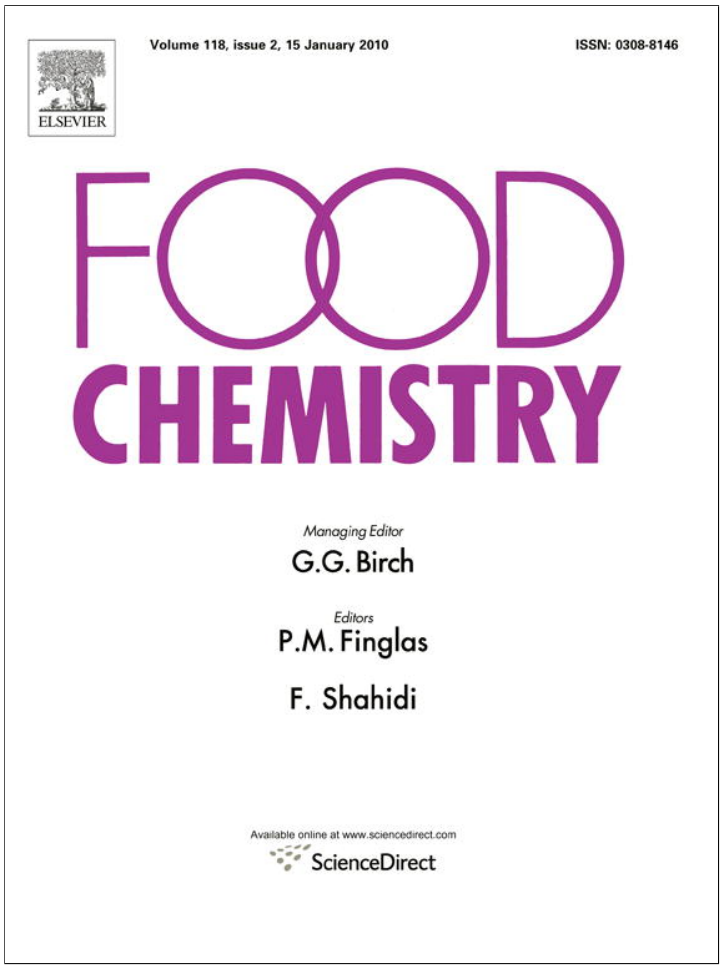

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Antioxidant and antiproliferative activities of phytochemicals from Quince (*Cydonia vulgaris*) peels

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ABSTRACT

Fifty-nine secondary metabolites have been isolated from *Cydonia vulgaris* peels and characterised on the basis of their spectroscopic features. Among them, five metabolites, 3β-(18-hydroxylinoleoyl)-28-hydroxyurs-12-ene (**12**), 3β-linoleoylurs-12-en-28-oic acid (**15**), 3β-oleoyl-24-hydroxy-24-ethylcholesta-5,28(29)-diene (**24**), tiglic acid 1-*O*-β-D-glucopyranoside (**35**), and 6,9-dihydroxymegastigmasta-5,7-dien-3-one 9-*O*-β-D-gentiobioside (**46**), have been isolated and elucidated for the first time. 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 antiproliferative activity of all the most abundant compounds by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) bioassay on murine B16-F1 melanoma cells has been also assessed.

1. Introduction

Plant-based foods provide, not only essential nutrients needed for life, but also other bioactive compounds for health promotion and disease prevention. In fact, fruit and vegetables contain significant amounts of phytochemicals that may afford desirable health benefits beyond basic nutrition and reduce the risk of chronic diseases (Liu, 2003). Numerous researches suggest that a wide variety of phytochemicals, such as phenolics and carotenoids, is able to prevent or slow down oxidative stress-induced damage leading to cancer. Kinghorn et al. (2004) reviewed the potential cancer chemopreventive effects of over 150 compounds in terms of the quinone reductase induction ability of flavonoids and withanolides and the cyclooxygenase-1 and -2 inhibitory activities of flavanones, flavones and stilbenoids. Epidemiological studies have shown that consumption of vegetables, mainly onion and garlic, reduces the risk of the intestinal stomach cancer (Gonzalez & Riboli, 2006).

In the investigation of health-protective compounds from edible plants, characteristic of the Campania Region (Italy), we identified several phytochemicals from local apple cultivars (Cefarelli et al., 2006; D'Abrosca, Fiorentino, Oriano, Monaco, & Pacifico, 2006; D'Abrosca, Pacifico, Cefarelli, Mastellone, & Fiorentino, 2007). Continuing this research we, recently, reported the isolation and antioxidant evaluation of carotenoid and phenol derivatives from *Cydonia vulgaris* fruits, also known as Quince (Fiorentino et al., 2006, 2007, 2008). *C. vulgaris* Pers. (sin. *C. oblonga* Mill.) is a small shrub belonging to the same family as apples and pears (Rosaceae). This species is the sole member of the genus. It is a small tree with bright golden yellow pome fruits, when mature. The fruit of *C. vulgaris*, known as Quince, resembles an apple, but differs in having many seeds in each carpel. Pomes of Quince, known in Italy as 'cotogna' apple, have hard flesh of high flavour, but very acid, and these are largely used for marmalade, liqueur, jelly and preserves. It has been reported that the leaves and fruits of Quince have some positive effects in the medical treatment of various conditions, including cardiovascular diseases, haemorroids, bronchial asthma, and cough (Yildirim, Oktay, & Bilaloğlu, 2001).

The present research reports the complete characterisation of the organic extracts of *C. vulgaris* peels. Among them, five metabolites, namely 3β-(18-hydroxylinoleoyl)-28-hydroxyurs-12-ene (**12**), 3β-linoleoylurs-12-en-28-oic acid (**15**), 3β-oleoyl-24-hydroxy-24-ethylcholesta-5,28(29)-diene (**24**), tiglic acid 1-*O*-β-D-glucopyranoside (**35**), and 6,9-dihydroxymegastigmasta-5,7-dien-3-one 9-*O*-β-D-gentiobioside (**46**), have been isolated and elucidated for the first time. 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)

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radical, and anion superoxide radical and to induce the reduction of Mo(VI) to Mo(V). The antiproliferative activities of terpenoid and isoprenoid constituents, as well as the most abundant polyphenol components, by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide bioassay on murine B16-F1 melanoma cells have also been assessed.

2. Materials and methods

2.1. Fruit collection and extraction

C. vulgaris Pers. (syn. *Cydonia 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 seven days in a refrigerated chamber at 4 °C in the dark. After removal of the ethanol, the peels were re-extracted first with Et_2O for seven days and then with petroleum ether (PE) for a further seven days. After distillation of the solvents in a 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).

2.2. General experimental procedures

NMR spectra were recorded at 300 MHz for ^1H and 75 MHz for ^{13}C on a Varian Mercury 300 Fourier transform NMR or 500 MHz for ^1H and 125 MHz for ^{13}C on a Varian Inova Fourier transform NMR spectrometer in $CDCl_3$, DMSO d_6 or CD_3OD, at 25 °C. Proton-detected heteronuclear correlations were measured using HSQC (optimised for $^1J_{HC} = 140$ Hz) and HMBC (optimised for $^nJ_{HC} = 8$ Hz). UV spectra were recorded on a UV-1700 Shimadzu spectrophotometer in MeOH solution. Optical rotations were measured on a Perkin–Elmer (Perkin–Elmer Co., Norwalk, CT) 141 in MeOH solution. Electron ionisation mass spectra (EIMS) were obtained with a HP 6890 instrument equipped with a MS 5973 N detector. Electrospray mass spectra were recorded using a Waters ZQ mass spectrometer (Waters Co., Milford Massachusetts, USA) equipped with an electrospray ionisation (ESI) probe operating in positive or negative ion mode. The scan range was *m*/*z* 80–2000. The HPLC apparatus consisted of a pump (Shimadzu LC-10AD), a refractive index detector (Shimadzu RID-10A) and a Shimadzu Chromatopac C-R6A recorder. Preparative HPLC was performed using NH_2 (Luna 10 μm, 250 × 10 mm i.d., Phenomenex, Torrance, CA, USA) and SiO_2 (Kromasil 10 μm, 250 × 10 mm i.d., Phenomenex, Torrance, CA, USA) columns. Analytical HPLC was performed using RP-8 (Luna 5 μm, 250 × 4.6 mm i.d., Phenomenex, Torrance, CA, USA) or Polar-RP-80A (Synergi 4 μm, 250 × 4.6 mm i.d., Phenomenex, Torrance, CA, USA) columns. Analytical TLC was performed on Merck Kieselgel 60 F254 or RP-18 F254 plates with 0.2 mm layer thickness. Spots were visualised 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 F254 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), Sephadex LH-20 (Pharmacia, Piscataway, U.S.A.), or on NH_2 silica (LiChroprep NH_2, 40–63 μm, Merck, Darmstadt, Germany).

2.3. Organic crude extracts fractionation and chemical characterisation

The EtOH extract (261.6 g) was dissolved in water and shaken 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 CC, eluting with MeOH/MeCN/H_2O solutions to obtain six fractions. The first, purified by RP-8 HPLC, eluting with MeOH/MeCN/H_2O (1:1:8), furnished pure **25** (10.0 mg). The second fraction was purified by preparative TLC, eluting with the organic lower phase of $CHCl_3$/MeOH/H_2O (13:7:3) biphasic solution to give pure compound **37** (19.6 mg). The third fraction was purified by RP-8 HPLC using MeOH/MeCN/0.1% TFA (1:2:8) solution as mobile phase, to obtain metabolite **43** (3.0 mg); the fourth fraction was resolved by TLC, eluting with the organic lower phase of $CHCl_3$/MeOH/H_2O (13:7:2) biphasic solution to obtain pure **35** (3.0 mg), **36** (1.0 mg), **41** (3.5 mg) and **46** (2.0 mg). The fifth fraction was chromatographed by NH_2 HPLC, using (as mobile phase) a MeCN/H_2O solution (9:1), to obtain compounds **42** (2.3 mg), **44** (7.3 mg), and **45** (6.0 mg). Finally, the sixth fraction contained pure metabolite **40** (9.4 mg).

Fraction B, eluted with MeOH/H_2O (1:4), was chromatographed by RP-8 CC, eluting with MeOH/MeCN/H_2O solutions. The fraction eluted with MeOH/MeCN/H_2O (1:1:8) gave two fractions, identified respectively as the carotenoid glycoside **38** (15.3 mg) and its isomer **39** (8.0 mg). The fraction eluted with MeOH/MeCN/H_2O (5:1:1) afforded pure **47** (12.0 mg). The fraction eluted with MeOH/MeCN/H_2O (7:1:1) furnished pure **27** (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 **26** (7.0 mg), and 4-hydroxybenzylamine **34** (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 obtain metabolite **28** (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 obtain pure rutin **33** (23.1 mg).

The EtOAc organic fraction (7.4 g) of the ethanol extract was pooled with the Et_2O crude extract (15.0 g) and chromatographed by SiO_2 fcc to obtain two fractions E–F. Fraction E, eluted with CH_2Cl_2 and re-chromatographed by SiO_2 fcc, eluting with $CHCl_3$/MeOH (19:1), furnished three fractions: the first one was purified by SiO_2 CC, eluting with hexane/methylethylketone [MEK] (4:1), to give the pure ursane triterpenes **14** (312.1 mg), and **18** (66.1 mg); the second mixture was resolved by SiO_2 HPLC [hexane/MEK (41:9)] to obtain pure compounds **16** (43.2 mg), and **17** (4.5 mg), while the third fraction was purified by preparative TLC, eluting with hexane/MEK (3:2) to obtain the oleanane triterpene **9** (31.5 mg). Fraction F, eluted with CH_2Cl_2/MeOH (1:1), was re-chromatographed by SiO_2 fcc, eluting with the organic lower phase of $CHCl_3$/MeOH/H_2O (13:7:5) to give a fraction which, purified by RP-8 HPLC-UV [MeOH/H_2O (1:149)], yielding to chlorogenic acids **28** (62.0 mg), **29** (6.0 mg), **30** (35.2 mg), and **31** (10.1 mg).

The PE crude extract (13.0 g) was chromatographed on SiO_2 by aspiration under vacuum to obtain three fractions G–I.

Fraction G, eluted with pure CH_2Cl_2, was chromatographed on NH_2 silica, yielding three fractions. The first one, eluted with EtOAc/PE (1:19), furnished pure **4** (2.8 mg), **5** (31.0 mg) and **12** (30.7 mg) and a mixture which was chromatographed by RP-8 HPLC [MeOH/MeCN (4:1)], to give pure metabolite **24** (3.6 mg). The fraction eluted with EtOAc/PE (1:9), was purified by NH_2 CC, to give pure compound **15** (53.0 mg). The third fraction, eluted with EtOAc/PE (1:4), furnished a mixture which was chromatographed on SiO_2 by preparative TLC, eluting with PE/$CHCl_3$/EtOAc (6:3:1) to give two spots. 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), while the second consisted in the acyl benzaldehydes **7a–c** (4.0 mg).

Fraction H, eluted with CH_2Cl_2/acetone [Me_2CO] (1:1), chromatographed by SiO_2 fcc, eluting with hexane/$CHCl_3$/Me_2CO (13:6:1), furnished four fractions. The first one 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 under 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 was purified by RP-8 CC to give pure **13** (30.1 mg), and mixtures A and B: mixture A was chromatographed by RP-8 CC [MeOH/MeCN (4:1)], to give pure metabolite **21** (61.7 mg); mixture B was purified by SiO_2 fcc [hexane/EtOAc (19:3)] to yield compound **20** (92.0 mg). The fourth fraction, chromatographed by RP-8 CC, furnished two fractions; the first was purified by RP-8 CC [MeOH/MeCN/H_2O (6:3:1)] to obtain pure **8** (119.8 mg), **11** (9.0 mg) and **32** (191.0 mg), while the second fraction was chromatographed by RP-8 HPLC, eluting with MeOH/MeCN/H_2O (7:2:1), to furnish metabolite **19** (4.9 mg).

Fraction I, eluted with CH_2Cl_2/MeOH (1:1), and chromatographed by SiO_2 fcc, furnished two fractions. The first, eluted with CH_2Cl_2/MeOH (19:1), was purified by preparative TLC [hexane/$CHCl_3$/MeOH (4:5:1)] to obtain compound **10** (7.8 mg), while the second fraction, eluted with CH_2Cl_2/MeOH (9:1), was chromatographed by preparative TLC [hexane/$CHCl_3$/MeOH (5:13:2)] to furnish metabolites **22** (21.0 mg) and **23** (26.0 mg).

2.4. Chemical characterisation

2.4.1. 3β-18-Hydroxylinoleoyl-uvaol (**12**)

$[\alpha]_D$ +23.9 (c = 0.82, $CHCl_3$); EIMS m/z 720 $[M]^+$; Anal. Calcd for $C_{48}H_{80}O_4$: C, 79.94; H, 11.18. Found: C, 80.01; H, 11.25. ^{1}H NMR (300 MHz, $CDCl_3$): see Table 1. ^{13}C NMR (75 MHz, $CDCl_3$): see Table 2.

2.4.2. 3β-Linoleoyl-ursolic acid (**15**)

$[\alpha]_D$ +30.6 (c = 0.15, $CHCl_3$); EIMS m/z 718 $[M]^+$; Anal. Calcd for $C_{48}H_{78}O_4$: C, 80.17; H, 10.93. Found: C, 80.22; H, 11.05; ^{1}H NMR (300 MHz, $CDCl_3$): see Table 1. ^{13}C NMR (75 MHz, $CDCl_3$): see Table 2.

Table 1
Selected ^{1}H NMR data of the new compounds **12**, **15** and **24** in $CDCl_3$ d = doublet, dd = doublet of doublet, m = multiplet, ov = overlapped, s = singlet, ob = obscured,; the J values are reported in the brackets.

Position	12	15	24
3	4.49 dd (8.1, 5.7)	4.49 dd (8.4, 5.5)	4.59 m
6	–		5.36 ov
12	5.12 t (3.6)	5.22 t (3.3)	
18		2.17 d (11.4)	0.68 s
19			1.01 s
21			0.95 d (6.6)
23	0.76 s	0.76 s	
24	0.98 s	0.85 s	
25	0.96 s	0.95 s	
26	0.84 s	0.84 s	0.88 ov
27	1.09 s	1.07 s	0.85 ob
28	3.52 d (10.8) 3.17 d (10.8)	–	5.74 d (18.0, 11.7)
29	0.83 d (6.7)	0.87 d (6.7)	5.27 dd (11.7, 1.5) 5.15 dd (18.0, 1.5)
30	0.90 d (6.6)	0.93 d (6.6)	
2′	2.29 t (7.5)	2.28 t (7.2)	2.35 t (7.2)
9′	5.34 ov	5.33 ov	5.36 ov
10′	5.34 ov	5.33 ov	5.36 ov
11′	2.76 t (5.7)	2.76 t (5.4)	2.00 m
12′	5.34 ov	5.33 ov	
13′	5.34 ov	5.33 ov	
18′	3.63 t (6.6)	0.88 ov	0.88 ov

2.4.3. 3β-Oleoyl-24-hydroxy-24-ethylcholesta-5,28(29)-diene (**24**)

$[\alpha]_D$ −25.9 (c = 0.19, $CHCl_3$); EIMS m/z 692 $[M]^+$; Anal. Calcd for $C_{47}H_{80}O_3$: C, 81.44; H, 11.63. Found: C, 81.52; H, 11.57. ^{1}H NMR (300 MHz, $CDCl_3$): see Table 1. ^{13}C NMR (75 MHz, $CDCl_3$): see Table 2.

2.4.4. Tiglic acid 1-O-β-D-glucopyranoside (**35**)

$[\alpha]_D$ +15.9 (c = 0.62, MeOH); ESIMS m/z 285 $[M+Na]^+$, 123 $[M-C_6H_5O_{10}+Na]^+$; Anal. Calcd for $C_{11}H_{18}O_7$: C, 50.38; H, 6.92. Found: C, 50.51; H, 6.77. ^{1}H NMR (300 MHz, CD_3OD): δ 7.00 (1H, dq, J = 6.9 and 1.5 Hz, H-3), 5.52 (1H, d, J = 7.2 Hz, H-1′), 3.83 (1H, dd, J = 12.0 and 1.8 Hz, H-6′a), 3.66 (1H, dd, J = 12.0 and 4.5 Hz, H-6′b), 3.50–3.33 (4H, ov, H-2′–H-5′), 1.84 (3H, d, J = 1.5 Hz, H-5), 1.82 (3H, d, J = 6.9 Hz, H-4). ^{13}C NMR (75 MHz, CD_3OD): δ 168.0 (C-1), 140.4 (C-3), 129.1 (C-2), 95.9 (C-1′), 78.8 (C-3′), 78.1 (C-5′), 74.0 (C-2′), 71.1 (C-4′), 62.3 (C-6′), 14.5 (C-4), 12.0 (C-5).

2.4.5. 6,9-Dihydroxymegastigmasta-5,7-dien-3-one 9-O-β-D-gentiobioside (**46**)

UV (EtOH) λ_{max} (log ε) 286.0 (2.66), $[\alpha]_D$ +10.5 (c = 0.15, MeOH); ESIMS: m/z 571 $[M+Na]^+$, 409 $[M-C_6H_5O_{10}+Na]^+$, 247 $[M-C_6H_5O_{10}-C_6H_5O_{10}+Na]^+$; Anal. Calcd for $C_{25}H_{40}O_{13}$: C, 54.74; H, 7.35. Found: C, 54.61; H, 7.47. ^{1}H NMR (300 MHz, CD_3OD): δ 5.84 (3H, ov, H-4, H-7, H-8), 4.24 (1H, d, J = 7.8. Hz, H-1′), 4.20 (1H, d, J = 7.6 Hz, H-1″), 4.13 dd (1H, dd, J = 11.9 and 2.1 Hz, H-6′), 3.81 (1H, dd, J = 11.9 and 5.3 Hz, H-6′), 2.52 (1H, d, J = 16.8 Hz, H-2), 2.17 (1H, d, J = 16.8 Hz, H-2), 1.92 (3H, d, J = 1.5 Hz, H-13), 1.30 and 1.29 (6H, s, H-11 and H-12), 1.03 (3H, d, J = 6.3 Hz, H-10). ^{13}C NMR (75 MHz, CD_3OD): δ 199.2 (C-3), 166.2 (C-5), 136.0 (C-8), 130.0 (C-4), 125.0 (C-7), 104.8 (C-1″), 102.2 (C-1′), 79.1 (C-9), 79.0 (C-6), 78.0 (C-3′, C-3″), 77.9 (C-5′, C-5″), 75.1 (C-2′, C-2″), 71.6 (C-4″), 71.4 (C-4′), 69.7 (C-6′), 62.7 (C-6″), 49.5 (C-2), 42.1 (C-1), 23.7 (C-12), 22.6 (C-13), 21.0 (C-10), 19.2 (C-11).

2.5. 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 stirring the solution 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. Organic upper phase (1 μl) was analysed by GC–MS, fitted with 30 m × 0.25 mm i.d., 0.2 μm Zebron ZB5MS, fused silica capillary column (Phenomenex, Torrance, CA, USA). The column oven temperature was held at 80 °C for 1 min, 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 EIMS spectra and by comparing their retention times with those of the standard fatty acid methyl esters (Supelco 37 Component FAME Mix).

2.6. DPPH radical-scavenging capacity

The DPPH radical-scavenging capacity of metabolites was measured according to the method of Brand-Williams, Cuvelier, and Berset (1995). DPPH· (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 a compound were added to 1.46 ml of DPPH· solution at room temperature. The absorbance at 517 nm was measured in a cuvette at 30 min vs blank (38 μl MeOH in 1.46 ml of DPPH· 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).

Table 2
^{13}C NMR data of the new compounds **12**, **15** and **24** in $CDCl_3$.

Position	12	15	24	Position	12	15	24
1	38.6	38.5	37.2	25	16.0	15.7	39.9
2	28.2	27.4	32.1	26	17.6	17.0	16.8
3	80.8	80.8	73.9	27	23.5	23.8	17.9
4	38.2	39.7	42.5	28	70.1	184.3	149.2
5	55.4	55.5	139.9	29	16.9	17.2	116.6
6	18.4	18.4	122.8	30	21.6	21.4	-
7	32.9	35.0	29.9	1′	173.9	173.9	173.6
8	40.2	39.7	32.1	2′	35.1	33.9	32.1
9	47.8	48.2	50.2	3′	22.9	24.9	25.3
10	37.0	37.9	36.4	4′	29.3–29.9	30.4–29.5	29.7–29.1
11	23.6	24.3	21.2	5′	29.3–29.9	30.4–29.5	29.7–29.1
12	125.1	125.9	39.9	6′	29.3–29.9	30.4–29.5	29.7–29.1
13	139.0	138.2	42.6	7′	29.3–29.9	30.4–29.5	29.7–29.1
14	42.2	42.1	56.9	8′	28.2	27.7	27.4
15	26.0	29.3	24.5	9′	130.1	130.2	130.2
16	35.4	25.4	28.5	10′	128.1	128.1	130.0
17	37.9	47.7	56.1	11′	25.4	25.8	27.4
18	54.2	52.7	12.1	12′	128.3	128.3	29.7–29.1
19	39.5	39.2	19.6	13′	130.2	130.4	29.7–29.1
20	39.6	39.0	36.1	14′	28.2	27.7	29.7–29.1
21	30.0	31.7	19.1	15′	29.3–29.9	30.4–29.5	29.7–29.1
22	30.8	37.1	35.0	16′	25.4	30.4–29.5	32.1
23	28.3	28.3	36.8	17′	33.0	22.8	22.9
24	17.0	17.3	89.3	18′	63.3	14.3	14.4

2.7. 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, Buchs, Switzerland) in the riboflavin–light–NBT system (Dasgupta & De, 2004). Each 3 ml reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine (Fluka, Buchs, Switzerland), 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 illumination from a fluorescent lamp. The analysis was carried out in triplicate and the results were expressed in terms of the percentage of radical-scavenging capacity (RCS).

2.8. Evaluation of total antioxidant capacity

Spectrophotometric evaluation of antioxidant capacity through the formation of a phosphomolybdenum complex was carried out according to Prieto, Pineda, and Aguilar (1999). Sample solutions (100 μl) containing reducing metabolites (0.4 μmol in 1 ml of dimethylsulphoxide) were combined in an Eppendorf tube with 1 ml of reagent solution (0.6 M sulphuric 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 were cooled to room temperature, the absorbance of 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).

2.9. Cell line and culture conditions

Murine melanoma B16-F1 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Freshly trypsinised cells were seeded and grown in RPMI-1640 medium supplemented with 10% (v/v) faetal bovine serum (FBS), 1% L-glutamine (v/v), 100 units/ml of penicillin and 100 μg/ml of streptomycin. The cells were grown at 37 °C in a humidified 5% CO_2 atmosphere.

2.10. MTT assay

To evaluate the effect of isolated metabolites on B16-F1 cells, the MTT colorimetric assay was performed as described by Mosmann (1983). The test is based upon the selective ability of living cells to reduce the yellow salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] to a purple-blue colour in soluble formazan precipitate. MTT was dissolved in phosphate buffer saline (PBS) at 5 mg/ml. Experiments were performed in 100 μl of media in sterile 96-well plates. After a 24 h incubation of B16-F1 cells at an initial density of 1 × 103 cells/well, medium was removed and replaced by 10% FCS medium containing test compounds (0–500 μM). After 24 h of incubation, stock MTT (Sigma–Aldrich) solution was added and plates were incubated at 37 °C in 5% CO_2 for 4 h. Blue crystals of MTT reduced by cells were dissolved with DMSO and cellular metabolism was determined by measuring the absorbance of samples at 570 nm in a microelisa reader.

3. Results and discussion

3.1. Chemical characterisation of phytochemicals

Structures of phytochemicals isolated from *C. vulgaris* extracts are shown in Figs. 1–4. The separation scheme of the secondary metabolites from the organic extracts of *C. vulgaris* is shown in Fig. 5. All of the structures were elucidated on the basis of 1D and 2D NMR experiments and by EI or ESI mass spectrometry. Compounds **1a–f** (Fig. 1) were identified as acyl derivatives of the *trans* isomer of the *p*-coumarylic alcohol. Compounds **2a–c** were identified as acyl derivatives of the *cis* isomer of the *p*-coumarylic alcohol. The structures of the fatty acid esters of *cis*-and *trans*-*p*-coumaryl alcohol were elucidated by GC–MS and ^{1}H and ^{13}C NMR spectroscopy after purification of the individual compounds by HPLC (Fiorentino et al., 2008). Compounds **3a**, **3b** and **4** were fatty acid esters characterised by the presence of a coniferyl alcohol moiety. In particular, metabolites **3a** and **b** were identified as acyl derivatives of the *E* isomer of the coniferyl alcohol, and compound **4** as the acyl derivative of its *Z* isomer. Compound **5**, identified for the first time in *C. vulgaris*, was characterised as *E*-

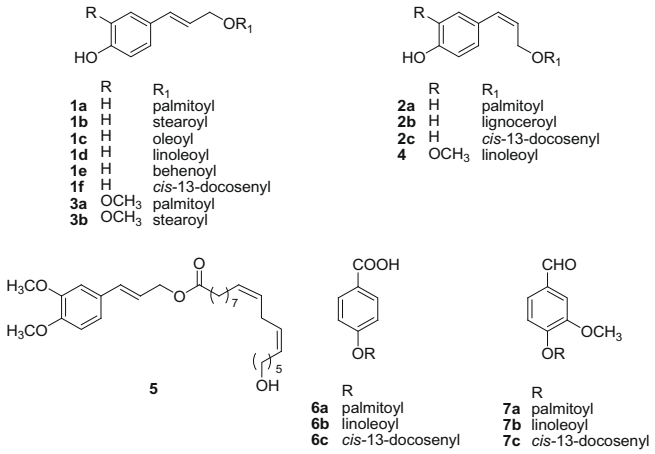


Fig. 1. Chemical structures of metabolites **1**–**7** from *C. vulgaris* peels.

3,4-dimethoxycinnamyl ω-hydroxylinoleate. Compounds **6a**–**c** were identified as 4-hydroxybenzoic acids, esterified through the 4-hydroxyl group to palmitic, linoleic and *cis*-13-docosenoic acids, respectively. Compounds **7a**–**c** were identified as 3-methoxy-4-hydroxy aldehydes, esterified by the hydroxyl group with the same fatty acid moiety as the previous compounds.

Compounds **8**–**10** (Fig. 2) were characterised as oleanane triterpenes: compound **8** was identified as oleananic acid (Kamiya, Yoshioka, Saiki, Ikuta, & Satake, 1997), while compounds **9** and **10** were identified as hederagenic acid (Bhandari & Rastogi, 1984) and its 2α-hydroxy derivative (Kojima & Ogura, 1988). The triterpenes **11**–**19** (Fig. 2) were characterised by the ursane skeleton. Compound **11** was identified as uvaol (Hota & Bapuji, 1994), and the new compound, **12**, as its acyl derivative. Its molecular formula was $C_{48}H_{80}O_4$, as suggested by the elemental analysis and the EIMS spectrum. The methyl signals in the ^1H and ^{13}C NMR spectra (Tables 1 and 2) were in good accordance with those of compound **11**, but some differences were evident. Besides, the H-12 proton at δ 5.12, in the ^1H NMR spectrum, further overlapped four olefinic protons, as a multiplet at δ 5.34, was present. In the same experiment, a methine at δ 4.49, and two methylenes as a triplet at δ 3.63 and as two doublets at δ 3.52 and 3.17 were also evident. In the upfield of the spectrum, seven methyls were detectable as five singlets at δ 1.09, 0.98, 0.96, 0.86 and two doublets at δ 0.90 and 0.83, besides signals due to doubly allylic protons at δ 2.76, a methylene at δ 2.29, methylene signals of the acyl chain in the range between 2.1 and 1.1 ppm, and a methylene triplet at δ 3.63. The downshifted H-3 proton at δ 4.49 indicated the linkage with an acyl group. The ^{13}C NMR and DEPT spectra were in good agreement with the presence of 48 carbons identified as 7 methyls, 23 methylenes, 11 methines and 7 tetrasubstituted carbons. These data led us to hypothesise the presence of the uvaol esterified with an unsaturated fatty acid. However, the absence of a methyl carbon, besides those of the triterpene, and the presence of the methylene at δ 63.3, suggested the presence of an ω-hydroxy fatty acid. The correlations in the HMBC experiment between the carbon at δ 173.9 (C-1′) with the protons at δ 4.49 (H-3), 2.29 (H-2′), and 1.62 (H-3′) confirmed this hypothesis. The acyl moiety was elucidated by GC–MS analysis of the methyl ester derivative of the fatty acid, obtained by reaction with KOH in MeOH, which showed a molecular peak at *m*/*z* 310. These data and the pattern of fragmentation, indicated the presence of the methyl ester of the ω-hydroxylinoleic acid, which was definitively confirmed by the 2D NMR data. Compounds **13** and **14** were identified as ursanaldehyde (Hota & Bapuji, 1994) and ursolic acid (Seo, Tomita, & Tori, 1975), respectively. The new compound **15** was identified as the 3-linoleoyl derivative of **14**. Its 1D NMR spectra (Tables 1 and 2) showed signals of the ursolic acid and signals attributable to a polyunsaturated fatty acid moiety. The downfield shift of the H-3 proton at δ 4.49 suggested the acylation point at the 3β-hydroxyl group of the triterpene. The acyl chain was confirmed by GC–MS analysis of the methyl derivatives obtained after methanolysis. Compounds **16**–**18** were characterised as 2α-hydroxy ursolic acid (Yamagishi et al., 1988), pomolic acid (Dong-Liang & Xiao-Ping, 1992) and

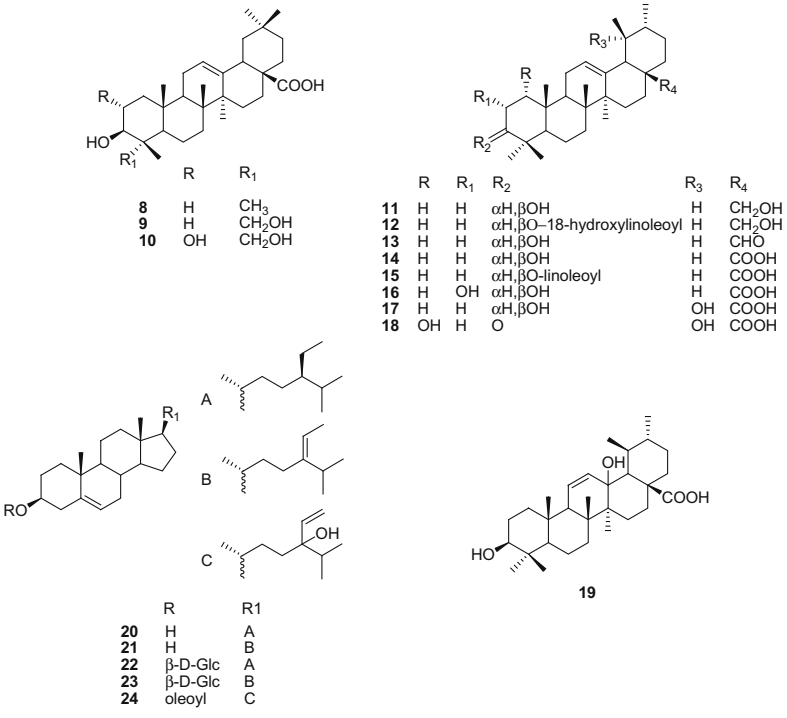


Fig. 2. Chemical structures of metabolites **8**–**24** from *C. vulgaris* peels.

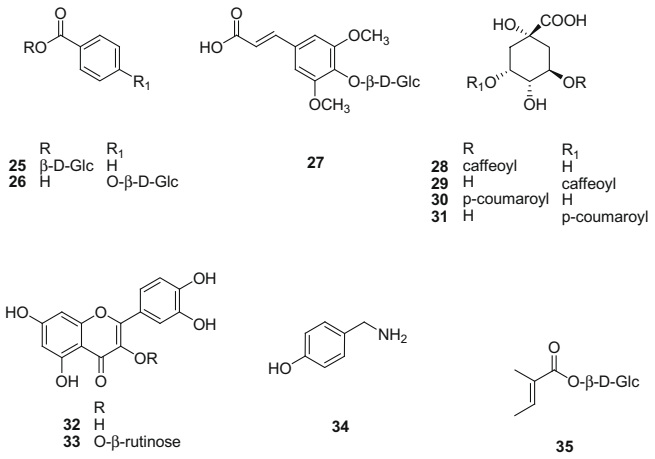


Fig. 3. Chemical structures of metabolites **25**–**35** from *C. vulgaris* peels.

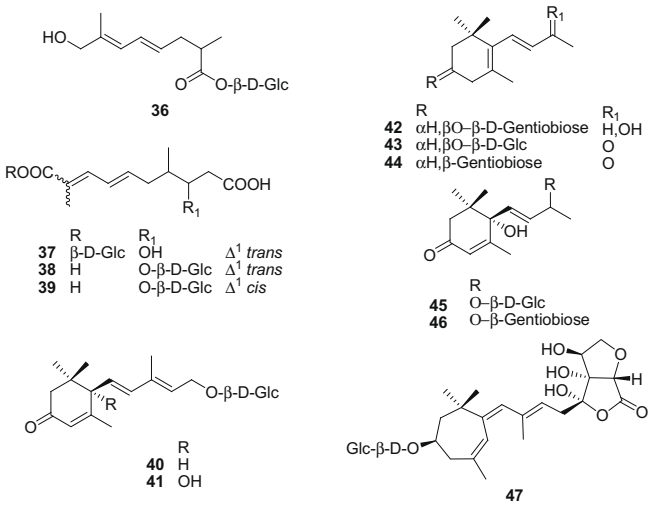
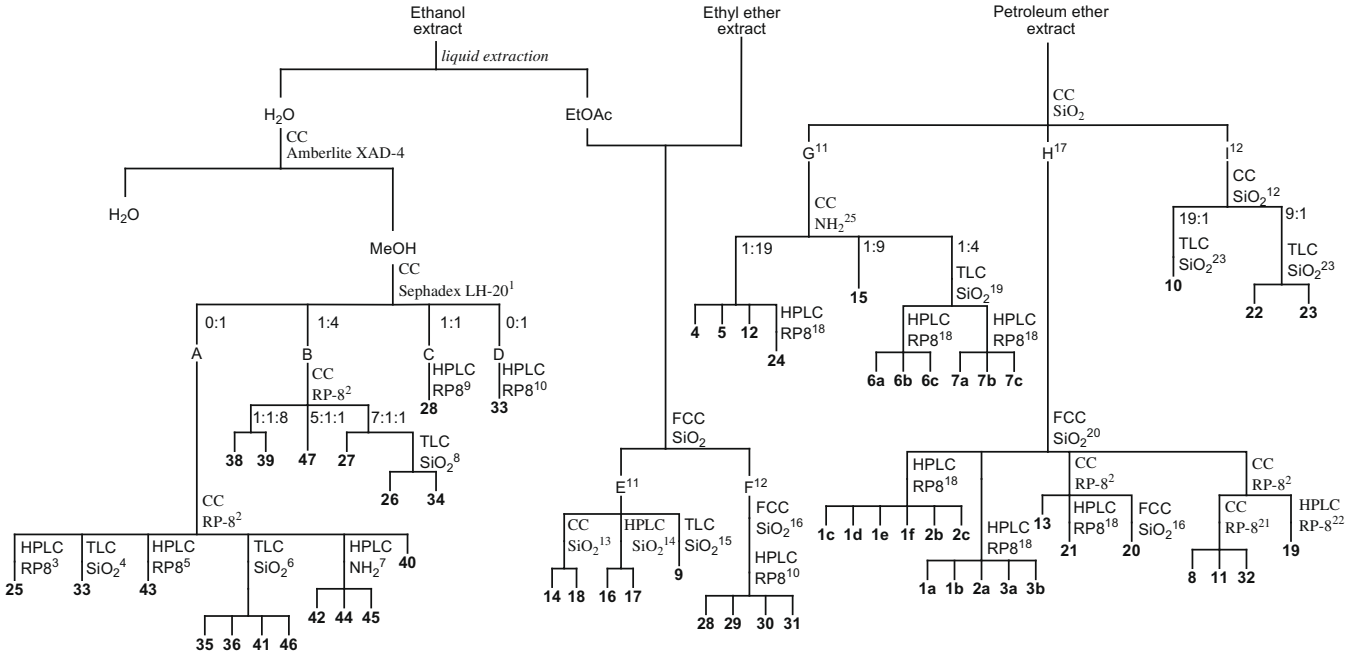


Fig. 4. Chemical structures of metabolites **36**–**47** from *C. vulgaris* peels.

annurcoic acid, respectively (D'Abrosca et al., 2006). Until now, this latter compound has been reported solely as a constituent of Annurca apple fruits. Finally, compound **19** was identified as 3β-13β-dihydroxyurs-11-en-28-oic acid (Huang, Sun, & Zhao, 1996).

All the steroids of *C. vulgaris* had a stigmastane skeleton (Fig. 2). Compounds **20** and **21** were identified as β-sitosterol (Wright et al., 1978) and fucosterol (Atta-ur-Rahman et al., 1999), while the glycosides **22** and **23** were characterised as 3-*O*-β-D-glucopyranoside derivatives (De Rosa, De Giulio, & Tommonaro, 1997; Tsai, Hsieh, Duh, & Chen, 1999). Compound **24** was characterised for the first time. It showed the molecular formula $C_{47}H_{80}O_3$ according to the elemental analysis and the EIMS spectrum showing the molecular peak at *m*/*z* 692. In the upfield region of the ^{1}H NMR spectrum (Table 1) methyl signals of a steroid were evident as two singlets at δ 0.68 and 1.01, and three methyl signals at δ 0.95, 0.87 and 0.85. In the downfield part of the spectrum, there were a multiplet at δ 4.59, and six olefin protons, three of which were present as an ABM spin system as three double doublets at δ 5.74, 5.27 and 5.15, suggesting the presence of a vinyl group, while the remaining protons were overlapped at δ 5.36. In the ^{13}C NMR (Table 2), there were a carboxyl carbon at δ 173.6, six olefinic carbons, confirming the presence of three double bonds, and two carbinol carbons at δ 73.9 and 89.3, besides other carbons ranging from 56.9 to 14.4 ppm. The DEPT experiment allowed us to identify all the carbons as 6 methyls, 25 methylenes, 11 methines and 5 quaternary carbons. The 2D NMR data allowed the complete assignment of all the protons and carbons in the molecule. In particular the tetrasubstituted carbinol at δ 89.3 correlated, in the HMBC experiment, with the vinyl protons, and with both the H-26 and H-27 methyls. These data suggested the presence of a hydroxyl group at the C-24 carbon and the double bond among the C-28 and C-29 carbons. ^{1}H and ^{13}C NMR values, relative to the steroidal skeleton, were in good accordance with the presence of a double bond at the C-5 carbon and an acyl group at the 3-hydroxyl, that was identified as oleic acid after reaction with KOH in MeOH and GC–MS analysis. The 2D NMR experiments confirmed our hypothesis.

Phenols **25**–**26** (Fig. 3) were identified as glucosides of benzoic acid, already identified from *Pinus contorta* needles (Higuchi & Donnelly, 1977) and from *Picea glauca* (Kraus & Spiteller, 1997); compound **27** was identified as synapyl acid 4-*O*-β-D-glucopyranoside (Beejmohun, Grand, Mesnard, Fliniaux, & Kovensky, 2004). Compounds **28**–**31** were characterised as chlorogenic acids, already reported as components of Hemerocallis (Clifford, Wu, & Kuhnert, 2006), while the flavones **32** and **33** were identified as quercetin and rutin, respectively (D'Abrosca et al., 2001). Compound **34** was identified as 4-hydroxybenzylamine (Lu, Rodriguez, Gu, & Silverman, 2003). Compound **35** was identified as the glucosyl derivative of tiglic acid. It showed a molecular formula $C_{11}H_{18}O_7$, as suggested by the ^{13}C NMR and ESIMS experiments. The ^{1}H NMR spectrum showed an olefinic quartet at δ 7.00, and two methyls as a singlet at δ 1.84 and a doublet at δ 1.82, a doublet of a sugar unity at δ 5.52, and a diastereotopic methylene as two double doublets at δ 3.83 and 3.66, besides four other protons geminal to oxygen in the range 3.50–3.33 ppm. The ^{13}C NMR spectrum revealed the presence of glucose, showing six carbinols at δ 95.9 (C-1′), 78.8, 78.1, 74.0, 71.1 and 62.3. The coupling constant of the anomeric proton was in good agreement with a β configuration for the C-1′ carbon. Furthermore, in the ^{13}C NMR spectrum, there were an ester carboxyl carbon at δ 168.0, two olefin signals as a methine at δ 140.4 and a tetrasubstituted carbon at δ 129.1 and two methyls at δ 14.5 and 12.0. The ^{13}C chemical shift values indicated the presence of a tiglic acid moiety in the molecule. Finally, the correlation between the carboxyl carbon and the anomeric proton confirmed the linkage of the glucose to the C-1 carbon of the aglycone trough an ester bond. Compounds **36**–**46** (Fig. 4) have been identified as carotenoid derivatives. Compound **36** was identified as (4*E*,6*E*)-8-hydroxy-2,7-dimethylocta-4,6-dienoic acid 1-*O*-β-D-glucopyranoside. This compound is reported as a precursor of isomeric marmelo lactones from Quince fruit (Winterhalter, Lutz, & Schreier, 1991). Carotenoids **37**–**40** (Fiorentino et al., 2006), **41** (Luts & Winterhalter, 1993), **42** (Winterhalter, Harmsen, & Trani, 1991) and **44** (Guldner & Winterhalter, 1991) were already reported as constituents of *C. vulgaris*, while compound **45** has been reported as a leaf constituent of *Alangium premnifolium* (Otsuka, Yao, Kamada, & Takeda, 1995). The new C_{13} norterpene glycoside, 46, had a molecular formula $C_{25}H_{40}O_{13}$ in accordance with ESIMS and ^{13}C NMR spectra. In the ^{1}H NMR, there were three overlapped protons at δ 5.84, two doublets at δ 4.20 and 4.24, two diastereotopic methylene protons at δ 2.52 and 2.17, three singlet methyls at δ 1.92, 1.29 and 1.30, and a doublet methyl at δ 1.03, besides other protons ranging from 4.18 to 3.10 ppm. In the ^{13}C NMR spectrum, 25 signals were evident, identified by a DEPT experiment, as four methyls, three methylenes, fourteen methines, and four tetrasubstituted carbons. The values of the carbinolic carbon were in good accordance with the presence of a gentiobiose moiety in the molecule. The remaining two carbinols were attributed to the C-6 and C-9 carbons on the basis of heterocorrelations shown in the HMBC experiment. In fact, the methyl at δ 1.92 correlated with the olefin carbons at δ 130.0 and 166.2, and with the tertiary carbinol at δ 79.0. The methyl doublet correlated with the carbons



[1]MeOH-H_2O; [2]MeOH/MeCN/H_2O; [3]MeOH/MeCN/H_2O (1:1:8); [4]$CHCl_3$/MeOH/H_2O (13:7:3); [5]MeOH/MeCN/0.1% TFA (1:2:8); [6]$CHCl_3$/MeOH/H_2O (13:7:2); [7]MeCN/H_2O (9:1); [8]$CHCl_3$/MeOH/EtOH/H_2O (10:7:5:5); [9]MeOH/H_2O (1:149); [10]MeOH/MeCN/H_2O (1:2:14); [11]CH_2Cl_2; [12]CH_2Cl_2/MeOH (1:1); [13]hexane/MEK (4:1); [14]hexane/MEK (41:9); [15]hexane/MEK (41:9); [16]$CHCl_3$/MeOH/H_2O (13:7:3); [17]CH_2Cl_2/Me_2CO (1:1); [18]MeOH/MeCN (4:1); [19]PE/CH_2Cl_2/etOAc (6:3:1); [20]hexane/$CHCl_3$/Me_2CO (13:6:1); [21]MeOH/MeCN/H_2O (6:3:1); [22]MeOH/MeCN/H_2O (7:2:1); [23]hexane/$CHCl_3$/Me_2CO (4:5:1); [24]hexane/$CHCl_3$/Me_2CO (5:13:2); [25]EtOAc/PE;

Fig. 5. Isolation scheme of the secondary metabolites from *C. vulgaris* extracts.

at δ 136.0 and 79.1. This latter showed a cross peak with the anomeric proton of the gentiobiose at δ 4.24. Furthermore, the carbonil carbon at δ 199.2, attributed to the C-3 carbon, correlated with the methylenes at δ 2.52 and 2.17 and with the proton at δ 5.84. These data allowed us to assign the structure of 6,9-dihydroxy-megastigmast-4,7-dien-3-one 9-*O*-β-D-gentiobioside. Finally, compound **47** was identified as cydonioside A (Fiorentino et al., 2007).

3.2. Antioxidant and radical-scavenging capacities

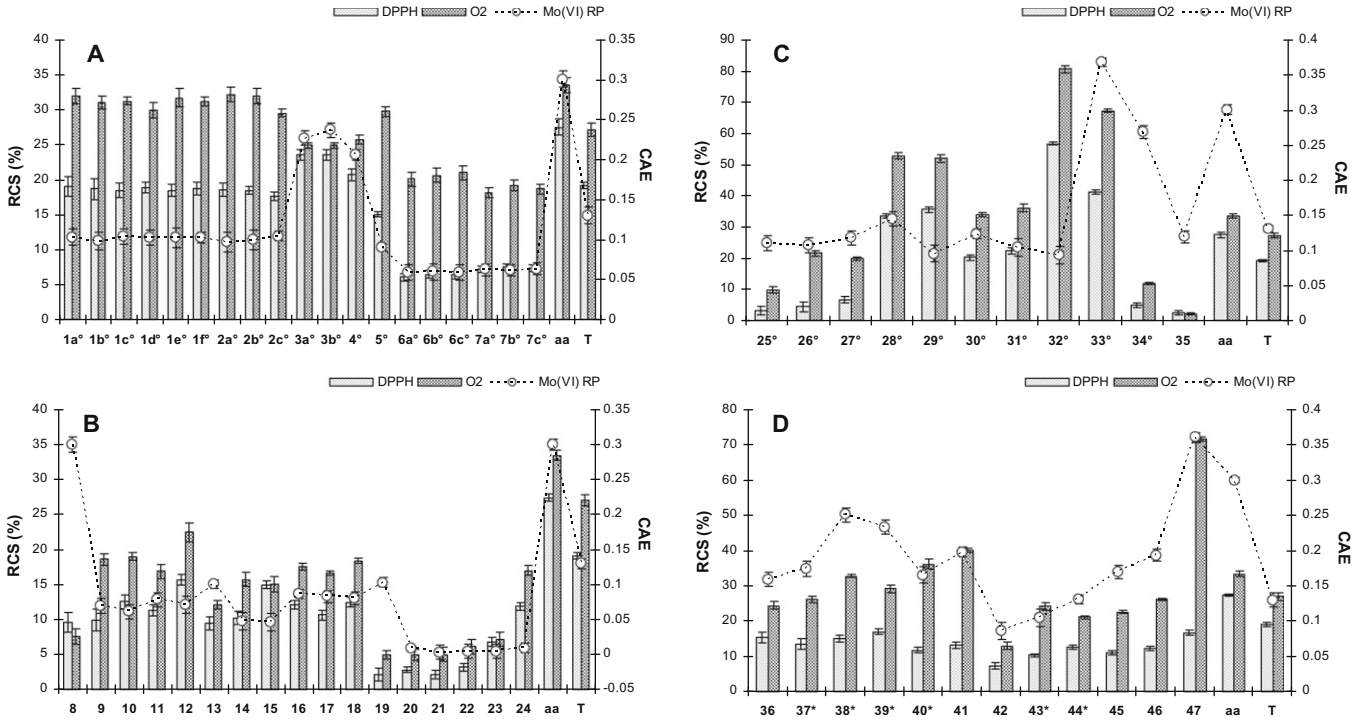
All the isolated metabolites were tested for their antioxidant capacity, using three different methods. Two of these methods estimate the radical-scavenging activities of the investigated substances against the DPPH radical and the anion superoxide radical; the remaining test evaluates the capacity to induce the Mo(VI) reduction. The standards used in all the methods were α-tocopherol and ascorbic acid, known natural antioxidants, and the results are shown in Fig. 6.

When radical-scavenging capacities were tested, the strongest antioxidant capacity was observed for the flavonol quercetin (**32**) and its 3-*O*-rutinoside (**33**). In particular, quercetin reduced the DPPH radical by 56.7% and the anion superoxide radical by 80.4%. The quinic acid derivatives, chlorogenic acid (**28**) and neochlorogenic acid (**29**) determined an average DPPH radical reduction of 35.0% and were able to scavenge O_2^- by 52.8% and 51.8%, respectively. Among triterpenes, compounds **12** and **15** were the most active substances. In particular, uvaol ω-hydroxylinoleate (**12**) was able to reduce the DPPH radical absorbance by 15.7% and the anion superoxide radical by 22.5%. The uvaol acyl derivative showed a higher activity than did uvaol (**11**). Carotenoid derivative metabolites appeared to be strong radical-scavenger substances, exhibiting activities comparable to that of the positive standards. All the other compounds showed weak inhibiting capacities. Spectrophotometric quantisation of the antioxidant capacity of tested metabolites, through the formation of a phosphomolybdenum complex, showed that, in the complex, the substances were feebly able to induce the reduction of Mo(VI) to Mo(V). Quercetin and rutin exhibit a massive reducing capacity, greater than that exercised by α-tocopherol and ascorbic acid. Good antioxidant activity was observed for the phenylpropanoid esters **3a**, **3b** and **4**: they are able to induce the reduction of Mo(VI) to Mo(V), much more so than α-tocopherol.

3.3. Antiproliferative activity

The most abundant compounds present in the Quince peels, were subjected to the MTT bioassay on murine B16-F1 melanoma cells. Besides the isoprenoid and carotenoid metabolites, the main polyphenols isolated from *C. vulgaris* (Fiorentino et al., 2008) have also been tested. Besides the flavonoids quercetin (**32**) and rutin (**33**), four caffeoyl and *p*-coumaroyl derivatives of quinic acid (**28**–**31**), as well as the ester dimethoxycoumaryl alcohol (**5**) and the sinapic acid glucoside (**27**), were subjected to the bioassay. The results of the bioassays are listed in Table 3.

A significant antiproliferative activity was recorded for the triterpenoids. In particular, the most active metabolite was ursolic acid, **14**, with an IC_{50} of 10.2 μM. Compounds **8**, **9**, **11**, **13**, **18**, **19** and **21** showed very good activities toward B16-F1 cells in the range 32.7–80.5 μM. The presence of the acyl chain in the triterpenoid derivatives, **12** and **15**, determined a lower activity than those of the related **11** and **14**, respectively. Furthermore, the highest IC_{50}, recorded for the ursane triterpene, **16**, suggested that the introduction of a 2α-hydroxyl function was able to reduce the ursane skeleton antiproliferative ability. Interestingly, annurcoic acid showed an IC_{50} value of 77.6 μM. This compound has been reported in the cv Annurca apple alone, and the presence in the *C. vulgaris* peels led us to suppose its occurrence also in other apple cultivars (Rosaceae).



°Data according to Fiorentino *et al*., 2006; *Data according to Fiorentino *et al*., 2008.

Fig. 6. Radical-scavenging and antioxidant capacities of phenolic esters **1**–**7** (A), triterpenes **8**–**19** and steroids **20**–**24** (B), polyphenols **25**–**34** (C) and carotenoid derivatives **36**–**47** (D) isolated from *C. vulgaris*. (A) Values are presented as percentage differences from control ±SD. AA = ascorbic acid; T = α-tocopherol. CAE = caffeic acid equivalent.

Among polyphenolic compounds, quercetin (**32**) and rutin (**33**) were found to exhibit strong effects on B16-F1 cells with IC_{50} values of 85.1 and 92.5 μM, respectively.

Antiproliferative activity of the triterpenoids has been extensively reported in the literature. He and Liu (2007) demonstrated the high antiproliferative capacity of 13 triterpenoids isolated from apple peels against human HepG2 liver cancer cells, MCF-7 breast cancer cells, and Caco-2 colon cancer cells. They suggested that these phytochemicals, characterised by ursane or oleane skeletons, may be partially responsible for the anticancer activities of whole apples. Zuco et al. (2002) showed that betulinic acid exerted a selective cytotoxicity on nine neoplastic cell lines, including melanomas, small- and non-small cell lung carcinomas, ovarian and cervical carcinomas in a very narrow range of doses (1.5–4.5 μg/ml).

It has been suggested that phytosterols possess antitumour properties in both animals and humans. Rats (Raicht, Cohen, Fazzini, Sarwal, & Takahashi, 1980) administered methylnitrosourea, a direct acting carcinogen, produced a significantly higher incidence of tumours after 28 weeks compared to rats administered the same compound and fed a diet containing 0.2% β-sitosterol.

Carotenoids have been implicated as important dietary phytonutrients having cancer preventive activity (Van Poppel, 1993). Little is known about carotenoid derivatives' antiproliferative capacity. The metabolite **38**, a C-12 carotenoid saccharide derivative, with a diene α,ω-diacid skeleton, already isolated from the plant Oryctanthus, has been identified as an inhibitor of the vascular endothelial growth factor (VEGF) receptor (Hedge et al., 2005).

There is increasing interest in plant-derived dietary polyphenols, because several representatives of these compounds proved to be proliferation inhibitors and apoptosis inducers in tumour cells. Quercetin, has been shown to exhibit anti-carcinogenic properties by inducing cell cycle arrest and apoptosis, and inhibiting proliferation in diverse cancer cell lines as single compounds (Choi et al., 2001; Richter, Ebermann, & Marian, 1999; Yoshida, Yamamoto, & Nikaido, 1992).

Table 3
Growth inhibition (IC_{50}) of phytochemicals against murine B16-F1 melanoma cells.

Compounds	IC_{50} (μM)	Compounds	IC_{50} (μM)	Compounds	IC_{50} (μM)
5	NE	**20**	330	**37**	NE
8	61.6	**21**	45.3	**38**	NE
9	32.7	**27**	NE	**39**	233
11	80.5	**28**	404	**40**	NE
12	479	**29**	NE	**42**	NE
13	50.0	**30**	197	**43**	NE
14	10.2	**31**	470	**44**	NE
15	NE	**32**	85.1		
16	355	**33**	92.6		
18	75.0	**35**	484		
19	77.6	**36**	NE		

NE = no effect.

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