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Free-Radical-Scavenging and Antioxidant Activities of Secondary Metabolites from Reddened Cv. Annurca Apple Fruits

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Forty-three secondary metabolites were isolated and characterized from cv. Annurca apple fruit, an apple variety cultivated in the south of Italy. This apple cultivar undergoes a typical reddening treatment after collection. All of the compounds were characterized on the basis of their spectroscopic data. The compounds were tested for their radical-scavenging and antioxidant activities by measuring their capacity to scavenge DPPH• (2,2′-diphenyl-1-picrylhydrazyl radical), H_2O_2, and NO (nitric oxide) and to inhibit the formation of methyl linoleate conjugated diene hydroperoxides or TBARS (thiobarbituric acid reactive species).

KEYWORDS: ***Malus domestica*** **cv. Annurca; secondary metabolites; triterpenes; phenolic fatty acid esters; polyphenols; free-radical-scavenging activity; antioxidant activity.**

INTRODUCTION

Free radicals are species with incomplete electron shells that make them more chemically reactive than those with complete electron shells. They are byproducts of metabolic processes. In cells, in fact, oxidation processes use oxygen to produce energy for biochemical reactions. During these reactions, the free radicals produced react with biomolecules such as proteins, lipids, and DNA, causing irreversible damages to the cells. Exposure to various environmental factors, including nitrogen dioxide and ozone in polluted air, heavy metals, halogenated hydrocarbons, ionizing radiation, and cigarette smoke, can increase free-radical formation.

Over time, such damage can become irreversible, and recent studies have demonstrated the involvement of free radicals in the pathology of human diseases such as atherosclerosis, cardiovascular diseases, and diabetes (*1*−*3*). Protective therapeutic intervention might include natural or synthetic pharmacologic agents with antioxidant activity. Antioxidants are often described as free-radical scavengers, meaning that they neutralize the electrical charge and prevent the free radicals from taking electrons from other molecules. Antioxidants are abundant in fruits and vegetables, as well as in other foods including nuts; grains; and some meats, poultry, and fish (*4*).

Among fruits, apples have historically shown medicinal properties. In particular, antioxidant activity is attributed to catechin and other flavonoids present in this fruit.

Annurca apple is a variety grown in the southern regions of Italy known for its high quality (*5*). The fruit is medium to small with a flat shape. The skin is thick, initially yellowish-green and becoming striped brilliant red blush when it ripens. The flesh is quite firm and strong, it has an average juiciness, it is sweet but slightly acidic, and it has an average amount of aromas and good flavor characteristics (*6*, *7*).

The unripe fruits are collected unreddened in the autumn, placed for about a month on a layer of straw or sawdust on the soil, and sprayed daily with water. When the sun-exposed surface of the fruit becomes red, the fruits are turned to redden the opposite side (*8*).

In the framework of the regional research center for the agro-alimentary productions of Campania, we have studied the chemical characteristics of the Annurca apple fruit. From the reddened peel, we isolated 12 phenolic fatty acids and several ursane-type triterpenes (*9*−*11*). The present research reports the complete characterization of the organic extracts of the fruits and their free-radical-scavenging and antioxidant capacities. The use of different methods is necessary in antioxidant activity assessment. The present work uses the combination of five different methods based on radicals (DPPH, H_2O_2, NO) and no radical species (MeLo and TBARS). The bioactivities were tested for all of the isolated phytochemicals.

MATERIAL AND METHODS

Fruit Collection. Annurca apple fruits were collected in Sant'Agata de' Goti, near Caserta (Italy) in October 2003 when the fruit had just been harvested (green peel), reddened on straw until November, and then stored in a climatic cell at 0 °C and 98% dampness.

General Experiment Procedures. Fourier transform NMR spectra were recorded at 300 MHz for ^{1}H and 75 MHz for ^{13}C in $CDCl_3$ or

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CD_3OD at 25 °C on a Varian Mercury 300 spectrometer. 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 obtained on a Perkin-Elmer Lambda 7 spectrophotometer in $CHCl_3$ or EtOH solution. CD spectra were obtained in MeOH solution on a Jasco J-715 spectrophotometer polarimeter. Optical rotations were measured on a Perkin-Elmer 343 polarimeter. Electronic impact mass spectra (EI-MS) were obtained with an HP 6890 spectrometer equipped with an MS 5973 N detector. The preparative 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 an RP-18 (Luna 10 μm, 250 × 10 mm i.d., Phenomenex) column. The analytical HPLC apparatus consisted of a pump (Beckman 127 System Gold), a UV−vis detector (Beckman 166), and a Shimadzu Chromatopac C-R6A recorder. HPLC was performed using RP-18 (Luna 5 μm, 250 × 4.6 mm i.d., Phenomenex) and RP-8 (Luna 5 μm, 250 × 4.6 mm i.d., Phenomenex) columns. Analytical TLC was performed on Merck Kieselgel 60 F_{254} or RP-18 F_{254} plates with a 0.2-mm layer thickness. Spots were visualized by illumination with 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 a 0.5- or 1-mm film thickness. Flash column chromatography (FCC) was performed on Merck Kieselgel 60 columns (230−400 mesh) at medium pressure. Column chromatography (CC) was performed on Merck Kieselgel 60 (70−240 mesh), Amberlite XAD-4 (Fluka), Amberlite XAD-7 (Fluka), or Sephadex LH-20 (Pharmacia) media.

Extraction and Isolation. The reddened Annurca apple fruit (5.21 kg) was sliced, frozen in liquid nitrogen, powdered in a mortar, and infused first in ethanol for 7 days and then in ethyl ether for 7 days. After removal of the solvents, we obtained crude alcoholic (1.5 g) and organic (4.2 g) extracts.

Organic Extract Fractionation. The ethyl ether extract was chromatographed on silica gel, eluting with chloroform and ethyl acetate solutions to obtain six fractions, denoted A−F.

Fraction A was chromatographed on SiO_2 by flash chromatography, and the fraction eluted with hexane/Me_2CO (9:1) was purified by HPLC with RP-18 plates eluting with MeOH/MeCN (4:1) to give pure compounds **3** (1.2 mg), **4** (1.5 mg), **5** (1.5 mg), **7** (1.7 mg), **10** (2.0 mg), **11** (1.2 mg), and **12** (1.5 mg). Fraction B was chromatographed on SiO_2 column by flash chromatography eluting with Me_2CO in hexane solution. The fraction eluted with Me_2CO/hexane (19:1) gave a fraction that, after purification by HPLC with RP-18 plates [MeOH/MeCN (4:1)], gave pure compounds **1** (1.3 mg), **2** (5.6 mg), **6** (1.4 mg), **8** (1.3 mg), and **9** (1.3 mg). Fraction C was chromatographed by CC on silica gel, eluting with Et_2O/petroleum ether (1:4), to obtain a fraction that, after purification by HPLC [SiO_2, MeCOEt/hexane (2:23)], gave compounds **13** (8.0 mg), **14** (7.0 mg), **15** (2.0 mg), **17** (3.0 mg), **19** (5.0 mg), and **23** (3.0 mg). Fraction D was chromatographed by FCC with Me_2CO/hexane (1:17) to give two fractions: the first was purified by HPLC with an RP-8 column, eluting with MeOH/MeCN/H_2O (7:2:1), to give compound **22** (13.0 mg); the second fraction was purified by HPLC with an RP-18 plate, eluting with MeOH/MeCN (4:1), to give compound **18** (15.0 mg). Fraction E was chromatographed on RP-18 silica, eluting with MeOH/MeCN/H_2O (11:4:5), to give a fraction that was purified by SiO_2 HPLC eluting with MeCOEt/hexane (9:41) to give compounds **26** (2.2 mg) and **27** (1.9 mg). Fraction F was chromatographed on RP-18 silica eluting with MeOH/MeCN/H_2O (5:4:1) to obtain pure ursolic acid **20** (610.0 mg) and two fractions. The first was purified by HPLC [SiO_2, MeCOEt/hexane (1:4)] to give oleanoic acid **16** (2.0 mg), pomolic acid **21** (6.0 mg), and annurcoic acid **24** (4.0 mg); the second was purified by SiO_2 HPLC eluting with MeCOEt/hexane (1:4) to give compound **25** (1.7 mg).

The ethanol extract was fractionated by liquid−liquid extraction. The aqueous fraction obtained was chromatographed on Amberlite XAD-4 and XAD-7 eluting first with H_2O and then with MeOH. The organic fraction was chromatographed on Sephadex LH-20 eluting with hydroalcholic solutions. Two of the obtained fractions were chromatographed by HPLC: the first eluting with MeOH/MeCN/H_2O (1:4:15) gave catechin **28** (2.0 mg), epicatechin **29** (5.0 mg), quercetin 3-*O*-β-D-glucopyranoside **30** (2.0 mg), rutin **31** (2.0 mg), phloridzin **32** (4.0 mg), phloretin-2′-xyloglucoside **33** (4.0 mg), chlorogenic acid **34** (3.0 mg), and triandrin **35** (1.0 mg); the second furnished some low-molecular-weight phenols identified as protocatechuic acid **36** (3.0 mg), cathecol **37** (6.0 mg), tyrosol **38** (3.0 mg), gentisic acid **39** (29.0 mg), 3,4-dimethoxyphenol **40** (2.2 mg), vanillic acid **41** (5.0 mg), benzoic acid **42** (7.0 mg), and salycilic acid **43** (3.0 mg).

Analytical HPLC Analyses of Annurca Samples. Samples of about 3 g each of the whole core-free Annurca apples were cut into small pieces, frozen in liquid nitrogen, powdered in a mortar, and lyophilized using an FTS System Flex-Dry instrument. The powders obtained were extracted with a Soxhlet apparatus for 4 h with $CHCl_3$. The solution was dried using an evaporator to obtain the single extracts, which were analyzed by UV HPLC. The residue was re-extracted with a Soxhlet apparatus for 4 h with EtOH.

One hundred milligrams of the EtOH extract was dissolved in 1 mL of MeOH and analyzed by UV−vis reverse-phase HPLC. HPLC analyses of phenols were performed on a Beckman Gold 127 liquid chromatograph. A reverse-phase Phenomenex Luna C-18 column (250 × 4.6 mm i.d., 5 μm) and a security guard column (4.0 × 2.0 mm i.d.) were used. The confirmation of the analysis was performed using a reverse-phase Phenomenex Luna C-8 column (250 × 4.6 mm i.d., 5 μm). The separation of phenols **36**−**43** was conducted using as the mobile phase a mixture of sol A (AcOH/H_2O, 1:99) and sol B (AcOH/MeOH, 1:99) at a gradient of A/B from 9:1 to 2:3 in 80 min; the flow rate was 0.7 mL min^{-1}, and detection was at 258 nm. The retention times (RT, min) of the compounds were as follows: 18.85 (**36**), 21.63 (**37**), 29.20 (**38**), 31.35 (**39**), 39.91 (**40**), 36.12 (**41**), 54.82 (**42**), 58.82 (**43**).

The HPLC analyses of compounds **28**−**34** were performed using as the mobile phase a mixture of sol A (H_2O) and sol B (MeCN/MeOH, 4:1) at a gradient of A/B from 49:1 to 7:3 in 20 min, then isocratic mode for 10 min, and finally 100% B for 5 min. The flow rate was 0.7 mL min^{-1}, and detection was at 260 nm. Retention times (min) of the compounds were as follows: 25.00 (**28**), 27.34 (**29**), 30.52 (**30**), 31.50 (**31**), 33.43 (**32**), 37.66 (**33**), 40.37 (**34**).

DPPH Radical-Scavenging Activity. The scavenging activities of the metabolites were measured according to the method of Brand-Williams et al. (*12*). The method is based on the reduction of methanolic DPPH• in the presence of a hydrogen-donating antioxidant.

DPPH• (Fluka) solution showed an absorption band at 515 nm and was intensely violet colored. The adsorption and color intensity decreased when DPPH• was reduced by an antioxidant compound. The remaining DPPH• corresponded inversely to the radical-scavenging activity of the antioxidant (*13*). DPPH• (2 mg) was dissolved in 54 mL of MeOH. The investigated metabolites were prepared by dissolving 0.1 mg of each compound in 1 mL of MeOH. Then, 38 μL of each solution containing compound was added to 1.462 mL of DPPH• solution at room temperature (*14*). The absorbance at 515 nm was measured in a cuvette at 5 and 30 min vs blank (38 μL of MeOH in 1.462 mL of DPPH• solution) using a UV-1601 Shimadzu spectrophotometer. The results are expressed in terms of the percentage reduction of the initial DPPH• adsorption by the test compounds

$$\text{percentage reduction of the initial DPPH}^{\bullet}\text{ adsorption} = \frac{A_{\text{DPPH}}(t) - A_{\text{sample}}(t)}{A_{\text{DPPH}}(t)} \times 100$$

where $A_{DPPH}(t)$ is the absorbance of DPPH• at time t and $A_{sample}(t)$ is the absorbance of the sample at the same time t.

H_2O_2 Scavenging Activity. The hydrogen peroxide scavenging activity was examined by the method of Pick and Keisari, modified by Bahorun at al. (*15*). In this case, 100 μL of water solution of isolated compounds (0.1 mg/mL) was added to 100 μL of 0.002% hydrogen peroxide. Then, 700 μL of 0.1 M phosphate buffer (pH 7.4) and 100 μL of 0.1 M sodium chloride were added. The reaction mixture was incubated for 20 min at 37 °C. Then, 1 mL of 0.2 mg/mL phenol red (Riedel) dye with 0.1 mg/mL horseradish peroxidase (Fluka) in 0.1 M phosphate buffer was added. After 15 min, 100 μL of 1 M NaOH was added, and the absorbance was measured at 610 nm using a UV-1601

Shimadzu spectrophotometer. The results are expressed in terms of the percentage reduction of H_2O_2 adsorption by the test compounds.

$$\text{percentage reduction of } H_2O_2 \text{ adsorption} = \frac{A_{H_2O_2} - A_{sample}(t)}{A_{H_2O_2}} \times 100$$

NO Scavenging Activity. NO generated from SNP (sodium nitroprusside, Fluka) was measured by the Griess reagent (*16*). For these experiments, 100 μL of water solution of the isolated metabolites (0.1 mg/mL) was added to 0.2 mL of 10 mM SNP and 1.8 mL of phosphate buffer, pH 7.4. The reaction mixture was incubated at 37 °C for 3 h. Then, 1.0 mL of the incubation mixture was removed and diluted with 1 mL of Griess reagent (1% sulfanilamide and 0.1% naphthyletylendiamine dihydrochloride in 5% H_3PO_4). The absorbance of the chromophore formed during the diazotination of nitrite with sulfanilamide and subsequent coupling with naphthylethylene diamine was observed at 540 nm and referred to the absorbance of a standard solution of sodium nitrite treated in the same way with Griess reagent.

Inhibition of Autoxidation of Methyl Linoleate. The oxidation of methyl linoleate (Sigma-Aldrich) results in conjugated diene (CD) hydroperoxides. The CD products absorb UV light in the wavelength range of 230−235 nm and can be determined spectrophotometrically by their maximum absorbance at 234 nm. An increase in the absorbance at 234 nm began almost immediately when methyl linoleate was kept at 60 °C. The increase was rapid initially until a maximum was obtained after 48 h. The antioxidant activity of each isolated compound was measured by its inhibition of methyl linoleate autoxidation in bulk phase (*17*). Methyl linoleate (0.1 mmol) containing the investigated metabolites or α-tocopherol, used as a reference (0.025 μmol; 0.025 mol % based on methyl linoleate), was placed in a test tube (1.5 cm in diameter) and incubated at 60 °C in the dark. After 48 h of incubation, each sample (1 μL) was withdrawn and dissolved in 1.0 mL of ethanol. The formation of hydroperoxides was monitored by measuring the formation of conjugated diene hydroperoxides spectrophotometrically at 234 nm. The amount of conjugated diene hydroperoxides was calculated using a molar absorptivity of 26000 (*18*).

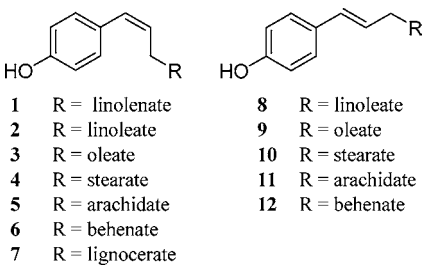
The antioxidant activity is expressed in terms of the percentage formation of methyl linoleate conjugated diene hydroperoxides (100%) after 48 h of oxidation (*19*).

Determination of TBARS. End products of polyunsaturated fatty acid oxygenation react with thiobarbituric acid (TBA, Fluka) to form a red adduct. Determination of thiobarbituric acid reactive substances (TBARS) was measured by the method of Sroka and Cisowski (*14*). TBA reagent was prepared as follows: For reagent A, 375 mg of TBA and 30 mg of tannic acid (Riedel) were dissolved in 30 mL of hot water; for reagent B, 15 g of thrichloracetic acid (Riedel) was dissolved in 70 mL of 0.3 M hydrogen chloride aqueous solution. Then, 30 mL of reagent A was mixed with 70 mL of reagent B. Next, 5.2 μL rapeseed oil were emulsified with 15.6 mg of Tween-40 (Fluka) initially dissolved in 2 mL of 0.2 M Tris-HCl buffer, pH 7.4 M. The emulsion was irradiated with UV light at 254 nm at 25 °C for 60 min. Then, 100 μL of water solution of test compounds (0.1 mg/mL) was added to 1 mL of the reaction mixture. The samples were irradiated with UV radiation for 30 min again. After addition of 2 mL of TBA reagent, all test tubes were placed into a boiling water bath for 15 min and then centrifuged using a Beckman GS-15R centrifuge for 3 min at 1500 *g*, and the supernatant was measured at 532 nm. Inhibition of lipid peroxidation was measured as a percentage vs blank containing no test compounds.

Data Analysis. The statistical significance of differences among the groups was determined by a Student's *t*-test, calculating mean values for growth inhibition. The level of significance was set at $P < 0.05$.

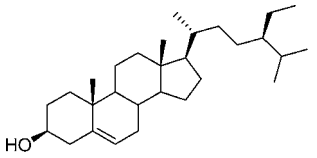
RESULTS AND DISCUSSION

The reddened whole fruits of cv. Annurca apples were sliced, frozen in liquid nitrogen, powdered in a mortar, and infused first in ethanol for 7 days and then in ethyl ether for 7 days. After removal of the solvents, crude organic extracts were obtained that were separated by chromatographic techniques (CC, TLC, HPLC) into their constituents. The pure compounds were characterized by EI-MS and by spectroscopic analysis using, in particular, 1D and 2D NMR experiments. Twelve *Z*- and *E*-*p*-coumaril fatty acid esters **1**−**12** (**Figure 1**), β-sitosterol **13** and 7β-hydroxystigmast-4-en-3-one **14** (**Figure 2**), two lupane triterpenes **15** and **16**, two oleanic triterpenes **17** and **18** (**Figure 3**), and nine ursane triterpenes **19**−**27** (**Figure 4**) were isolated from the ethyl ether extract and identified.

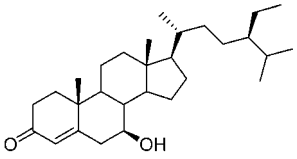


1 R = linolenate
2 R = linoleate
3 R = oleate
4 R = stearate
5 R = arachidate
6 R = behenate
7 R = lignocerate
8 R = linoleate
9 R = oleate
10 R = stearate
11 R = arachidate
12 R = behenate

Figure 1. Structures of the coumaryl fatty acid esters **1**–**12** from reddened Annurca apple.

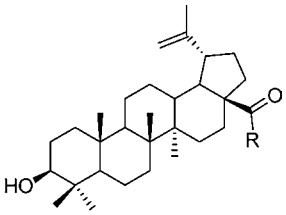


13 *β-sitosterol*

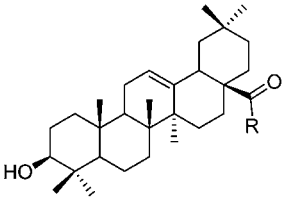


14 *7β-hydroxystigmast-4-en-3-one*

Figure 2. Structures of the steroids **13** and **14** from reddened Annurca apple.



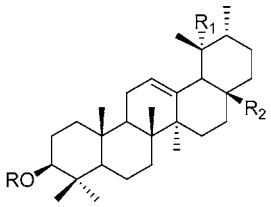
15 *betulinic aldehyde* R = H
16 *betulinic acid* R = OH



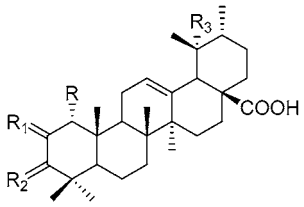
17 *oleanic aldehyde* R = H
18 *oleanic acid* R = OH

Figure 3. Structures of the lupane (**15**–**16**) and oleane (**17**–**18**) triterpenes from reddened Annurca apple.

The structures of the 12 fatty acid esters of *Z*- and *E*-*p*-coumaryl alcohol **1**−**12** were elucidated by GC-MS and ^1H and ^{13}C NMR spectroscopy after purification of the individual compounds by HPLC (*9*). HPLC analysis allowed the esters to be localized in the fruit peel. During reddening of the fruit, there was a substantial increase in the amount of esters and particu-



		R	R_1	R_2
19	*ursanaldehyde*	H	H	CHO
20	*ursolic acid*	H	H	COOH
21	*pomolic acid*	H	OH	COOH
22	*uvaol*	H	H	CH_2OH
23	*uvaol acetate*	Ac	H	CH_2OH

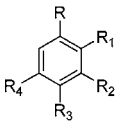


		R	R_1	R_2	R_3
24	*annurcoic acid*	OH	H,H	O	OH
25	*annurconic acid*	OH	H,H	O	H
26	*2-oxopomolic acid*	H	O	βOH,αH	OH
27	*3-epi-2-oxopomolic acid*	H	O	αOH,βH	OH

Figure 4. Structures of the ursane triterpenes **19**–**27** from reddened Annurca apple.

larly in molecular species with unsaturated fatty acids. The *β*-sitosterol **13** was found in large quantities in this apple. Aside from 7-*β*-hydroxystigmast-4-en-3-one **14**, no other sterols were found in the Et_2O extract. Lupane and oleane triterpenes were present as aldehydes or acids at the C-28 position. Also, ursane triterpenes are characterized by the oxidation of the C-28 carbon. Compounds **19** and **20** were identified as ursolaldehyde (*20*) and ursolic acid (*21*), respectively. Compound **21** was characterized as pomolic acid (*22*), and compound **22** as uvaol, already reported by Siddiqui et al. (*23*) as a constituent of *Nerium oleander*. Compound **23** was identified as the acetyl derivative of compound **22**. Compound **26** was identified as 2-oxo-pomolic acid, an unusual triterpene already isolated from infected *Malus pumila* (*24*). Compounds **24**, **25**, and **27** were isolated and identified for the first time from the cv. Annurca apples (*10*, *11*).

Polyphenols from the ethanolic extract of cv. Annurca apple were identified, by NMR data, as catechin **28** (*25*), epicatechin **29** (*26*), quercetin 3-*O*-*β*-D-glucopyranoside **30** (*27*), rutin **31** (*27*), phloridzin **32** (*28*), phloridzin-6′-xiloside **33** (*29*), chlorogenic acid **34** (*30*), and triandrin **35** (*31*) (**Figure 5**). Compound **28** showed an $[\alpha]_D$ value (c, 0.16) of − 17.5 and a positive Cotton effect of $\Delta\epsilon_{280nm}$ +3.2 nm, in accordance with the presence of (−)-catechin (*32*). Low-molecular-weight phenols (**Figure 6**) were identified by ^1H and ^{13}C NMR spectroscopy



		R	R_1	R_2	R_3	R_4
36	*protocatechuic acid*	COOH	H	OH	OH	H
37	*cathecol*	OH	OH	H	H	H
38	*tyrosol*	CH_2CH_2OH	H	H	OH	H
39	*gentisic acid*	COOH	OH	H	H	OH
40	*3,4-dimethoxy phenol*	OH	H	OCH_3	OCH_3	H
41	*vanillic acid*	COOH	H	OCH_3	OH	H
42	*benzoic acid*	COOH	H	H	H	H
43	*salicilic acid*	COOH	OH	H	H	H

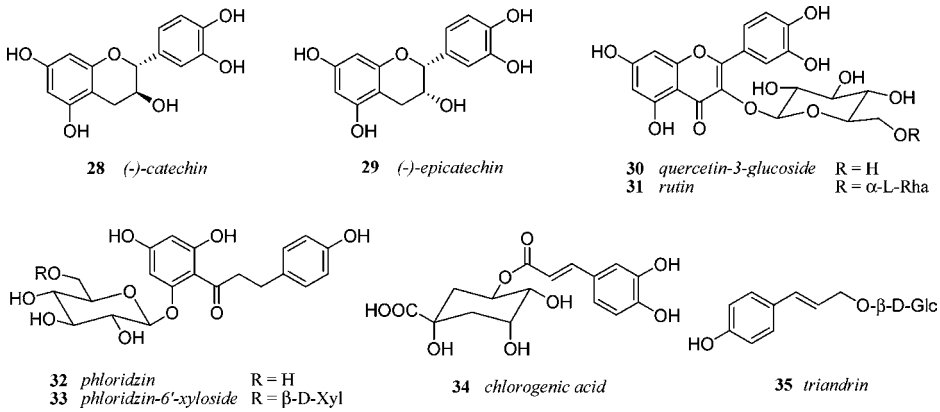
Figure 6. Structures of the low-molecular-weight phenols from reddened Annurca apple.

as protocatechuic acid **36**, cathecol **37**, tyrosol **38**, gentisic acid **39**, 3,4-dimethoxyphenol **40**, vanillic acid **41**, benzoic acid **42**, and salycilic acid **43**.

Analytical HPLC allowed the average abundance of each aromatic compound in the extracts to be estimated (**Figures 7** and **8**). The terpenes were quantized by a gravimetric method. Ursolic acid was the most abundant species; it was found at a level of 11.72 mg/100 g of fresh weight.

The metabolites isolated from the ethyl ether and ethanolic extracts were tested for their antioxidant activity. Evaluation of antioxidant activity was performed using five different methods. Three of these methods estimate the radical-scavenging activities of the investigated substances against the DPPH radical, the pro-oxidant hydrogen peroxide, and nitric oxide; the remaining tests evaluate the capacity to inhibit peroxidative processes by measuring the formation of methyl linoleate (MeLo) conjugated diene hydroperoxides and of TBARS substances. The standard used in all the methods was α-tocopherol, a known natural antioxidant, and the results are reported in **Table 1**.

All of the metabolites showed inhibiting activity against the autoxidation of methyl linoleate. The strongest activity was observed for the phenolic compounds. *p*-Coumaryl esters of fatty acids showed a different activity. Evaluation of the results allows us to state that the activity was influenced by chemical and stereochemical functions: the presence of the phenolic hydroxyl in the position para with respect to the side chain, the stereochemistry of the double bond of the alcoholic moiety, and the length and unsaturation of the fatty acids. Strong inhibition was observed for *E* isomers; particular activity was found for compounds **1**, **3**, and **6**, which inhibited methyl linoleate autoxidation by 80%. Moderate inhibiting activity was observed from triterpenoid with no ursanic skeleton, except for oleanic aldheyde **17**, which showed an antioxidative power of 96%. Instead, pomolic acid appeared as the strongest antioxidant compound among the ursanic triterpenes. It inhibits 75% of the



28 *(-)-catechin* **29** *(-)-epicatechin* **30** *quercetin-3-glucoside* R = H; **31** *rutin* R = α-L-Rha

32 *phloridzin* R = H; **33** *phloridzin-6′-xyloside* R = β-D-Xyl **34** *chlorogenic acid* **35** *triandrin*

Figure 5. Structures of the main polyphenols isolated from reddened Annurca apple.

Figure 7. Quantitative analysis of *p*-coumaryl fatty acid esters and isoprenoids from reddened Annurca apple (mg/100 g of fresh weight).

autoxidation of MeLo. All of the flavonoidic metabolites caused an acceptable inhibition of the formation of hydroperoxides. The strongest activities were observed for catechin **29** and its isomer **30**.

When DPPH radical scavenging was tested, the strongest activity was observed for (*E*)-*p*-coumaryl oleate **9**. It reduced the radical by 78% after 30 min of incubation. Also in this test, *E* isomers showed a greater activity. Among triterpenic metabolites, betulinic acid **16** and its corresponding aldheyde **15** showed fast inhibiting activities. In fact, they were able to reduce radical absorbance by 50% after 5 min. Whereas uvaol reduced DPPH radical by 56%, its acetylated derivative showed a lower activity (26%). Flavonoidic compounds appeared to be strong reducing substances. Catechin **29** reduced DPPH radical by 60% after 5 min. Low-molecular-weight phenols showed strong activities, exhibiting an average inhibition of 50%.

All of the isolated metabolites showed hydrogen peroxide scavenging activity. Among compounds **1**−**12**, metabolites **5**, **7**, and **8** showed the highest activities. They were able to reduce hydrogen peroxide by 76%, 73%, and 77% respectively. Mild scavenging activity, with the exception of oleanic aldheyde **17** and ursanes, was observed for the triterpenes; compound **14** reduced the pro-oxidant by only 5%. Among ursanic triterpenes, ursolic acid **20** had the strongest reducing activity (78%). Compound **22** showed a weaker activity than compound **21**.

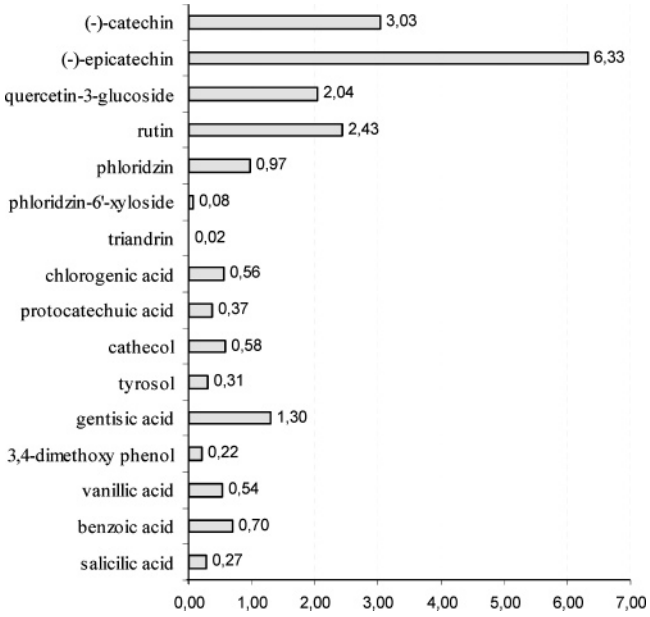


Figure 8. Quantitative analysis of polyphenols from reddened Annurca apples (mg/100 g of fresh weight).

These data suggest that the presence of an acetylic function establishes a reduction of scavenging activity. Flavonoidic compounds reduced hydrogen peroxide by 80%. Among these, glycoside **34** scavenged 86% of the pro-oxidant. These results seem to confirm that the radical-scavenging activity was influenced by the hydroxyl functions on the carbon skeleton.

NO generated from SNP in aqueous solution at physiological pH reacts with oxygen to form nitrite ions. The compounds that exhibit NO scavenging activity inhibit nitrite formation by competing with oxygen to react with NO. This leads to the reduction of the nitrite concentration in the assay medium. All of the compounds exhibited strong NO radical-scavenging activities. The activity seems to be correlated with the presence of a phenolic moiety. The mechanism of the antioxidant activity of phenols is widely known to involve their ability to act as free-radical scavengers, leading to the formation of phenoxyl radicals (*33*).

Malondialdehyde (MDA), an end product of the oxygenation of polyunsaturated fatty acids, is commonly used as a biomarker for assessing lipid peroxidation. MDA reacts with thiobarbituric acid to form a pink/red adduct. Isolated *p*-coumaryl esters of fatty acids showed inhibiting activity in the formation of reactive species to thiobarbituric acid.

Antioxidant activity was correlated with the structural properties of the tested molecules. Among metabolites **13**−**18**, notable activity was observed for oleanic aldehyde (87% inhibition) and its corresponding acid (80%). Ursanic triterpenes showed a strong antioxidant activity. Ursolic acid **20** had a strong inhibiting power.

All of the flavonoid metabolites exhibited activities comparable to that of the positive standard. It was evident that the antioxidant power was closely correlated with the structure of tested molecules. Regioisomer metabolites **18** and **20** showed distinct activities in all of the tests. The difference in antioxidative power might be linked to the differing substitution of methyls at C-19 and C-20 the on pentacyclic skeleton.

Few studies have been conduced on the antioxidant and radical-scavenging activities of phytosterols and terpenes (*34*, *35*). Schimke and co-workers described the antioxidant activity of the terpenoids from *Ginkgo biloba*, reporting that these

Table 1. Radical-Scavenging and Antioxidant Activities of the Secondary Metabolites of Cv. Annurca Apples[a]

	scavenging activity (%)					
	DPPH•					
	5 min	15 min	H_2O_2	NO	hydroperoxide CD formation (%)	TBARS determination (%)
1	17.9 ± 0.048[i]	25.6 ± 0.035[ii]	45.5 ± 0.063	78.1 ± 0.011[ii]	21.0 ± 0.011[ii]	78.9 ± 0.001[i]
2	20.5 ± 0.011[i]	26.9 ± 0.017[ii]	60.2 ± 0.272	80.4 ± 0.010[ii]	64.5 ± 0.011[ii]	45.7 ± 0.008[i]
3	19.5 ± 0.028[i]	27.6± 0.028[ii]	67.7 ± 0.063	65.1 ± 0.038[ii]	18.0 ± 0.011[i]	80.1 ± 0.011[i]
4	18.7 ± 0.051[i]	27.4± 0.017[ii]	67.8 ± 0.113	83.3 ± 0.017[ii]	94.8 ± 0.011[i]	87.4 ± 0.002[i]
5	29.9 ± 0.030[i]	38.9± 0.008[ii]	75.8 ± 0.058	83.1 ± 0.006	44.7 ± 0.011[i]	42.7 ± 0.013[i]
6	23.2 ± 0.008[i]	31.6 ± 0.015[ii]	68.0 ± 0.153	80.7 ± 0.003[i]	29.1 ± 0.011[i]	75.2 ± 0.033[i]
7	21.2 ± 0.004[i]	30.8 ± 0.015[ii]	73.1 ± 0.099	84.1 ± 0.006	61.8 ± 0.011[ii]	44.0 ± 0.010[i]
8	21.7 ± 0.003[i]	45.6 ± 0.008[ii]	77.2 ± 0.025	79.3 ± 0.010[ii]	57.0 ± 0.011[ii]	58.0 ± 0.001[i]
9	64.5 ± 0.031[i]	73.2 ± 0.011[ii]	37.5 ± 0.537	80.8 ± 0.001[i]	98.5 ± 0.011[ii]	74.4 ± 0.012[i]
10	20.3 ± 0.028[i]	30.7 ± 0.007[ii]	37.5 ± 0.114	72.8 ± 0.008[i]	29.5 ± 0.011[ii]	82.1 ± 0.024[i]
11	58.6 ± 0.051[i]	63.7 ± 0.018[ii]	65.3 ± 0.100	80.5 ± 0.008[ii]	73.7 ± 0.011[ii]	53.7 ± 0.001[i]
12	29.3 ± 0.025[i]	37.9± 0.007 [ii]	57.4 ± 0.152	54.5 ± 0.008[i]	78.5 ± 0.011[ii]	67.4 ± 0.009[i]
13	37.1 ± 0.007[i]	38.0 ± 0.004[ii]	43.4 ± 0.497	82.9 ± 0.003	64.5 ± 0.015[ii]	53.7 ± 0.001[i]
14	13.8 ± 0.021[i]	16.2 ± 0.013[ii]	5.13 ± 0.388	58.2 ± 0.004[i]	84.5 ± 0.047[ii]	52.8 ± 0.004[i]
15	52.7 ± 0.023[i]	55.4 ± 0.037[ii]	ND	ND	22.9 ± 0.027[i]	ND
16	56.5 ± 0.013[i]	58.4 ± 0.017[ii]	24.9 ± 0.470	76.1 ± 0.004[i]	33.8 ± 0.017[i]	63.1 ± 0.029[i]
17	38.6 ± 0.007[ii]	42.7 ± 0.041[ii]	66.6 ± 0.086	80.2 ± 0.001[i]	4.4 ± 0.021[i]	87.3 ± 0.001[i]
18	28.5 ± 0.006[ii]	32.2 ± 0.023[ii]	36.3 ± 0.581	71.5 ± 0.014[i]	84.3 ± 0.030[ii]	79.8 ± 0.009[i]
19	21.7 ± 0.017[ii]	27.0 ± 0.013[ii]	42.5 ± 0.368	81.4 ± 0.006[ii]	24.3 ± 0.024[ii]	45.5 ± 0.003[i]
20	46.2 ± 0.023[ii]	49.5 ± 0.028[ii]	78.4 ± 0.027	70.1 ± 0.010[i]	88.1 ± 0.008[ii]	91.9 ± 0.017[i]
21	30.8 ± 0.007[ii]	31.2 ± 0.013[ii]	67.5 ± 0.045	63.9 ± 0.011[i]	18.6 ± 0.018[ii]	58.5 ± 0.054[i]
22	50.1 ± 0.039[ii]	52.7 ± 0.024[ii]	34.6 ± 0.432	80.9 ± 0.001[i]	32.2 ± 0.020[ii]	77.5 ± 0.001[i]
23	17. 7 ± 0.013[ii]	22.7 ± 0.027[ii]	53.5 ± 0.254	59.8 ± 0.021[i]	32.7 ± 0.007[ii]	58.9 ± 0.024[i]
24	23.0 ± 0.006[ii]	26.3 ± 0.008[ii]	67.4 ± 0.044	81.6 ± 0.008	48.0 ± 0.017[ii]	53.9 ± 0.087[i]
25	27.8 ± 0.023[ii]	33.1 ± 0.018[ii]	64.0 ± 0.071	66.1 ± 0.013[i]	30.0 ± 0.032[ii]	67.5 ± 0.008[i]
26	27.5 ± 0.007[ii]	33.0 ± 0.062[ii]	66.1 ± 0.111	65.4 ± 0.003[i]	34.2± 0.009[ii]	88.7 ± 0.001[i]
27	26.3 ± 0.037[ii]	32.1 ± 0.006[ii]	74.2 ± 0.079	61.0 ± 0.016[i]	32.1 ± 0.021[ii]	76.8 ± 0.066[i]
28	51.8 ± 0.013[ii]	57.4 ± 0.017[ii]	45.5 ± 0.005	79.3 ± 0.001[i]	18.8 ± 0.017[ii]	44.0 ± 0.015[i]
29	60.4 ± 0.017[ii]	61.9 ± 0.006[ii]	86.7 ± 0.011	79.2 ± 0.007[i]	12.2 ± 0.031[ii]	55.8 ± 0.041[i]
30	51.8 ± 0.008[ii]	56.1 ± 0.007[ii]	86.2 ± 0.004	75.7 ± 0.001[i]	5.8 ± 0.027[ii]	48.4 ± 0.020[i]
31	48.6 ± 0.018[ii]	51.8 ± 0.008[ii]	87.5 ± 0.015	78.7 ± 0.007[i]	17.0 ± 0.013[ii]	57.7 ± 0.004[i]
32	50.9 ± 0.015[ii]	54.4 ± 0.007[ii]	85.1 ± 0.027	77.6 ± 0.008[i]	15.4 ± 0.013[ii]	62.7 ± 0.005[i]
33	45.3 ± 0.009[ii]	53.1 ± 0.006[ii]	74.1 ± 0.026	81.2 ± 0.001[i]	27.9 ± 0.013[ii]	45.6 ± 0.008[i]
34	51.2 ± 0.006[ii]	54.6 ± 0.006[ii]	87.9 ± 0.004	78.9 ± 0.008[i]	19.8 ± 0.010[i]	53.6 ± 0.009[i]
35	47.3 ± 0.008[ii]	64.6 ± 0.018[ii]	41.5 ± 0.006	77.5 ± 0.004[i]	47.2 ± 0.001[i]	65.3 ± 0.002[i]
36	61.2 ± 0.022[ii]	88.9 ± 0.011[ii]	57.8 ± 0.018	75.5 ± 0.003[i]	39.5 ± 0.055[i]	76.5 ± 0.002[i]
37	15.3 ± 0.042[ii]	24.5 ± 0.035[ii]	65.8 ± 0.059	75.8 ± 0.028[i]	53.4 ± 0.016[i]	58.0 ± 0.012[i]
38	57. 8 ± 0.012[ii]	71.8 ± 0.027[ii]	60.8 ± 0.017	79.4 ± 0.003[i]	27.1 ± 0.020[i]	81.6 ± 0.013[i]
39	46.0 ± 0.006[ii]	50.5 ± 0.002[ii]	53.5 ± 0.020	77.9 ± 0.003[i]	41.4 ± 0.001[i]	43.7 ± 0.055[i]
40	18.0 ± 0.016[ii]	27.1 ± 0.015[ii]	38.2 ± 0.030	78.8 ± 0.005[i]	38.0 ± 0.030[i]	67.5 ± 0.003[i]
41	29.8 ± 0.043[ii]	34.3 ± 0.126[ii]	61.7 ± 0.017	76.1 ± 0.016[i]	63.4 ± 0.001[i]	72.7 ± 0.005[i]
42	27.7 ± 0.002[ii]	35.7 ± 0.006[ii]	48.8 ± 0.026	42.4 ± 0.041[i]	32.8 ± 0.011[i]	67.9 ± 0.001[i]
43	46.1 ± 0.026[ii]	56.6 ± 0.030[ii]	72.5 ± 0.013	87.1 ± 0.025[i]	41.4 ± 0.017[i]	72.9 ± 0.004[i]
control	20.3 ± 0.006[ii]	23.7 ± 0.021[ii]	60.4 ± 0.038	83.6 ± 0.001[i]	26.6 ± 0.011[ii]	53.1 ± 0.002[i]

[a] Values are presented as percentage differences from control and are significantly different with $P > 0.05$ for Student's t-test: (i) $P > 0.01$, (ii) $0.01 < P < 0.05$

compounds exhibit remarkable solubility in hydrophobic regions such as lipids in which superoxides might be able to persist for a longer time because of the lack of protonation, the latter leading to the hydroperoxyl radical and subsequent dismutation with the formation of oxygen and hydrogen peroxide. Thus, the reaction of lipophilic compounds with superoxides should be considered in a lipophilic environment (*36*).

The overproduction of radical reactive species leads to lipidic peroxidation, harming the membranes of biological systems and causing serious pathologies such as cancer, senescence, and inflammation. The research into natural products as health-protecting factors against oxidative damage is an interesting field. Diverse studies have shown that natural products have a large range of biological activities such as antitumor and antiviral activities. Triterpenes have been reported as antitumor natural products, and recently pomolic acid was tested on the human leukemic cell line K562 (*37*). Ursolic acid isolated from apple peels was found to show growth-inhibiting activity against the four tumor cell lines HL-60, BGC, Bel-7402, and HeLa. Structural modifications were performed on the C-3, C-28, and C-11 positions of ursolic acid, and the cytotoxicity of the derivatives was evaluated. The SAR revealed that triterpenes containing two hydrogen-bond-forming groups (a H donor and a carbonyl group) at positions 3 and 28 exhibit cytotoxic activity. The configuration at C-3 was found to be important for the activity (*38*).

Phytochemicals, including phenolics and flavonoids, are suggested to be bioactive compounds contributing to the health benefits of apples. Recently, Liu et al. (*39*) showed that whole apple extracts inhibit mammary cancer growth in a rat model; thus, consumption of apples might be an effective strategy for cancer protection. The radical-scavenging and antioxidant properties of isolated metabolites demonstrate that the antioxidant activity of cv. Annurca apples cannot be ascribed exclusively to one class of secondary metabolites. The synergy of several natural product classes renders cv. Annurca apple a true source of wellbeing.

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