-(β-D-glucopyranosyloxy)-4-methoxycinnamic acid.

Compounds **3** and **4** had UV spectra similar to that of compound **1**, with maxima at 203 and 328–329 nm. Both compounds exhibited the same pseudo-molecular-ion peak at *m*/*z* 515 ($[M-H]^-$), in their HR-ESI-MS, indicating the molecular formula $C_{25}H_{24}O_{12}$, which corresponds to the mass of quinic acid with two attached caffeoyl moieties. The signals of the H-atoms H–C(3), H–C(4), and H–C(5) in compound **3** were observed between 3.96 and 5.42 ppm, while the same H-atoms in compound **4** resonated between 4.37 and 5.62 ppm (*Table 2*). This fact, along with the comparison of the rest of spectroscopic data with those reported in [16a][17], allowed identification of **3** and **4** as 3,5-di-*O*-caffeoylquinic acid (3,5-di-CQA) and 4,5-di-*O*-caffeoylquinic acid (4,5-di-CQA), respectively.

The more polar compound **5** was assigned a molecular formula of $C_{34}H_{30}O_{15}$ by HR-ESI-MS (pseudo-molecular-ion peak at *m*/*z* 677 ($[M-H]^-$)), which was consistent with the presence of a third unit of caffeic acid moiety on quinic acid. This was confirmed by ^{1}H- and ^{13}C-NMR analysis [22]. Six *doublet*s in the ^{1}H-NMR spectrum with coupling constants of *ca.* 15.9 Hz appeared for the (*E*)-olefinic H-atoms H–C(7′) and H–C(8′), H–C(7″) and H–C(8″), and H–C(7‴) and H–C(8‴) of the three caffeoyl groups (*Table 2*). The signals of H–C(3) (equatorial), H–C(4) (axial) and H–C(5) (axial) of the quinic acid moiety were assigned according to their multiplicity and their spin–spin coupling constants. The locations of caffeoyl substituents were deduced by comparative analysis of the chemical shifts of the H-atoms in free quinic acid [22] and those in the quinic acid moiety of compounds **3** and **4**. The three signals for H-atoms H–C(3), H–C(4), and H–C(5) were shifted downfield 1.58, 1.93, and 1.67 ppm, respectively, indicating esterification at C(3), C(4), and C(5), allowing us to identify compound **5** as 3,4,5-tri-*O*-caffeoylquinic acid (3,4,5-tri-CQA) [18].

Radical-Scavenging Activity of the Isolated Compounds. The antioxidant activities of the five isolated compounds from *A. herba-alba* were determined by the DPPH radical scavenging assay as described above. In *Table 4*, the EC_{50} values for pure compounds **1**–**5** and reference compounds (rosmarinic acid and BHT) are compiled as the average of three replicates. Among these compounds, the most active one is the 3,4,5-tri-*O*-caffeoylquinic acid (**5**), followed by 4,5-di-*O*-caffeoylquinic (**4**), and 3,5-di-

Table 4. *DPPH Radical Scavenging Activity* (expressed as efficient concentration, EC_{50}) *of the Isolated Compounds from* Artemisia herba-alba

Compound	EC_{50} [μg/ml][a])
Rosmarinic acid (reference compound)	6.4
3,4,5-Tri-*O*-caffeoylquinic acid (**5**)	10.0
4,5-Di-*O*-caffeoylquinic acid (**4**)	14.1
3,5-Di-*O*-caffeoylquinic acid (**3**)	14.3
5-*O*-Caffeoylquinic acid (**1**)	25.2
BHT[b]) (reference compound)	93.6
(*E*)-2-(β-D-Glucopyranosyloxy)-4-methoxycinnamic acid (**2**)	220.8

[a]) EC_{50} Values are means of three replicates and RSD is less than 1%. [b]) 2,6-Di-(*tert*-butyl)-4-methylphenol.

O-caffeoylquinic (**3**) acids, of which the EC_{50} values are almost equal. Since the potency of a molecule for scavenging the radical is due to the number of OH H-atoms available for donation, a greater number of caffeoyl moieties present in the molecule increases the antioxidant activity. On the other hand, as expected, the glycosyl group of compound **2** does not contribute effectively to the radical-scavenging activity of the molecule. All the CQAs have slightly lower radical-scavenging activities (10.0 – 25.2 μg/ml) than the natural antioxidant rosmarinic acid (6.4 μg/ml) and much higher activities than the synthetic antioxidant BHT (93.6 μg/ml). It means that Algerian *A. herba-alba* could be an interesting source of natural antioxidants that deserve further studies of the previously demonstrated chemodiversity of this plant.

Experimental Part

Sample Collection and Storage. The aerial parts of *A. herba-alba* used in this study were collected from M'sila region, located 400 km southeast of Alger, in high table-lands of Algeria in June 2008 at the flowering stage. The plant was authenticated by Dr. *R. Amirouche*, and a voucher specimen (HAM/48/2008) was deposited with the Herbarium of the Vegetal Biology Departament, University of Sciences and Technology Houari Boumediene, Alger. The sample was stored one month in a dry and dark place at r.t. Just before starting the extraction, the plant material was finely crushed.

Chemicals. The solvents used for extraction and chromatographic separation (hexane, CH_2Cl_2, AcOEt, BuOH, EtOH, and $CHCl_3$) were glass-distilled prior to use. MeOH used for radical-scavenging activity assays was of HPLC grade. All aq. solns. were prepared with pure H_2O produced by *Milli-Q* water (1.8 MΩ) system (*Millipore*). AcOH used for slight acidification of HPLC solvents (*vide supra*) was of synthetic grade (*Scharlau*). Deuterated MeOH was used to prepare solns. of purified compounds for NMR analysis. The following reagents were used for radical scavenging assays: 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$; 95%, *Sigma–Aldrich*, ES-Madrid), rosmarinic acid (96%, *Sigma–Aldrich*, ES-Madrid), and 2,6-di-(*tert*-butyl)-4-methylphenol (BHT; 99%, *Sigma–Aldrich*, ES-Madrid).

Solvent Extraction of Antioxidants. The vegetal material (1.0 kg) of *A. herba-alba* was extracted by maceration during 24 h with 96% EtOH (2 l) at r.t., and concentrated to give a dark residue (**N0**; 59.93 g). This crude extract was re-dissolved in 200 ml of 10% aq. NaCl soln. and extracted with CH_2Cl_2 (3 × 200 ml) to remove non-phenolic content (**N1**; 35.72 g). The aq. layer was again extracted with AcOEt (3 × 200 ml), and precipitation occurred. At that point, we had a solid (**N3**; 6.64 g), an org. layer, from which AcOEt was removed under vacuum to give 2.84 g of extract (**N2**), and an aq. layer. Then, the aq. phase was acidified by addition of 5% aq. HCl soln. to pH 3 and again re-extracted with AcOEt (3 × 150 ml). This org. layer yielded, after evaporation under vacuum, the extract **N4** (1.41 g). Finally, the remaining aq. layer was extracted with BuOH (3 × 200 ml). The BuOH was evaporated to dryness under vacuum at temp. not higher than 40° to give the extract **N5** (4.97 g) (*Fig. 1*).

HPLC Conditions. HPLC Analyses were performed with a anal. reversed-phase (RP)-HPLC column (*Spherisorb ODS-2* (250 mm × 3 mm i.d., 5 μm), *Waters Chromatography Division*, Milford, MA, USA) on a *Waters 600E* instrument (*Waters Chromatography Division*, Milford, MA, USA) equipped with a diode array detection (DAD), scan range, 190 – 800 nm (*Waters CapLC 2996* photodiode array detector, *Waters Chromatography Division*, Milford, MA, USA), and operating temp., 30°. A typical sample for injection was prepared in MeOH at a concentration of 10 mg/ml, and the injection volume was 10 μl. The separation was obtained by a step gradient with MeOH/AcOH, 99.8 : 0.2 (solvent *A*) and H_2O/AcOH 99.8 : 0.2, (solvent *B*) at a flow rate of 0.7 ml/min: linear gradient from 10 to 70% *A* in 35 min; linear gradient from 70 to 100% *A* in 5 min; isocratic condition of 100% of *A* for 5 min, and another 5 min to return to the initial conditions. The total run time excluding equilibration was 50 min. Prep. HPLC separations were performed using a prep. RP-HPLC column (*Spherisorb ODS-2* column, 250 mm × 10 mm i.d., 5 μm, *Waters Chromatography Division*, Milford, MA, USA) on the instrument described above, with solvent *A* and solvent *B* at a flow rate of 5 ml/min.

HPLC–DPPH Instrumental Set-up. A block scheme of the instrument used to perform the on-line HPLC–DAD–DPPH assays is depicted in *Fig. 2*. The HPLC equipment and conditions used have been described above. The MeOH soln. of DPPH$^{\bullet}$ (4.7×10^{-5} M) was introduced in the system with a post-column pump (*Waters Reagent Manager*, *Waters Chromatography Division*, Milford, MA, USA) at a flow rate of 0.5 ml/min. A six-way switching valve (*Waters Switching Valve*, *Waters Chromatography Division*, Milford, MA, USA) was mounted between the HPLC column and the detector described above. A reaction coil (3-m long and of 0.50-mm internal diameter) was installed between the DPPH$^{\bullet}$ pump and the DAD. Switching valve, DPPH$^{\bullet}$ pump, and coil where joined by a T-connection. Thus, the switching valve allows the eluted compounds from the HPLC column to reach the detector, in a first run, to give a 'normal' HPLC chromatogram, and the valve also allows the eluted compounds to be directed to the reaction coil, in a second run, where both antioxidants and DPPH$^{\bullet}$ react to lead to a decrease of absorbance at 515 nm (absorbance of DPPH$^{\bullet}$) detected in the DAD ('inverted' HPLC chromatogram).

On-line HPLC–DAD–DPPH Assays. On-line HPLC radical-scavenging assays of the extracts **N0**, **N2**, **N3**, **N4**, **N5** were performed using the methodology of *van Beek* and co-workers [6], modified as described above. To obtain 'inverted' HPLC chromatograms, a MeOH soln. of DPPH$^{\bullet}$ was continuously introduced into the reaction coil, and then, a MeOH soln. of a given extract is injected in the HPLC equipment. Thus, the introduced separated compounds were directed by the switching valve to the reaction coil where the post-column reaction occurs. *Fig. 3* outlines the final result for extract **N2**.

Chromatographic Fractionation and Isolation. Chromatographic separations were performed on *Scharlau* silica gel (230–400 mesh) and *Merck* silica gel (70–230 mesh) with the solvent systems indicated. Solvent systems are reported as *v/v* percent ratios. All fractions were monitored by HPLC (see above) and TLC with *Merck* silica gel *60* F_{254} precoated aluminum sheets (0.25 mm).

The AcOEt extract **N2** (2.84 g) was chromatographed by open column chromatography on silica gel, with CH_2Cl_2/EtOH mixtures of increasing polarity. Fractions of 175 ml each were collected, monitored by TLC and HPLC, pooled, and evaporated to give five major fractions: *N2a* (356 mg), *N2b* (1.214 g), *N2c* (341 mg), *N2d* (309 mg), and *N2e* (145 mg). *Fr. N2c* was submitted to prep. RP-HPLC with a linear gradient from 30 to 70% of solvent *A* in 30 min to yield pure compound **3** (15.5 mg). *Fr. N2e* was separated by prep. RP-HPLC with a linear gradient from 30 to 70% of solvent *A* in 30 min to yield pure compound **4** (7.7 mg).

Precipitate **N3** (6.64 g) was subjected to open column chromatography on silica gel (130 g) with CH_2Cl_2/EtOH/HCOOH (0.2 ml of HCOOH for every 250 ml of CH_2Cl_2/EtOH mixture) mixtures of increasing polarity. Fractions of 125 ml each were collected, monitored by TLC and HPLC, pooled, and evaporated to give five major fractions: *N3a* (85 mg), *N3b* (757 mg), *N3c* (391 mg), *N3d* (1.256 g), and *N3e* (1.179 g). *Fr. N3d* was again chromatographed on silica gel (50 g) with CH_2Cl_2/EtOH/HCOOH (0.2 ml of HCOOH for every 250 ml of CH_2Cl_2/EtOH mixture) mixtures of increasing polarity. Fractions of 50 ml each were also collected, monitored by TLC and HPLC, pooled, and evaporated to give five major fractions: $N3d_1$ (133 mg), $N3d_2$ (279 mg), $N3d_3$ (594 mg), $N3d_4$ (156 mg), and $N3d_5$ (56 mg). Eventually, *Fr.* $N3d_2$ (279 mg) was submitted to prep. RP-HPLC with a linear gradient from 40 to 70% of solvent *A* in 20 min, to yield pure compounds **3** (70 mg) and **5** (34 mg).

The AcOEt extract (pH 3) **N4** (1.41 g) was separated by open column chromatography on silica gel (150 g), with CH_2Cl_2/EtOH mixtures of increasing polarity. Fractions of 50 ml each were collected, monitored by TLC and HPLC, pooled, and evaporated to give six fractions: *N4a* (166 mg), *N4b* (174 mg), *N4c* (128 mg), *N4d* (291 mg), *N4e* (456 mg), and *N4f* (72 mg). *Fr. N4c* was subjected to prep. RP-HPLC with a linear gradient from 30 to 70% of solvent *A* in 30 min to yield pure compound **2** (5.1 mg). An aliquot of *Fr. N4e* (150 mg) was purified by prep. RP-HPLC with a linear gradient from 20 to 50% of solvent *A* in 30 min to yield pure compound **1** (6.3 mg).

Identification of Isolated Compounds. Optical rotations were recorded using MeOH as solvent with a *Perkin–Elmer 241* polarimeter (*Perkin–Elmer Instruments*, Norwalk, CT, USA), in a 10-cm 2-ml cell. UV Spectra were obtained in MeOH on a *Varian Cary 4000* UV/VIS spectrophotometer (*Varian Inc.*, Palo Alto, CA, USA); λ_{max} (log ε) in nm. IR Spectra were recorded in *Single Reflection ATR System*, on a *Bruker Tensor 27 FTIR* spectrometer (*Bruker Optik GmbH*, D-Ettlingen); $\tilde{\nu}$ in cm^{-1}. ^{1}H- (400 MHz), ^{13}C- (100 MHz), and 2D-NMR (400/100 MHz) spectra were recorded on a *Bruker Avance 400* spectrometer (*Bruker BioSpin GmbH*, D-Rheinstetten); δ in ppm rel. to Me_4Si as internal standard, *J* in

Hz. High-resolution mass spectra (HR-ESI-MS) were obtained on a LC002FMS equipment composed of an *Alliance 2795 Waters* HPLC chromatograph fitted to a *Micromass LCT Premier* mass spectrometer, with an electrospray ionization (ESI) source and a time-of-flight (TOF) analyzer (*Waters Corp.*, Milford, MA, USA); in *m/z*.

*5-*O*-Caffeoylquinic Acid* (=*(1*S*,3*R*,4*R*,5*R*)-3-{[(2*E*)-3-(3,4-Dihydroxyphenyl)-1-oxoprop-2-en-1-yl]oxy}-1,4,5-trihydroxycyclohexanecarboxylic Acid*; **1**). Colorless syrup. $[\alpha]_D^{25} = -7.6$ ($c = 0.32$, MeOH). UV: 203 (0.75), 327 (0.38). IR (neat): 3342 (OH), 2957, 2926 (R–COOH), 1730 (ester C=O), 1601 (α,β-unsaturated ester C=O), 1457 (arom. C–C), 1377 (arom. O–H), 1271 (arom. C–OH), 1182 (ester C–O), 1124, 1072 (C–OH). ^{1}H- and ^{13}C-NMR: see *Table 2*. HR-ESI-MS: 353.1905 ($[M-H]^-$, $C_{16}H_{18}O_9^-$; calc. 353.0873).

*(*E*)-2-(β-*D*-Glucopyranosyloxy)-4-methoxycinnamic Acid* (=*(2*E*)-3-[2-(β-*D*-Glucopyranosyloxy)-4-methoxyphenyl]prop-2-enoic Acid*; **2**). Colorless syrup. $[\alpha]_D^{25} = -70.6$ ($c = 0.26$, MeOH). UV: 203 (0.80), 279 (0.30), 309 (0.26). IR (neat): 3327 (OH), 1786 (acid C=O), 1632 (C=C–COOH, α,β-unsaturated acid), 1160 (ether), 1006 (pyranyloxy), 1074 (CH_2–OH). ^{1}H- and ^{13}C-NMR: see *Table 3*. HR-ESI-MS: 355.1020 ($[M-H]^-$, $C_{16}H_{20}O_9^-$; calc. 355.1029).

*3,5-Di-*O*-caffeoylquinic Acid* (=*(3*R*,5*R*)-3,5-Bis{[(2*E*)-3-(3,4-dihydroxyphenyl)-1-oxoprop-2-en-1-yl]oxy}-1,4-dihydroxycyclohexanecarboxylic Acid*; **3**). Colorless syrup. $[\alpha]_D^{25} = -123.2$ ($c = 0.78$, MeOH). UV: 203 (0.76), 328 (0.56). IR (neat): 3373 (O–H), 2916, 2849 (R–COOH), 1733 (ester C=O), 1600 (α,β-unsaturated ester C=O), 1469 (arom. C–C), 1375 (arom. O–H), 1271 (arom C–OH), 1181 (ester C–O), 1121 (C–OH). ^{1}H- and ^{13}C-NMR: see *Table 2*. HR-ESI-MS: 515.1169 ($[M-H]^-$, $C_{25}H_{24}O_{12}^-$; calc. 515.1190).

*4,5-Di-*O*-caffeoylquinic Acid* (=*(1*S*,3*R*,4*R*,5*R*)-3,4-Bis{[(2*E*)-3-(3,4-dihydroxyphenyl)-1-oxoprop-2-en-1-yl]oxy}-1,5-dihydroxycyclohexanecarboxylic Acid*; **4**). Colorless syrup. $[\alpha]_D^{25} = -199.6$ ($c = 0.39$, MeOH). UV: 203 (0.81), 329 (0.59). IR (neat): 3373 (O–H), 2916, 2849 (R–COOH), 1697 (ester C=O) 1597 (α,β-unsaturated ester C=O), 1466 (arom C–C) 1375 (arom. O–H), 1268 (arom C–OH), 1162 (ester C–O). ^{1}H- and ^{13}C-NMR: see *Table 2*. HR-ESI-MS: 515.1207 ($[M-H]^-$, $C_{25}H_{24}O_{12}^-$; calc. 515.1190).

*3,4,5-Tri-*O*-caffeoylquinic Acid* (=*(3*R*,5*R*)-3,4,5-Tris{[(2*E*)-3-(3,4-dihydroxyphenyl)-1-oxoprop-2-en-1-yl]oxy}-1-hydroxycyclohexanecarboxylic Acid*; **5**). Colourless syrup. $[\alpha]_D^{25} = -285.1$ ($c = 1.15$, MeOH). UV: 216 (0.84), 329 (0.84). IR (neat): 3369 (O–H), 2959 (R–COOH), 1691 (ester C=O), 1629, 1601 (α,β-unsaturated ester C=O), 1445 (arom. C–C) 1365 (arom. O–H), 1274 (arom. C–OH), 1179, 1160 (ester C–O), 1115 (C–OH). ^{1}H- (400 MHz) and ^{13}C-NMR (100 MHz): see *Table 2*. HR-ESI-MS: 677.1507 ($[M-H]^-$, $C_{34}H_{30}O_{15}^-$; calc. 677.1507).

Free-Radical Scavenging Activity. Radical scavenging activities of extracts **N0**, **N2**, **N3**, **N4**, and **N5**, purified compounds **1**–**5**, and reference compounds (rosmarinic acid and BHT) were determined by spectrophotometric measurements according to the ability of the tested samples to scavenge the free radical DPPH$^\bullet$. This activity was determined using the method employed by *Brand–Williams et al.* [23] and modified as follows: MeOH solns. (1.2 ml) of a given compound, at different concentrations, were mixed in a 1-cm path-length disposable plastic cuvette with 2.4 ml of MeOH soln. of DPPH$^\bullet$ (*ca.* 7×10^{-5} M; absorbance at 515 nm of 0.80 ± 0.03 AU; the exact DPPH$^\bullet$ concentration was calculated from a calibration curve). Triplicate samples were shaken and allowed to stand for 15 min in the dark at r.t., and the decrease of absorbance at 515 nm was measured with a *Perkin–Elmer* UV/VIS spectrophotometer *Lambda 19* (*Perkin–Elmer Instruments*, Norwalk, CT, USA).

We wish to thank the *Junta de Andalucía*, Spain for general financial support. We also acknowledge the *Université Mouloud Mammeri*, Algeria, for supporting a research stay of *N. Dahmani-Hamzaoui* at the University of Jaén, Spain.

REFERENCES

[1] S. Sen, R. Chakraborty, C. Sridhar, Y. S. R. Reddy, B. De, *Int. J. Pharm. Sci. Rev. Res.* **2010**, *3*, 91.
[2] N. Gharavi, S. Haggarty, A. O. S. El-kadi, *Curr. Drug Metab.* **2007**, *8*, 1.

[3] B. Dimitrios, *Trends Food Sci. Technol.* **2006**, *17*, 505.
[4] a) T. A. van Beek, K. K. R. Tetala, I. I. Koleva, A. Dapkevicius, V. Exarchou, S. M. F. Jeurissen, F. W. Claassen, E. J. C. van der Klift, *Phytochem. Rev.* **2009**, *8*, 387; b) S.-Y. Shi, Y.-P. Zhang, X.-Y. Jiang, X.-Q. Chen, K.-L. Huang, H.-H. Zhou, *Trends Anal. Chem.* **2009**, *28*, 865.
[5] H. A. G. Niederländer, T. A. van Beek, A. Bartasiute, I. I. Koleva, *J. Chromatogr., A* **2008**, *1210*, 121.
[6] a) I. I. Koleva, H. A. G. Niederländer, T. A. van Beek, *Anal. Chem.* **2000**, *72*, 2323; b) A. Dapkevicius, T. A. van Beek, H. A. G. Niederländer, *J. Chromatogr., A* **2001**, *912*, 73.
[7] A. Pukalskas, P. R. Venskutonis, I. Dijkgraaf, T. A. van Beek, *Food Chem.* **2010**, *122*, 804.
[8] M. Pérez-Bonilla, S. Salido, T. A. van Beek, P. de Waard, P. J. Linares-Palomino, A. Sánchez, J. Altarejos, *Food Chem*. **2011**, *124*, 36; H. Zbidi, S. Salido, J. Altarejos, M. Pérez-Bonilla, A. Bartegi, J. A. Rosado, G. M. Salido, *Blood Cells Mol. Dis.* **2009**, *42*, 279; M. Pérez-Bonilla, S. Salido, T. A. van Beek, P. J. Linares-Palomino, J. Altarejos, M. Nogueras, A. Sánchez, *J. Chromatogr., A* **2006**, *1112*, 311.
[9] S. Djebaïli, 'Phytosociologie et Écologie', Office des Publications Universitaires, Alger, 1984.
[10] J. Valnet, 'Aromathérapie: Traitement des Maladies par les Essences des Plantes', Maloine Editions, Paris, 1984.
[11] A. E.-H. H. Mohamed, M. A. El-Sayed, M. E. Hegazy, S. E. Helaly, A. M. Esmail, N. S. Mohamed, *Rec. Nat. Prod.* **2010**, *4*, 1.
[12] B. M. Lawrence, *Perfum. Flavor.* **2009**, *34*, 54.
[13] S. Salido, L. R. Valenzuela, J. Altarejos, M. Nogueras, A. Sánchez, E. Cano, *Biochem. Syst. Ecol.* **2004**, *32*, 265; S. Salido, J. Altarejos, M. Nogueras, A. Sánchez, *J. Essent. Oil Res.* **2001**, *13*, 221.
[14] N. Dahmani-Hamzaoui, A. Baaliouamer, *J. Essent. Oil Res.* **2010**, *22*, 514; N. Dahmani-Hamzaoui, A. Baaliouamer, *Riv. Ital. EPPOS* **2005**, *40*, 7.
[15] M. Repčák, A. Pastirová, J. Imrich, V. Švehlíková, P. Mártonfi, *Plant Breed* **2001**, *120*, 188.
[16] a) G. F. Pauli, U. Kuczkowiak, A. Nahrstedt, *Magn. Reson. Chem.* **1999**, *37*, 827; b) G. F. Pauli, F. Poetsch, A. Nahrstedt, *Phytochem. Anal.* **1998**, *9*, 177.
[17] C. A. Simões-Pires, E. F. Queiroz, A. T. Henriques, K. Hostettmann, *Phytochem. Anal.* **2005**, *16*, 307.
[18] I. Agata, S. Goto, T. Hatano, S. Nishibe, T. Okuda, *Phytochemistry* **1993**, *33*, 508.
[19] IUPAC Commission on the Nomenclature of Organic Chemistry (CNOC) and IUPAC-IUB Commission on Biochemical Nomenclature (CBN), Nomenclature of Cyclitols, Recommendations, *Biochem. J.* **1976**, *153*, 23.
[20] M. Haribal, P. Feeny, C. C. Lester, *Phytochemistry* **1998**, *49*, 103.
[21] N. Nakatani, S.-i. Kayano, H. Kikuzaki, K. Sumino, K. Katagiri, T. Mitani, *J. Agric. Food Chem.* **2000**, *48*, 5512.
[22] I. Merfort, *Phytochemistry* **1992**, *31*, 2111.
[23] W. Brand-Williams, M. E. Cuvelier, C. Berset, *Food Sci. Technol.* **1995**, *28*, 25.

Received September 15, 2011