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Isolation and Structure Elucidation of Antioxidant Polyphenols from Quince (*Cydonia vulgaris*) Peels

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Thirteen new compounds, as well as 16 already known, have been isolated from organic extracts of peels of *Cydonia vulgaris*, a fruit of a shrub belonging to the same tribe as the apple. All of the structures were elucidated by EI- or ESI-MS and ^{1}H and ^{13}C NMR after purification of individual compounds by HPLC. Thirteen fatty acid esters of cinnamyl alcohols, three fatty acid esters of hydroxybenzoic acid, three fatty acid esters of hydroxybenzaldehyde, three glucosides of aromatic acids, four chlorogenic acids, two flavonols, and a benzylamine have been identified. The fatty acid moieties have been identified by GC-MS analysis of the methanolysis products. All of the compounds were tested for their radical scavenging and antioxidant activities by measuring their capacity to scavenge the 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical and anion superoxide radical and to induce the reduction of Mo(VI) to Mo(V). The chlorogenic acids and the flavonols exhibited more antioxidant and radical scavenger capacity than the positive standards α-tocopherol and ascorbic acid. The results of the tests were analyzed by cluster analysis that grouped all of the compounds on the basis of the substituents on the aromatic ring.

KEYWORDS: ***Cydonia vulgaris*; polyphenols; structural elucidation; antioxidant activity; structure–activity relationship**

INTRODUCTION

Antioxidants are chemicals that reduce oxidative damage to cells and biomolecules due to reactive oxygen species (ROS). They prevent injury to blood vessel membranes, helping to optimize blood flow to the heart and brain, defend against cancer-causing agents, and help lower the risk of cardiovascular disease, diabetes, and dementia, including Alzheimer's disease (*1–4*). Antioxidants are found in cereals, fruits, vegetables, and fish, or they can be taken in the form of dietary supplements. In fact, research suggests that a diet rich in fruits, vegetables, and cereals has additional benefits, because of lesser known but potent antioxidants in food that may have a combined effect greater than that of any single nutrient or individual antioxidant supplement. Among dietary antioxidants, polyphenols, naturally occurring in vegetables, fruits, tea, red wine, extra virgin olive oil, etc., are the most abundant ones. Recent studies showed that polyphenols are promising compounds that may help to control oxidative stress and consequently inflammatory response (*5*). Furthermore, it has been demonstrated that olive oil and red wine antioxidant polyphenols at nutritionally relevant concentrations transcriptionally inhibit endothelial adhesion molecule expression, thus partially explaining atheroprotection afforded by Mediterranean diets (*6*).

The search for antioxidant activity of fruit and vegetable constituents can increase the nutritional value of the food, leading to the discovery of new phytochemicals useful for the manufacture of functional foods. These products, defined as any modified food or food ingredient, which may provide a health benefit beyond the traditional nutrients it contains (*7*), are being developed and subjected to scientific evaluation. In the investigation of antioxidant metabolites from food plants growing in the Campania region (Italy), we isolated and characterized several antioxidants from local apple cultivars (*8–10*). Continuing this research we recently reported the isolation and antioxidant evaluation of carotenoid derivatives from *Cydonia vulgaris* fruits, commonly known as quince (*11, 12*). The '*cotogna*' apple pomes resemble an apple with a hard flesh of high flavor, but very acid, and are largely used for marmalade, liqueur, jelly, and preserves preparation. The high pectin content allows its use in the food industry as thickener. It has been reported that the leaves and fruits of quince have some positive

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Table 1. ^{13}C NMR Data of Metabolites **1a**, **1f**, **2a**, **2c**, **3b**, and **4**

	1a	1f	2a	2c	3b	4
1	173.2	173.2	173.2	173.2	173.9	173.9
2	34.1	34.1	34.1	34.1	34.1	33.8
3	24.9	24.9	24.9	24.9	24.9	24.9
4	29.9–29.1	29.8−29.2	29.9–29.1	29.8–29.2	29.9–29.1	30.4–29.5
5	29.9–29.1	29.8–29.2	29.9–29.1	29.8–29.2	29.9–29.1	30.4–29.5
6	29.9–29.1	29.8–29.2	29.9–29.1	29.8–29.2	29.9–29.1	30.4–29.5
7	29.9–29.1	29.8–29.2	29.9–29.1	29.8–29.2	29.9–29.1	30.4–29.5
8	29.9–29.1	29.8–29.2	29.9–29.1	29.8–29.2	29.9–29.1	27.7
9	29.9–29.1	29.8–29.2	29.9–29.1	29.8–29.2	29.9–29.1	130.1–127.9
10	29.9–29.1	29.8–29.2	29.9–29.1	29.8–29.2	29.9–29.1	130.1–127.9
11	29.9–29.1	29.8–29.2	29.9–29.1	29.8–29.2	29.9–29.1	25.8
12	29.9–29.1	27.3	29.9–29.1	27.3	29.9–29.1	130.1–127.9
13	29.9–29.1	130.3	29.9–29.1	130.3	29.9–29.1	130.1–127.9
14	30.9	130.1	30.9	130.1	29.9–29.1	27.7
15	22.5	27.3	22.5	27.3	29.9–29.1	30.4–29.5
16	14.3	29.8–29.2	14.3	29.8–29.2	30.9	30.4–29.5
17		29.8–29.2		29.8–29.2	22.7	22.8
18		29.8–29.2		29.8–29.2	14.5	14.2
19		29.8–29.2		29.8–29.2		
20		32.0		32.0		
21		23.0		23.0		
22		14.2		14.2		
1′	129.4	129.4	129.4	129.4	127.5	127.5
2′	130.2	130.2	130.2	130.2	111.7	111.7
3′	115.9	115.9	115.9	115.9	149.4	149.4
4′	156.4	156.4	156.4	156.4	150.7	150.7
5′	115.9	115.9	115.9	115.9	116.3	116.3
6′	130.2	130.2	130.2	130.2	124.1	124.1
7′	133.9	133.9	133.9	133.9	132.0	132.0
8′	130.0	130.0	130.0	130.0	123.9	123.9
9′	62.6	62.6	62.6	62.6	61.2	61.2
OMe					55.6	55.7

effects in the medical treatment of various conditions including cardiovascular diseases, hemorroids, bronchial asthma, and cough (*13*). In this study we have isolated, structurally characterized, and determined the antioxidant properties of the polyphenol components in the peel of the fruit.

MATERIALS AND METHODS

Fruit Collection and Extraction. *C. vulgaris* Pers. (syn *C. oblonga* Mill.) fruits were collected in Durazzano, near Caserta (Italy), in October 2005 when the fruit had just been harvested. The fruits were sliced, and the peels (3.1 kg) were infused in ethanol (5.0 L) for 7 days in a refrigerated chamber at 4 °C in the dark. After removal of the ethanolic solution, the peels were re-extracted first with Et_2O for 7 days and then with petroleum ether (PE) for a further 7 days. After distillation of the solvents under vacuum, we obtained the EtOH crude extract (261.6 g), Et_2O crude extract (15.0 g), and a PE crude extract (13.0 g).

General Experimental Procedures. Fourier transform NMR spectra were recorded at 300 MHz for ^1H and at 75 MHz for ^{13}C on a Varian Mercury 300 spectrometer in $CDCl_3$, DMSO, or CD_3OD, at 25 °C. Proton-detected heteronuclear correlations were measured using HSQC (optimized for $^1J_{HC} = 140$ Hz) and HMBC (optimized for $^nJ_{HC} = 8$ Hz). UV spectra were recorded on a UV-1700 Shimadzu spectrophotometer in MeOH solutions. Optical rotations were measured on a Perkin-Elmer 141 polarimeter in MeOH solutions. Electrospray mass spectra were recorded using a Waters ZQ mass spectrometer (Waters Co., Milford, MA) equipped with an electrospray ionization (ESI) probe operating in positive or negative ion mode. The scan range was *m/z* 80–2000. The preparative HPLC apparatus consisted of a pump (Shimadzu LC-10AD), a refractive index detector (Shimadzu RID-10A), and a Shimadzu C-R6A Chromatopac recorder. Preparative HPLC was performed using a 250 × 10 mm i.d., 10 μm, Phenomenex Luna RP-18 column (Phenomenex, Torrance, CA). Analytical HPLC was performed using a 250 × 4.6 mm i.d., 5 μm Phenomenex Luna RP-18 and RP-8 columns. Analytical TLC was performed on Merck Kieselgel 60 F_{254} or RP-18 F_{254} plates with 0.2 mm layer thickness. Spots were visualized by UV light or by spraying with H_2SO_4/AcOH/H_2O (1:20:4). The plates were then heated for 5 min at 110 °C. Preparative TLC was performed on Merck Kieselgel 60 F_{254} plates, with 0.5 or 1.0 mm film thickness. Flash column chromatography (FCC) was performed on Merck Kieselgel 60 (230–400 mesh) at medium pressure. Column chromatography (CC) was performed on Merck Kieselgel 60 (70–240 mesh), Amberlite XAD-4 (Fluka, Buchs, Switzerland), or Sephadex LH-20 (Pharmacia, Piscataway, NJ).

Organic Crude Extract Fractionation. EtOH extract (261.6 g) was dissolved in water and beat with EtOAc to obtain an organic (8 g) and an aqueous fractions. The aqueous fraction (190.0 g) was chromatographed on Amberlite XAD-4 eluting with water first and then with MeOH. The methanolic fraction was chromatographed on Sephadex LH-20 eluting with water–methanol solutions to obtain fractions A−D. Fraction A, eluted with H_2O, was chromatographed on RP-8 silica gel to obtain a fraction which, purified by RP8 HPLC eluting with MeOH/MeCN/H_2O (1:1:8), furnished pure **8** (10.0 mg). Fraction B, eluted with MeOH/H_2O (1:3), was chromatographed on RP-8 silica eluting with MeOH/MeCN/H_2O (7:1:1) to have pure **10** (12.0 mg) and a mixture, which was resolved by TLC chromatography eluting with the organic phase of $CHCl_3$/MeOH/EtOH/H_2O (10:7:5:5) to obtain the glucoside **9** (7.0 mg) and 4-hydroxybenzylamine **17** (5.0 mg). Fraction C, eluted with MeOH/H_2O (1:1), was purified by RP-8 HPLC-UV using as mobile phase a MeOH/H_2O solution (1:149) to have metabolite **11** (11.0 mg). Fraction D, eluted with pure MeOH, was chromatographed by RP-8 HPLC-UV eluting with MeOH/MeCN/H_2O (1:2:14) to have pure rutin **16** (23.1 mg).

The Et_2O crude extract was joined together with the EtOAc organic fraction of the EtOH crude extract and chromatographed on SiO_2 by flash column chromatography (FCC). The fraction eluted with CH_2Cl_2/MeOH (1:1) was chromatographed by FCC on SiO_2 eluting with the organic phase of $CHCl_3$/MeOH/H_2O (13:7:5) to give a fraction that was purified by RP-8 HPLC-UV using as mobile phase a MeOH/H_2O solution (1:149) and led to chlorogenic acids **11** (20.0 mg), **12** (35.2 mg), **13** (6.0 mg), and **14** (10.1 mg).

The PE crude extract was chromatographed on SiO_2 by aspiration under vacuum to obtain two fractions, E and F. Fraction E, eluted with

Table 2. NMR Data of Metabolite **5** Registered in $CDCl_3$

	δ_H[a] (J values in Hz)	COSY	δ_C	DEPT	HMBC (H→C)
1			129.5	C	
2	6.94 d (1.8)	6	109.1	CH	1, 3, 4, 6, 7
3			149.3	C	
4			149.4	C	
5	6.91 dd (8.4, 1.8)	6	111.3	CH	3, 4, 6
6	6.81 d (8.4)	2, 5	120.2	CH	1, 2, 4
7	6.59 d (15.9)	8	134.4	CH	1, 2, 6, 8, 9
8	6.16 dt (15.9, 6.7)	7, 9	121.5	CH	1, 9
9	4.71 dd (6.6, 1.5)	8	65.3	CH_2	1′, 7, 8
OMe	3.89 s		56.0	CH_3	3
OMe	3.87 s		56.1	CH_3	4
1′			173.9	C	
2′	2.34 t (7.8)	3′	34.6	CH_2	1′, 3′
3′	2.03 ov	2′, 4′	22.9	CH_2	
4′−7′	1.30 ov		29.3–29.9	CH_2	
8′	2.02 ov		27.4	CH_2	9′, 10′
9′	5.34 ov		130.1	CH	8′, 11′
10′	5.34 ov	11′	128.1	CH	11′
11′	2.76 t (5.4)	10′, 12′	25.9	CH_2	10′, 12′
12′	5.34 ov	11′	128.3	CH	11′
13′	5.34 ov		130.0	CH	12′, 14′
14′	2.02 ov		27.4	CH_2	12′, 13′
15′	1.29 ov		29.8	CH_2	
16′	1.65 ov	15′	25.2	CH_2	18′
17′	1.65 ov	18′	33.0	CH_2	
18′	3.63 t (6.6)	17′	63.3	CH_2	16′, 17′

[a] d, doublet; dd, double doublet; dt, double triplet; s, singlet; t, triplet; ov, overlapped.

CH_2Cl_2, was chromatographed on NH_2 silica, obtaining two fractions. The first one eluted with EtOAc/PE (1:19) furnished pure **4** (2.8 mg) and **5** (31.0 mg).

Z-Coniferyl linoleate (**4**): ^{1}H NMR ($CDCl_3$) δ 7.18 (d, J = 1.8 Hz, H-2′), 6.80 (d, J = 7.8 Hz, H-5′), 7.05 (dd, J = 7.8 and 1.8 Hz, H-6′), 6.58 (1H, d, J = 11.0 Hz, H-7′), 5.70 (1H, dt, J = 11.0 and 6.4 Hz, H-8′), 5.36 (8H, m, H-9, H-10, H-12 and H-13), 4.83 (2H, dd, J = 6.4 and 1.5 Hz, H-9′), 2.77 (2H, m, H-11), 2.33 (2H, t, J = 7.9 Hz, H-2), 2.05 (4H, m, H-8 and H-14), 1.30 (16H, m, H-3−H-7 and H-15−H-17), 0.88 (3H, t, J = 6.9 Hz, H-18); ^{13}C NMR ($CDCl_3$) see **Table 1**; EI-MS, m/z 442 $[M]^+$. Anal. Calcd for $C_{28}H_{42}O_4$: C, 75.98; H, 9.56. Found: C, 76.01; H, 9.55.

E-3,4-Dimethoxycinnamyl ω-hydroxylinoleate (**5**): ^{1}H- and ^{13}C NMR ($CDCl_3$): see **Table 2**; EI-MS: m/z 472 $[M]^+$. Anal. Calcd for $C_{28}H_{42}O_4$: 73.69; H, 9.38. Found: C, 73.71; H, 9.35.

The second fraction eluted with EtOAc/PE (1:4) furnished a mixture that was chromatographed on SiO_2 by preparative TLC eluting with PE/$CHCl_3$/EtOAc (6:3:1) to give two bands. The first contained the acyl benzoic acids **6a**−**c** (3.0 mg), which were separated by RP-18 HPLC eluting with MeOH/MeCN (4:1), whereas the second band consisted of the acyl benzaldehydes **7a**−**c** (4 mg), which were purified in the same conditions as the compounds **6a**−**c**.

4-(Palmitoyloxy)benzoic acid (**6a**): ^{1}H NMR ($CDCl_3$) δ 7.80 (2H, d, J = 8.4 Hz, H-3′ and H-7′), 6.96 (2H, d, J = 8.4 Hz, H-4′ and H-6′), 2.36 (2H, t, J = 7.8 Hz, H-2), 1.30 (26H, m, H-3 - H-15), 0.88 (3H, t, J = 6.9 Hz, H-16); EI-MS, m/z 376 $[M]^+$. Anal. Calcd for $C_{23}H_{36}O_4$: C, 73.37; H, 9.65. Found: C, 73.41; H, 9.65.

4-(Linoleoyloxy)benzoic acid (**6b**): ^{1}H NMR ($CDCl_3$) δ 7.80 (2H, d, J = 8.4 Hz, H-3′ and H-7′), 6.96 (2H, d, J = 8.4 Hz, H-4′ and H-6′), 5.36 (8H, m, H-9, H-10, H-12, and H-13), 2.77 (2H, m, H-11), 2.33 (2H, t, J = 7.9 Hz, H-2), 2.05 (4H, m, H-8 and H-14), 1.30 (16H, m, H-3−H-7 and H-15−H-17), 0.88 (3H, t, J = 6.9 Hz, H-18); EI-MS, m/z 376 $[M]^+$. Anal. Calcd for $C_{25}H_{36}O_4$: C, 74.96; H, 9.06. Found: C, 75.00; H, 9.05.

(*Z*)-4-(Docos-13-enoyloxy)benzoic acid (**6c**): ^{1}H NMR ($CDCl_3$) δ 7.80 (2H, *d*, J = 8.4 Hz, H-3′ and H-7′), 6.96 (2H, d, J = 8.4 Hz, H-4′ and H-6′), 5.34 (4H, m, H-13 and H-14), 2.35 (2H, t, J = 7.2 Hz, H-2), 2.00 (4H, m, H-12 and H-15), 1.29 (30H, m, H-3−H-11 and H-16−H-21), 0.88 (3H, t, J = 6.7 Hz, H-22); EI-MS, m/z 458 $[M]^+$. Anal. Calcd for $C_{25}H_{36}O_4$: C, 75.94; H, 10.11. Found: C, 75.90; H, 10.07.

3-Methoxy-4-(palmitoyloxy)benzaldehyde (**7a**): ^{1}H NMR ($CDCl_3$) δ 9.83 (1H, s, H-1′), 7.42 (1H, d, J = 1.8 Hz, H-3′), 7.44 (1H, dd, J = 8.2 and 1.8 Hz, H-7′), 7.03 (1H, d, J = 8.2 Hz, H-6′), 3.98 (3H, s, OMe), 2.36 (2H, t, J = 7.8 Hz, H-2), 1.30 (26H, m, H-3−H-15), 0.88 (3H, t, J = 6.9 Hz, H-16); EI-MS, m/z 390 $[M]^+$. Anal. Calcd for $C_{24}H_{38}O_4$: C, 73.81; H, 9.81. Found: C, 73.84; H, 9.85.

3-Methoxy-4-(linoleoyloxy)benzaldehyde (**7b**): ^{1}H NMR ($CDCl_3$) δ 9.83 (1H, s, H-1′), 7.42 (1H, d, J = 1.8 Hz, H-3′), 7.44 (1H, dd, J = 8.2 and 1.8 Hz, H-7′), 7.03 (1H, d, J = 8.2 Hz, H-6′), 3.98 (3H, s, OMe), 5.36 (8H, m, H-9, H-10, H-12. and H-13), 2.77 (2H, m, H-11), 2.33 (2H, t, J = 7.9 Hz, H-2), 2.05 (4H, m, H-8 and H-14), 1.30 (16H, m, H-3−H-7 and H-15−H-17), 0.88 (3H, t, J = 6.9 Hz, H-18); EI-MS, m/z 416 $[M]^+$. Anal. Calcd for $C_{26}H_{40}O_4$: C, 74.96; H, 9.65. Found: C, 74.94; H, 9.65.

(*Z*)-3-Methoxy-4-(docos-13-enoyloxy)benzaldehyde (**7c**): ^{1}H NMR ($CDCl_3$) δ 9.83 (1H, s, H-1′), 7.42 (1H, d, J = 1.8 Hz, H-3′), 7.44 (1H, dd, J = 8.2 and 1.8 Hz, H-7′), 7.03 (1H, d, J = 8.2 Hz, H-6′), 5.34 (4H, m, H-13 and H-14), 3.98 (3H, s, OMe), 2.35 (2H, t, J = 7.2 Hz, H-2), 2.00 (4H, m, H-12 and H-15), 1.29 (30H, m, H-3−H-11 and H-16−H-21), 0.88 (3H, t, J = 6.7 Hz, H-22); EI-MS, m/z 444 $[M]^+$. Anal. Calcd for $C_{28}H_{44}O_4$: C, 75.63; H, 9.97. Found: C 75.60; H, 9.95.

Fraction F, eluted with CH_2Cl_2/Me_2CO (1:1), chromatographed on SiO_2 by FCC eluting with hexane/$CHCl_3$/Me_2CO (13:6:1), furnished three fractions: the first was purified by RP-18 HPLC eluting with MeOH/MeCN (4:1) to give pure **1c** (2.5 mg), **1d** (2.1 mg), **1e** (2.2 mg), **1f** (1.8 mg), **2b** (2.9 mg), and **2c** (3.1 mg); the second fraction, purified in the same conditions, gave **1a** (2.6 mg), **1b** (2.3 mg), **2a** (3.2 mg), **3a** (1.0 mg), and **3b** (1.1 mg); the third fraction contained pure quercetin **15** (21.0 mg).

E-*p*-Coumaryl palmitate (**1a**): ^{1}H NMR ($CDCl_3$) δ 7.28 (2H, d, J = 8.7 Hz, H-2′ and H-6′), 6.78 (2H, d, J = 8.7 Hz, H-3′ and H-5′), 6.58 (1H, d, J = 15.9 Hz, H-7′), 6.15 (1H, dt, J = 15.9 and 6.9 Hz, H-8′), 4.70 (2H, dd, J = 6.9 and 2.2 Hz, H-9′), 2.37 (2H, t, J = 7.8 Hz, H-2), 1.27 (26H, m, H-3−H-15), 0.88 (3H, t, J = 6.7 Hz, H-16); ^{13}C NMR ($CDCl_3$) see **Table 1**; EI-MS, m/z 388 $[M]^+$. Anal. Calcd for $C_{25}H_{40}O_3$: C, 77.27; H, 10.38. Found: C, 77.30; H, 10.40.

E-*p*-Coumaryl 13-*cis*-docosenoate (**1f**): ^{1}H NMR ($CDCl_3$) δ 7.28 (2H, d, J = 8.7 Hz, H-2′ and H-6′), 6.78 (2H, d, J = 8.7 Hz, H-3′ and H-5′), 6.58 (1H, d, J = 15.9 Hz, H-7′), 6.15 (1H, dt, J = 15.9 and 6.9 Hz, H-8′), 5.34 (4H, m, H-13 and H-14), 4.70 (2H, dd, J = 6.9 and 2.2 Hz, H-9′), 2.35 (2H, t, J = 7.2 Hz, H-2), 2.00 (4H, m, H-12 and H-15), 1.29 (30H, m, H-3−H-11 and H-16−H-21), 0.88 (3H, t, J = 6.7 Hz, H-22); ^{13}C NMR ($CDCl_3$) see **Table 1**; EI-MS, m/z 470 $[M]^+$. Anal. Calcd for $C_{31}H_{50}O_3$: C, 79.10; H, 10.71. Found C, 79.14; H, 10.68.

Z-*p*-Coumaryl palmitate (**2a**): ^{1}H NMR ($CDCl_3$) δ 7.28 (2H, d, J = 8.7 Hz, H-2′ and H-6′), 6.82 (2H, d, J = 8.7 Hz, H-3′ and H-5′), 6.58 (1H, d, J = 12.0 Hz, H-7′), 5.70 (1H, dt, J = 12.0 and 6.2 Hz, H-8′), 4.85 (2H, d, J = 6.2 Hz, H-9′), 2.37 (2H, t, J = 7.8 Hz, H-2), 1.27 (26H, m, H-3−H-15), 0.88 (3H, t, J = 6.7 Hz, H-16); ^{13}C NMR ($CDCl_3$) see **Table 1**; EI-MS, m/z 388 $[M]^+$. Anal. Calcd for $C_{25}H_{40}O_3$: C, 77.27; H, 10.38. Found: C, 77.29; H, 10.36.

Z-*p*-Coumaryl 13-*cis*-docosenoate (**2c**): ^{1}H NMR ($CDCl_3$) δ 7.28 (2H, d, J = 8.7 Hz, H-2′ and H-6′), 6.82 (2H, d, J = 8.7 Hz, H-3′ and H-5′), 6.58 (1H, d, J = 12.0 Hz, H-7′), 5.70 (1H, dt, J = 12.0 and 6.2 Hz, H-8′), 5.34 (4H, m, H-13 and H-14), 4.85 (2H, d, J = 6.2 Hz, H-9′), 2.35 (2H, t, J = 7.2 Hz, H-2), 2.00 (4H, m, H-12 and H-15), 1.29 (30H, m, H-3−H-11 and H-16−H-21), 0.88 (3H, t, J = 6.7 Hz, H-22); ^{13}C NMR ($CDCl_3$) see **Table 1**; EI-MS, m/z 470 $[M]^+$. Anal. Calcd for $C_{31}H_{50}O_3$: C, 79.10; H, 10.71. Found: C, 79.08; H, 10.70.

E-Coniferyl stearate (**3b**): ^{1}H NMR ($CDCl_3$) δ 7.18 (d, J = 1.8 Hz, H-2′), 6.80 (d, J = 7.8 Hz, H-5′), 7.05 (dd, J = 7.8 and 1.8 Hz, H-6′), 6.58 (1H, d, J = 11.0 Hz, H-7′), 5.70 (1H, dt, J = 11.0 and 6.4 Hz, H-8′), 4.83 (2H, dd, J = 6.4 and 1.5 Hz, H-9′), 2.37 (2H, t, J = 7.8 Hz, H-2), 1.27 (30H, m, H-3−H-17), 0.89 (3H, t, J = 6.7 Hz, H-18). ^{13}C NMR ($CDCl_3$) see **Table 1**; EI-MS, m/z 446 $[M]^+$. Anal. Calcd for $C_{28}H_{46}O_4$: C, 75.29; H, 10.38. Found: C, 75.31; H, 10.41.

GC-MS Analyses of Fatty Acid Moieties. Pure ester (0.1 mg) was dissolved in 0.2 mL of 2 N KOH in methanol in a 1 mL vial. After the

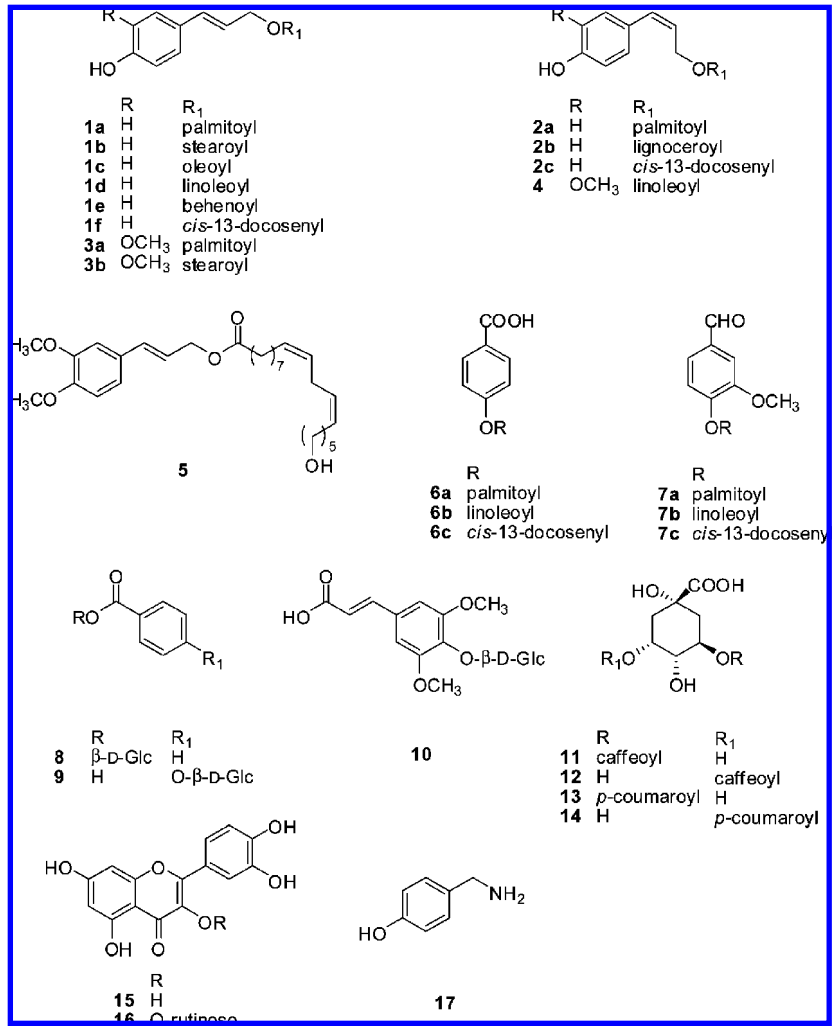

Figure 1. Structures of phenolics and derivatives isolated from *Cydonia vulgaris*.

solution had been stirred for 30 min, heptane (0.8 mL) was added. The solution was mixed by a vortex mixer and centrifuged, using a Beckman GS-15R centrifuge, for 10 min at 4000 rpm. The organic upper phase (1 μL) was analyzed by GC-MS fitted with a 30 m × 0.25 mm i.d., 0.2 μm Zebron ZB5MS, fused silica capillary column (Phenomenex). The column oven was held at 80 °C for 1 min and then increased to 260 °C at 10 °C/min. Injector and detector temperatures were 250 and 150 °C, respectively, the carrier gas was He, and the flow rate was 1.0 mL/min. The fatty acid methyl esters were identified on the basis of their EI-MS spectra and by comparison of their retention times with those of the standard fatty acid methyl esters (Supelco 37 Component FAME Mix).

DPPH Radical Scavenging Capacity. The scavenging capacity of metabolites was measured according to the method of Brand-Williams (*14*). The method was based on the reduction of methanolic DPPH$^{\bullet}$ in the presence of a hydrogen-donating antioxidant. DPPH$^{\bullet}$ (Fluka) solution showed an absorption band at 517 nm and was intensely violet colored. The adsorption and color decreased when DPPH$^{\bullet}$ was reduced by an antioxidant compound. The remaining DPPH$^{\bullet}$ corresponded inversely to the radical scavenging activity of the antioxidant (*15*). DPPH$^{\bullet}$ (2 mg) was dissolved in 54 mL of MeOH. The investigated metabolites were prepared by dissolving 0.4 μmol of each compound in 1 mL of MeOH. Then 38 μL of each solution containing compound was added to 1.462 mL of DPPH$^{\bullet}$ solution at room temperature (*16*). The absorbance at 517 nm was measured in a cuvette at 30 min versus blank (38 μL of MeOH in 1.462 mL of DPPH$^{\bullet}$ solution) using a UV-1700 Shimadzu spectrophotometer. The analysis was carried out in triplicate, and the results were expressed in terms of the percentage of radical scavenging capacity (RCS).

Superoxide Radical Scavenging Activity. The assay of superoxide radical scavenging capacity was based on the capacity of each isolated metabolite (0.4 mM) to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) (Fluka) in the riboflavin−light−NBT system (*17*). Each 3 mL reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine (Fluka), 2 μM riboflavin (Riedel-de Haën, Seelze, Germany), 100 μM EDTA (Carlo Erba Reagents, Rodano, Milano, Italy), 75 μM NBT, and 100 μL of sample solution. The production was followed by monitoring the increase in absorbance at 560 nm after 10 min of illumination from a fluorescent lamp. The analysis was carried out in triplicate, and the results were expressed in terms of the percentage of RCS.

Evaluation of Total Antioxidant Capacity. Spectrophotometric evaluation of antioxidant capacity through the formation of a phosphomolybdenum complex was carried out according to the method of Prieto et al. (*18*). Sample solutions (100 μL) containing reducing metabolites (0.4 μmol in 1 mL of dimethyl sulfoxide) were combined in an Eppendorf tube with 1 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95 °C for 90 min. After the samples had cooled to room temperature, the absorbance of an aqueous solution of each was measured at 820 nm against a blank. The analysis was carried out in triplicate, and the antioxidant activity was expressed as caffeic acid equivalents (CAE), an antioxidant substance significantly better than α-tocopherol (*19*).

Statistical Treatment. A numerical clustering of data bioassay was made on the basis of percentage differences from control. An average linkage agglomeration criterion and the Euclidean distance as dis-

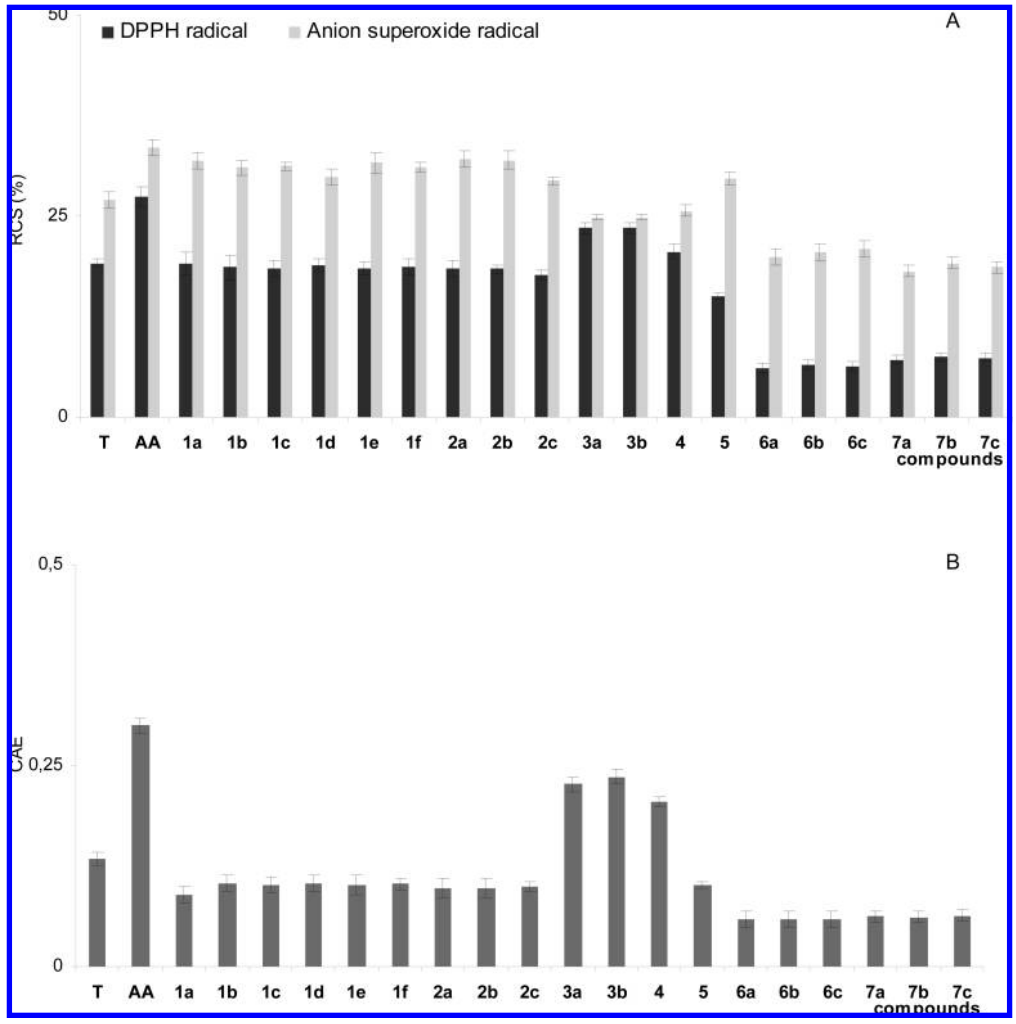

Figure 2. Antioxidant capacity of metabolites **1a**−**f**, **2a**−**c**, **3a**,**b**, **4**, **5**, **6a**−**c**, and **7a**−**-c**: (**A**) radical scavenging capacity (RCS, %) on DPPH radical and anion superoxide radical; (**B**) reducing capacity Mo(VI) as caffeic acid equivalents (CAE). AA, ascorbic acid; T, α-tocopherol.

similarity coefficient were applied by the software NTSYSpc, Numerical Taxonomy and Multivariate Analysis System, version 2.2 (*20*).

RESULTS AND DISCUSSION

Chemical Characterization of Phytochemicals. The chemical structures of phytochemicals isolated from the organic extracts of *C. vulgaris* are reported in **Figure 1**. All of the structures were elucidated on the basis of 1D and 2D NMR experiments and by EI or ESI mass spectrometry.

Compounds **1b**−**e** and **2b** have been already characterized from 'Annurca' apples (*21*), and **3a** was reported as an antioxidant metabolites isolated from *Platycodon grandiflorum* (*22*). The NMR data of **1a** and **1f** showed signals due to a *trans*-*p*-coumaric alcohol esterified with fatty acids. The GC-MS analysis of the methanolysis product, obtained by reaction with 10% KOH in MeOH, led to the identification of the acyl moieties as palmitic acid and *cis*-13-docosenoic acid for **1a** and **1f**, respectively. Compounds **2a** and **2c** were characterized by the presence, in the ^{1}H NMR, of signals indicating the presence of the cis isomer of the *p*-coumarylic alcohol. Also, for **2a** and **2c**, the esterified fatty acids were identified by GC-MS as palmitic and *cis*-13-docosenoic acids, respectively.

Compounds **3b** and **4** were characterized by the presence of *trans*- and *cis*-coniferyl alcohol, respectively; the fatty acids were identified by GC-MS as stearic and linoleic acids, respectively.

Compound **5** showed a molecular formula of $C_{29}H_{44}O_5$ calculated on the basis of the EI-MS spectrum and elemental analysis. The ^{1}H NMR spectrum showed three aromatic protons of a 1,2,4-trisubstituted benzene at δ 6.94, 6.91, and 6.81, two olefinic protons as a doublet at δ 6.59, and a double triplet at δ 6.16, besides another four overlapped protons at δ 5.34. In the carbinolic region of the spectrum were evident two methylenes as a doublet at δ 4.71, a triplet at δ 3.63, and two methoxyls at δ 3.87 and 3.89. Finally, in the upfield region were evident a double–doublet at δ 2.76 and a triplet at δ 2.34, besides other protons due to a fatty acid moiety ranging from 2.1 to 0.8 ppm. In the ^{13}C NMR spectrum were evident an ester carbon at δ 173.9, 12 sp^2 carbons ranging from 149 to 109 ppm, 2 methylenes at δ 65.3 and 63.3, 2 methoxyls at δ 56.1 and 56.0, and other partially overlapped signals ranging from 35 and 14 ppm. A series of 2D homo-NMR (DQCOSY, TOCSY) and hetero-NMR (HMQC, HSQC-TOCSY) experiments allowed the assignment of all of the overlapped proton and carbon signals. In fact, in the HSQC-TOCSY were evident correlations between the carbon at δ 121.5, correlated in the HSQC with the proton at δ 6.16, and the protons at δ 6.59 and 4.71, which were bonded to the carbons at δ 134.4 and 65.3, respectively. The carbon at δ 134.4 was correlated, in the HMBC experiment, to the protons at δ 4.71 and with the doublet at δ 6.94 and the double–doublet at δ 6.91. The latter showed, in the same experiment, correlations with the carbons at δ 109.1, 120.2, 129.5, and 149.4. This carbon showed cross peaks with the methoxyl at δ 3.87. The proton at δ 6.94 showed heterocorrelation, in the HMBC experiment, with the carbons at δ 111.3, 120.2, and 149.3, which

was in turn correlated with the methoxyl at δ 3.89. These data suggested the presence of a 3,4-dimethoxycinnamoyl alcohol. The methylene at δ 4.71 showed, in the HMBC experiment, correlation with the carbon at δ 173.9 and with the olefin carbons of the side chain of the phenylpropane moiety. The NMR data led us to hypothesize the presence of an unsaturated fatty acid. The presence of a triplet at δ 2.76 and their correlations with four olefin carbons confirmed the hypothesis. However, the absence of a methyl carbon and the presence of the methylene at δ 63.3 indicated the presence of an ω-hydroxy fatty acid. The structure was elucidated by GC-MS analysis. In fact, the methyl ester derivative of the fatty acid, obtained by reaction with KOH in MeOH, showed a molecular peak at *m/z* 310. These data indicated the presence of the methyl ester of the ω-hydroxylinoleic acid, which was definitively confirmed by the 2D NMR data.

Compounds **6a**−**c** showed in the ^{1}H NMR spectrum two signals of an AA′BB′ spin system at δ 6.96 and 7.80 besides protons of a fatty acid moiety. The ^{13}C NMR showed signals of a carboxyl carbon at δ 179.2, which was correlated in the HMBC experiment with the protons at δ 7.80. These data suggested the presence of a 4-hydroxybenzoic acid esterified through the 4-hydroxyl group to a fatty acid. This was identified for the compounds **6a**−**c** by GC-MS analysis as palmitic, linoleic, and *cis*-13-docosenoic acids. Compounds **7a**−**c** showed the same fatty acids as the previous compounds, but in the ^{1}H NMR were evident an aldehyde at δ 9.83 and three aromatic protons at δ 7.44, 7.42, and 7.03, besides a methoxyl at δ 3.98. These data were in accordance with the presence of a 3-methoxy-4-hydroxy aldehyde esterified by the hydroxyl group with the acid moiety.

Phenols **8**−**10** were identified as glucosides: compound **8** was characterized as a benzoic acid esterified with a β-D-glucopyranose, already identified from *Pinus contorta* needles (*23*), whereas the 4-*O*-β-D-glucopyranosyloxybenzoic acid (**9**) was reported as component of the galls and shoots of *Picea glauca* (*24*); compound **10** was identified as sinapyl acid 4-*O*-β-D-glucopyranoside (*25*).

Compounds **11**−**14** were characterized as chlorogenic acids already reported as components of *Hemerocallis* (*26*), whereas the flavones **15** and **16** were identified as quercetin and rutin, respectively (*27*). Finally, compound **17** was identified as 4-hydroxybenzylamine (*28*).

Antioxidant and Radical Scavenging Capacities. All of the metabolites isolated from *C. vulgaris* fruits were tested for their antioxidant activity by measuring their ability to scavenge free radicals, DPPH$^{\bullet}$ and $O_2^{\bullet -}$, and by evaluating their capacity to induce the formation of a phosphomolybdenum complex. The antioxidant capacity of compounds was compared with those shown by two known natural antioxidants, ascorbic acid (AA) and α-tocopherol (T), treated in the same way. The results are reported in **Figure 2**.

The determination of the antioxidant activity of *E*- and *Z*-*p*-coumaryl esters of fatty acid (compounds **1a**−**f** and **2a**−**c**) showed that these metabolites have high antioxidative properties. These compounds showed an inhibition of the radical superoxide similar to that of ascorbic acid and higher than that measured for the α-tocopherol (**Figure 2A**). A good antioxidant activity was observed for the phenylpropanoid esters **3a**, **3b**, and **4**,; they are be able to induce the reduction of Mo(VI) to Mo(V) much more than α-tocopherol (**Figure 2B**). These data were in good accordance with published papers reporting that the antioxidant activity of a phenylpropanoid compounds is closely

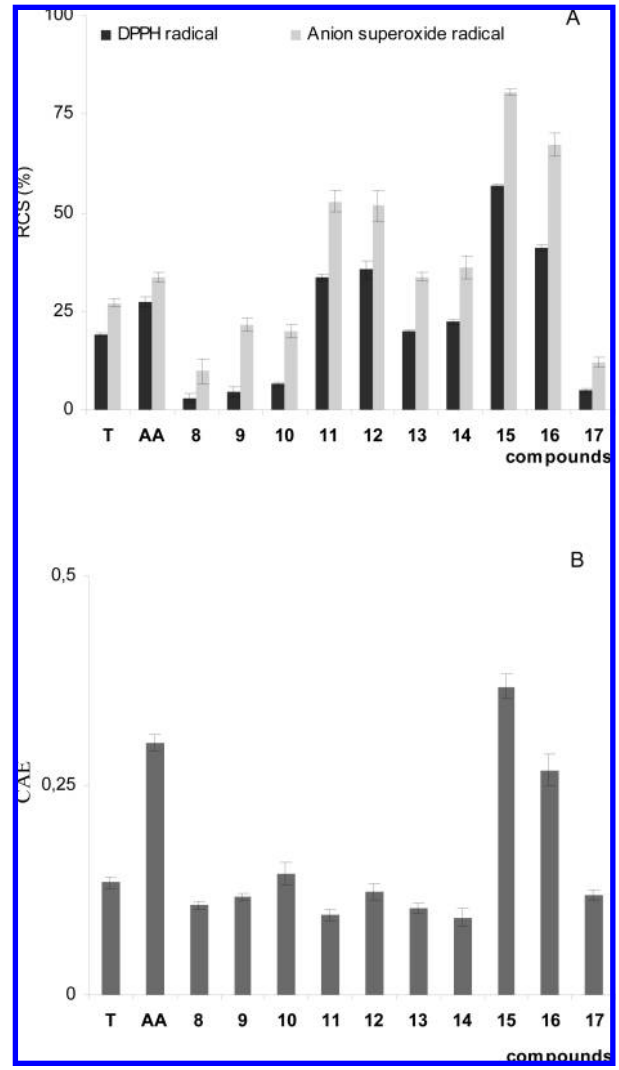

Figure 3. Antioxidant capacity of metabolites **8**−**17**: (**A**) radical scavenging capacity (RCS, %) on DPPH radical and anion superoxide radical; (**B**) reducing capacity Mo(VI) as caffeic acid equivalents (CAE). AA, ascorbic acid; T, α-tocopherol.

associated with its chemical structure, such as substitutions on the aromatic ring and side chain (*29*–*31*).

By testing compounds **13** and **14**, identified as 3-*O*-*p*-coumaroylquinic acid and 5-*O*-*p*-coumaroylquinic acid, respectively, an antioxidant activity was observed comparable to that of *E*- and *Z*-*p*-coumaryl esters of fatty acid. Evaluation of these results led us to conclude that the activity of these two groups of compounds was influenced by the presence of the *p*-hydroxyphenolic moiety. In all our antioxidant tests, a very good activity was observed for chlorogenic acid and neochlorogenic acid (compounds **11** and **12**). These metabolites reduce the DPPH radical by 33 and 35%, respectively, and scavenged superoxide radical by 51% (**Figure 3A**). The catechol moiety is responsible for the hydrogen-donating activity, whereas quinic acid shows no contribution to this activity (*32*).

Among isolated metabolites, the strongest antioxidant activity was observed for flavonoids (compounds **15** and **16**). Quercetin **15** scavenges $O_2^{\bullet -}$ and DPPH$^{\bullet}$ by 80 and 57%, respectively (**Figure 3A**). Furthermore, quercetin is able to induce the reduction of Mo(VI) to Mo(V); it exhibits a reducing activity (0.370 CAE) greater than that α-tocopherol and ascorbic acid (**Figure 3B**). Rutin **16** showed a very strong antioxidant activity; it reduces $O_2^{\bullet -}$, DPPH$^{\bullet}$, and Mo(VI) by 67%, 41%, and 0.268 CAE.

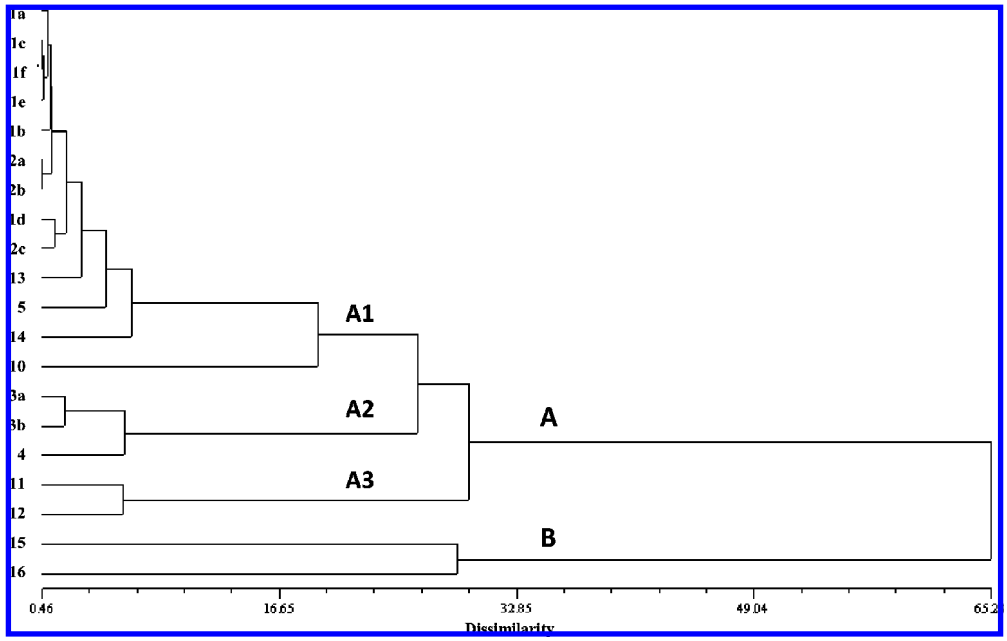

Figure 4. Dendrogram of the 29 phenols from *C. vulgaris* based on registered radical scavenging capacities.

As shown in **Figure 4**, the cluster analysis of biological data produced a dendrogram with two principal groups of metabolites (A and B). Cluster A can be divided into three subclusters that grouped all of the metabolites on the basis of their chemical structures. In fact, subcluster A1 included, besides compound **5**, the *p*-coumaryl esters and the chlorogenic acids **13** and **14**, all characterized by the presence of a 4-hydroxyphenyl moiety in the molecule; subcluster A2 grouped all of the phenols constituted by a guaiacyl group; moreover, subcluster A3 included the metabolites with a catechol moiety in their structures. These data indicated the role of the phenolic moiety in the antioxidant and radical scavenging activities. Finally, cluster B included the two flavonols **15** and **16**. In all our tests, the antioxidant activity of compounds **16** was lower than that of metabolite **15**; these data indicate that the glycosidic moiety did not contribute to the antioxidant ability. In the literature, it was reported that the *o*-dihydroxy (catechol) structure in the B-ring, the 2,3-double bond in conjunction with a 4-oxo function, and the additional presence of a 3-hydroxyl group in the C-ring, as in quercetin, were three structural groups important for maximal antioxidant potential of flavonoids (*33*).

Significant antioxidant, antitumoral, antiviral, and antibiotic capacities are frequently reported for plant phenols (*34*). They have often been identified as active principles of numerous folk herbal medicines. The regular intake of fruits and vegetables has been highly recommended, because the plant phenols and polyphenols they contain are thought to play important roles in long-term health and reduction in the risk of chronic and degenerative diseases.

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