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Antioxidant Properties of Sour Cherries (*Prunus cerasus* L.): Role of Colorless Phytochemicals from the Methanolic Extract of Ripe Fruits

SIMONA PICCOLELLA, ANTONIO FIORENTINO,* SEVERINA PACIFICO, BRIGIDA D'ABROSCA, PIERA UZZO, AND PIETRO MONACO

Dipartimento di Scienze della Vita, Laboratory of Phytochemistry, Seconda Università degli Studi di Napoli, via Vivaldi 43, I-81100 Caserta, Italy

Many edible plant metabolites are known to be useful as cellular antioxidants. In the search for antioxidative chemicals from native fruits of the Campania region of Italy, *Prunus cerasus* L., an acidic cherry widely used for culinary purposes, has been studied. Fruit crude extracts (MeOH, EtOAc, and hexane) were submitted to an antioxidative screening using specific assay media characterized from the presence of highly reactive radical species (DPPH$^•$, ABTS$^{•+}$, $O_2^{•-}$, NO) or lipoperoxidation markers. The reducing power of the samples was also determined. It was observed that the most polar extracts in MeOH and EtOAc were able to exercise a massive and dose-increasing antioxidative capacity. The peculiar efficacy of the same extracts was revealed by investigating their protein and deoxyribose oxidation capacity. A preliminary analysis of total phenol, flavonoid, and anthocyanin contents together with biological screening data put the basis on *P. cerasus* fruit phytochemical investigation of methanolic extract. Twenty secondary metabolites were isolated and characterized by spectroscopic (especially 1D and 2D NMR) and spectrometric techniques. 1-(4-Hydroxyphenyl)-1,2-ethanediol-1,2-bis-1-*O*-β-D-glucopyranoside (**3**), (4-hydroxy-3-methoxyphenyl)methanol-1-*O*-β-D-gentiobioside (**4**), epicatechin-3-malate (**14**), and epicatechin-3-(1″-methyl)malate (**15**) were isolated for the first time. All of the compounds were evaluated for their radical scavenging activity on DPPH$^•$, $O_2^{•-}$, and NO. Flavonoids and quinic acid derivatives were found to be the more antioxidative substances.

KEYWORDS: ***Prunus cerasus;*** **antioxidant activity; radical scavenging activity; phytochemicals; flavonoids**

INTRODUCTION

Antioxidant compounds play an important role as health-protective factors. Most of the antioxidant compounds in a typical diet derive from plant sources and belong to various classes of compounds with a wide variety of physical and chemical properties. They can delay or inhibit lipid oxidation, by inhibiting the initiation or propagation of oxidizing chain reactions, and are also involved in scavenging free radicals. Primary sources of naturally occurring antioxidants are fruits and vegetables. Antioxidants from plant food sources, such as vitamin C, vitamin E, phenolic acids, and flavonoids, have been recognized as having the potential to reduce risk for chronic diseases including tumor and heart disease, helping to neutralize free radicals, which are unstable molecules linked to the development of such diseases (*1*). Flavonoids and other phenolics are important in the plant for normal growth development and defense against infection and injury. Flavonoids also partly provide plant colors present in flowers, fruits, and leaves. They generally occur as glycosylated derivatives in plants, although conjugation with inorganic acid and malonylation are also known (*2*). The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. In addition, they have metal chelation potential (*3*).

Recently our research group began to study some native pome fruits of the Campania region of Italy, such as *Malus domestica* cv. Annurca (*4–7*), *M. domestica* cv. 'Limoncella' (*8*), and *Cydonia vulgaris* (*9, 10*), showing that they are a rich resource of metabolites with an important antioxidant capacity toward free radicals. In this investigation we determined the chemical composition of *Prunus cerasus* L. (Rosaceae), an autochthonous edible species, also known as sour cherry, a deciduous shrub native to much of Europe and southwestern Asia having white flowers and tart red fruits. Sour cherry fruits are heart-shaped drupes with color ranging from light to dark red. It was widely

* Corresponding author (telephone +39 0823274576; fax +39 0823274571; e-mail antonio.fiorentino@unina2.it).

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demonstrated that tart cherry's anthocyanins, responsible for the fruit color, have the potential to inhibit tumor growth, slow cardiovascular diseases, and possibly retard the aging process (*11, 12*). The phytochemical investigation of the American cherry cultivars Montmorency and Balaton led to the isolation of several antioxidative secondary metabolites (*13*).

In this investigation we determined the chemical and antioxidant properties of *P. cerasus* L. crude extracts (MeOH, EtOAc, and hexane) as total phenol, flavonoid, and anthocyanin contents, reducing power determination, and protein and deoxyribose oxidation capacity. Antioxidative screening of the three extracts was performed by evaluating the capacity to scavenge radical species 2,2′-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$), cation 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) radical ($ABTS^{\bullet +}$), anion superoxide radical ($O_2^{\bullet -}$), and nitric oxide (NO) and to inhibit the synthesis of thiobarbituric acid reactive species (TBARS). The chromatographic analysis of the methanolic extract led us to isolate 20 secondary metabolites, identified through spectroscopic and spectrometric techniques. An evaluation of the metabolites' radical scavenging capacity was carried out using three different methods.

MATERIALS AND METHODS

Isolation and Characterization of Compounds. Fourier transform NMR spectra were recorded at 300 MHz for ^1H and at 75 MHz for ^{13}C in $CDCl_3$ or CD_3OD at 25 °C on a Varian Mercury 300 spectrometer (Varian, Palo Alto, CA). Proton-detected heteronuclear correlations were measured using HSQC (optimized for $^1J_{HC} = 145$ Hz) and HMBC (optimized 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 ionization mass spectra (EI-MS) were obtained with a HP 6890 instrument equipped with a MS 5973 N detector. Electrospray mass spectra were recorded using a Waters Z-Q mass spectrometer (Waters Co., Milford, MA) equipped with an electrospray ionization (ESI) probe.

The preparative HPLC apparatus consisted of a pump LC-10AD (Shimadzu, Japan), a refractive index detector (Shimadzu RID-10A), and a Shimadzu Chromatopac C-R6A recorder. Preparative HPLC was performed using a 250 × 10 mm i.d., 10 μm, Luna RP-8 (Phenomenex, Torrance, CA) column. Analytical TLC was performed on Merck Kieselgel (Darmstadt, Germany) 60 F_{254} or RP-8 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 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), reversed phase silica gel 100 C8 (230–400 mesh) (Fluka, Buchs, Switzerland), Fluka Amberlite XAD-4, or Sephadex LH-20 (Pharmacia, Piscataway, NJ) media.

Plant Material, Extraction, and Isolation of Metabolites. *P. cerasus* fresh fruits (5.0 kg) were collected in June 2006 in Durazzano (Benevento, Italy), stoned, and deprived of the stalk. Stoned fruits (4.0 kg) were infused in MeOH (3.5 L), then in ethyl acetate (EtOAc, 4.0 L), and finally in hexane (4.0 L), each one for 7 days. After the removal of the solvents by Rotavapor, we obtained the alcoholic (0.4 kg) and the organic (30.0 and 2.8 g, respectively) crude extracts. The methanolic extract was dissolved in water and then shaken by liquid–liquid extraction using EtOAc as extracting solvent, obtaining an organic (fraction A) and an aqueous fraction (fraction B). Fraction A was dried with Na_2SO_4 and concentrated under vacuum, yielding 30.4 g of crude residue. Then it was chromatographed by flash column chromatography (FCC) eluting with the organic phase of $CHCl_3$/MeOH/H_2O solutions to have four fractions I, II, III, and IV.

Fraction I was chromatographed by FCC on silica gel eluting with MeOH/$CHCl_3$ solutions with an increasing polarity. Fractions from 12 to 15, eluted with MeOH/$CHCl_3$ (1:9), were chromatographed on silica gel column using as mobile phase MeOH/$CHCl_3$ (7:93) to obtain metabolite **13** (28.1 mg). Fractions from 18 to 22 were subjected to a TLC eluting with the organic phase of a $CHCl_3$/MeOH/EtOH/H_2O (10:7:5:7) solution. The eluate was purified first by RP-8 HPLC-UV eluting with MeOH/MeCN/H_2O (1:2:14) and then filtered on a NH_2 Sep-Pak with MeCN/H_2O (22:3) and pure MeOH to obtain compounds **10** (2.5 mg) and **11** (2.7 mg). Fractions from 33 to 35, eluted with pure MeOH, were subjected to a RP-8 CC, and the eluate was purified by RP-8 HPLC-UV using as mobile phase a H_2O/MeOH solution (149:1), to have metabolites **16** (1.7 mg) and **18** (1.5 mg).

Fraction II was chromatographed on a RP-8 column eluting first with MeOH/MeCN/H_2O (1:1:5) to obtain compound **12** (10.9 mg) and then with MeOH/MeCN/H_2O (1:1:7) followed by a TLC to give pure compounds **5**, **6**, **7**, and **8** (10.4, 3.3, 1.7, and 8.5 mg, respectively).

Fraction III was chromatographed on Sephadex LH-20 using as mobile phase H_2O and MeOH/H_2O solutions with descending polarity. Fractions from 3 to 10, eluted with MeOH/H_2O (1:4), were rechromatographed on TLC eluting with the organic phase of a $CHCl_3$/MeOH/H_2O (13:9:3) to obtain metabolites **19** (12.1 mg), **17** (2.1 mg), and **20** (3.0 mg).

Fraction IV was subjected to a RP-8 column chromatography eluting with MeOH/MeCN/H_2O (1:1:3) to give pure compounds **14** (52.9 mg) and **15** (3.1 mg). Data for epicatechin-3-malate **14** (light yellow oil): UV (MeOH) λ_{max} (nm) (log ϵ) 217.0 (2.93); ^1H NMR spectroscopic data (300 MHz, CD_3OD), δ 6.96 (1H, d, J = 1.5, H-2′), 6.80 (1H, dd, J = 1.5 and 8.1 Hz, H-6′), 6.75 (1H, d, J = 8.1 Hz, H-5′), 5.93 (1H, d, J = 2.1, H-8), 5.90 (1H, d, J = 2.1 Hz, H-6), 4.80 (1H, s, H-2), 4.48 (1H, dd, J = 4.5 and 7.8 Hz, H-2″), 4.17 (1H, m, H-3), 2.86 (1H, dd, J = 4.8 and 16.5 Hz, H-4), 2.80 (1H, dd, J = 4.5 and 15.9 Hz, H-3″), 2.74 (1H, dd, J = 3.0 and 16.5 Hz, H-4), 2.64 (1H, dd, J = 7.8 and 15.9 Hz, H-3″); ^{13}C NMR spectroscopic data (75 MHz, CD_3OD), δ 177.1 (C, C-1″), 174.1 (C, C-4″), 157.9 (C, C-7), 157.6 (C, C-5), 157.3 (C, C-9), 145.9 (C, C-3′), 145.7 (C, C-4′), 132.2 (C, C-1′), 119.4 (CH, C-6′), 116.0 (CH, C-5′), 115.3 (CH, C-2′), 100.0 (C, C-10), 96.4, (CH, C-8), 95.9 (CH, C-6), 79.8 (CH, C-2), 68.4 (CH, C-2″), 67.4 (CH, C-3), 40.0 (CH_2, C-4), 29.2 (CH_2, C-3″); ESI-MS, m/z 429 $[M + Na]^+$, 315 $[M - C_4H_4O_4 + Na]^+$; $[\alpha]_D$ −25.5 (c 0.51, MeOH). Anal. Calcd for $C_{19}H_{18}O_{10}$: C, 56.16; H, 4.46. Found: C, 56.34; H, 4.57. Data for epicatechin-3-(1″-methyl)malate **15** (light yellow oil): UV (MeOH) λ_{max} (nm) (log ϵ) 216.0 (2.57); ^1H NMR spectroscopic data (300 MHz, CD_3OD), δ 6.99 (1H, d, J = 1.7, H-2′), 6.79 (1H, dd, J = 1.7 and 8.1 Hz, H-6′), 6.76 (1H, d, J = 8.1 Hz, H-5′), 5.94 (1H, d, J = 2.1, H-8), 5.92 (1H, d, J = 2.1 Hz, H-6), 4.80 (1H, s, H-2), 4.46 (1H, dd, J = 4.5 and 7.9 Hz, H-2″), 4.16 (1H, m, H-3), 3.77 (3H, s, OMe), 2.87 (1H, dd, J = 4.2 and 16.5 Hz, H-4), 2.80 (1H, dd, J = 4.5 and 15.9 Hz, H-3″) 2.74 (1H, dd, J = 3.0 and 16.5 Hz, H-4), 2.63 (1H, dd, J = 7.9 and 15.9 Hz H-3″); ^{13}C NMR spectroscopic data (75 MHz, CD_3OD), δ 174.1 (C, C-4″), 169.9 (C, C-1′), 158.9 (C, C-7), 157.7 (C, C-5), 157.5 (C, C-9), 146.0 (C, C-3′), 145.8 (C, C-4′), 132.0 (C, C-1′), 120.1 (CH, C-6′), 116.2 (CH, C-5′), 115.4 (CH, C-2′), 100.6 (C, C-10), 96.4, (CH, C-8), 96.0 (CH, C-6), 79.9 (CH, C-2), 68.5 (CH, C-2″), 67.4 (CH, C-3), 56.2 (CH_3, OMe), 39.9 (CH_2, C-4), 30.0 (CH_2, C-3″); ESI-MS m/z, 443 $[M + Na]^+$, 313 $[M + Na - C_5H_6O_4]^+$; $[\alpha]_D$ −19.2 (c 0.17, MeOH). Anal. Calcd for $C_{20}H_{20}O_{10}$: C, 57.14; H, 4.80. Found: C, 57.33; H, 4.72.

Fraction B was chromatographed on Amberlite XAD-4 eluting first with H_2O and then with MeOH. Fractions 10 and 11, eluted with MeOH, were chromatographed on Sephadex LH-20 eluting with H_2O and H_2O/MeOH solutions with descending polarity. Fractions 19–21, eluted with pure H_2O, were rechromatographed by RP-18 CC, obtaining three fractions. The first one, eluted with pure H_2O, was chromatographed on TLC eluting with n-BuOH/i-PrOH/EtOH/H_2O (6:2:1:8) to have metabolites **2** (1.0 mg) and **4** (5.7 mg). Data for (4-hydroxy-3-methoxyphenyl)methanol-1-O-β-D-gentiobioside (**4**, amorphous powder): UV (MeOH) λ_{max} (nm) (log ϵ) 434.5 (0.80), 280.5 (2.06), 228.5 (2.50); ^1H NMR spectroscopic data (300 MHz, CD_3OD), δ 7.00 (1H, d, J = 1.6 Hz, H-2′), 6.82 (1H, dd, J = 7.8 Hz, H-5′), 6.75 (1H, d, J = 1.6 and 7.8 Hz, H-4′), 4.55 (2H, d, J = 6.3 Hz, H-1), 4.28 (2H, d, J = 7.5 Hz, Glc1 and Glc1′), 4.06 (1H, dd J = 2.4 and 10.6 Hz, Glc6), 3.84 (1H, dd, J = 2.4 and 10.5 Hz, Glc6′), 3.82 (3H, s, OMe), 3.65

(1H, dd, $J = 5.4$ and 10.6 Hz, Glc6), 3.59 (1H, dd, $J = 5.4$ and 10.8 Hz, Glc6′), 3.20–3.82 (8H, ov, saccharidic protons); ^{13}C NMR spectroscopic data (75 MHz, CD_3OD), δ 149.9 (C, C-3′), 147.9 (C, C-4′) 134.0 (C, C-1′), 121.0 (CH, C-6′), 116.0 (CH, C-5′), 112.0 (CH, C-2′), 105.0 (CH, Glc1 and Glc1′), 77.9 (CH, Glc3, Glc3′, and Glc5′), 77.8 (CH_2, C-1), 75.6 (CH, Glc2 and Glc2′), 75.2 (CH, Glc5), 72.5 (CH, Glc4), 72.0 (CH, Glc4′), 64.0 (CH_2, Glc6), 62.6 (CH_2, Glc6′), 56.3 (CH_3, OMe;. ESI-MS, m/z 501 $[M + Na]^+$, 339 $[M - C_6H_{10}O_5 + Na]^+$, 177 $[M - C_{12}H_{20}O_{10} + Na]^+$; $[\alpha]_D$ +1.7 (c 0.03, MeOH). Anal. Calcd for $C_{20}H_{30}O_{13}$: C, 50.21; H, 6.32. Found: C, 50.43; H, 6.24. The second one, eluted with CH_3CN/H_2O (1:9), was subjected to TLC using as mobile phase the organic phase of a n-BuOH/i-PrOH/H_2O (2:1:3) solution. In this way we isolated compounds **3** (3.8 mg) and **9** (11.1 mg). Data for 1-(4-hydroxyphenyl)ethane-1,2-diol-1,2-bis-O-β-D-glucopyranoside (**3**, amorphous powder): UV (MeOH) λ_{max} (nm) (log ϵ) 435.0 (1.10), 277.0 (1.63), 225.0 (2.47); ^{1}H NMR spectroscopic data (300 MHz, CD_3OD), δ 7.19 (2H, d, $J = 8{,}4$ Hz, H-2′ and H-6′), 6.75 (2H, d, $J = 8.4$ Hz, H-3′ and H-5′), 4.54 (1H, dd, $J = 1.5$ and 6.9 Hz, H-1), 4.20 (1H, d, $J = 2.4$ Hz, Glc1), 4.18 (1H, d, $J = 2{,}1$ Hz, Glc1′), 3.81 (1H, ov, H-2), 3.30 (1H, ov, H-2), 3.20–3.82 (12H, ov, saccharide protons); ^{13}C NMR spectroscopic data (75 MHz, CD_3OD), δ 158.5 (C, C-4′), 133.5 (C, C-1′), 129.3 (CH, C-2′ and C-6′), 116.1 (CH, C-3′ and C-5′), 105.0 (CH, Glc1′), 104.6 (CH, Glc1), 77.9 (CH, Glc3, Glc5, Glc3′, and Glc5′), 76.2 (CH, C-1), 75.9 (CH, Glc2 and Glc2′), 72.5 (CH_2, C-2), 71.5 (CH, Glc4 and Glc4′), 62.7 (CH_2, Glc6′), 62.6 (CH_2, Glc6); ESI-MS, m/z 501 $[M + Na]^+$, 339 $[M - C_6H_{10}O_5 + Na]^+$; $[\alpha]_D$ +26.7 (c 0.62, MeOH). Anal. Calcd for $C_{20}H_{30}O_{13}$: C, 50.21; H, 6.32. Found: C, 50.12; H, 6.09. The third fraction, eluted with a MeOH/MeCN/H_2O (8:1:1) solution. was identified as metabolite **1** (60.6 mg).

Determination of Total Phenols. The amount of total phenols in methanolic extract was determined according to the Folin−Ciocalteu procedure (*14*). A share of the methanolic extract (10 mg in 1.0 mL) was introduced into test tubes: 1.0 mL of Folin−Ciocalteu's reagent and 8 mL of sodium carbonate (7.5%) were added. The tubes were mixed and allowed to stand for 3 h. Then, each aliquot (750 μL) was poured into 750 μL of water. Absorption at 765 nm was measured. The total phenols of the samples are expressed as milligrams of gallic acid equivalents (GAE) per 100 g of fresh material and reported as phenol milligrams per 100 g of fresh product. All samples were prepared in triplicate.

Determination of Total Flavonoids. The flavonoid content of methanolic extracts was measured using a colorimetric assay developed by Zhishen et al. (*15*). A known volume (0.5 mL) of the extract or standard solution of catechin was added to a 10 mL volumetric flask. Distilled water was added to make a volume of 5 mL. At 0 time, 0.3 mL of $NaNO_2$ (5%, w/v) was added to the flask. After 5 min, 0.6 mL of $AlCl_3$ (10%, w/v) was added, and after 6 min, 2 mL of NaOH (1.0 M) was added to the mixture followed by the addition of 2.1 mL of distilled water. Absorbance was read at 510 nm against the blank (water), and flavonoid content is expressed as milligrams of catechin equivalents (CA) per 100 g of fresh material and reported as flavonoid milligrams per 100 g of fresh product. All samples were prepared in triplicate.

Determination of Total Anthocyanins. Total anthocyanin content of methanolic extract was measured using a colorimetric assay. The extract (1.0 mg) was dissolved in acetate buffer (25 mM, pH 4.5, 1.0 mL). Sample measurement absorbancies were read at 520 nm against a blank cell containing deionized water. Anthocyanin content is expressed as milligrams of kouromanin (cyanidin-3-O-glucoside) equivalents (KO) per 100 g of fresh material and reported as anthocyanin milligrams per 100 g of fresh product. All samples were prepared in triplicate.

DPPH Radical Scavenging Activity. The DPPH radical scavenging method (*16*) was based on the reduction of methanolic DPPH radical in the presence of a hydrogen-donating antioxidant. $DPPH^{\bullet}$ (Fluka Chemie, Buchs, Switzerland) solution showed an absorption band at 515 nm and was intensely violet colored. The adsorption and color intensity decreased when $DPPH^{\bullet}$ was reduced by an antioxidant compound. The remaining DPPH radical corresponded inversely to the radical- scavenging activity of the antioxidant. Evaluating the scavenging activity of the extracts, their aliquots (12.5, 25, 50, 100, 200, 300, 500 μg), dissolved in 1 mL of MeOH, were added to 1 mL of $DPPH^{\bullet}$ (0.1 mM) solution at room temperature. The absorbance at 515 nm was measured at 30 min against a blank (1.0 mL of MeOH in 1.0 mL of $DPPH^{\bullet}$ solution).

To determine the $DPPH^{\bullet}$ scavenging activity of pure isolated metabolites, prepared by dissolving 0.1 mg of each compound in 1 mL of MeOH, 100 μL of each solution containing the compound was added to 1.4 mL of DPPH radical solution at room temperature. The absorbance at 515 nm was measured at 30 min against a blank (100 μL of MeOH in 1.4 mL of DPPH radical solution) using a UV-1700 Shimadzu spectrophotometer. The analyses were carried out in triplicate. The results are expressed in terms of the percentage reduction of the initial DPPH radical adsorption by the test samples. The DPPH radical scavenging activity has been compared with those exercised by α-tocopherol (Fluka Chemie) and ascorbic acid (Fluka Chemie) used as standards.

$O_2^{\bullet -}$ Scavenging Activity. The assay of superoxide radical scavenging activity was based on the capacity of antioxidative substances to inhibit the photochemical reduction of nitroblue tetrazolium (Fluka Chemie) in the riboflavin−light−nitroblue tetrazolium system (*17*). To test the scavenging capacity of *P. cerasus* extracts, rates of 12.5, 25, 50, 100, 200, 300, and 500 μg were dissolved in deionized water (1.0 mL). Each 3.0 mL of reaction mixture contained sodium phosphate buffer (50 mM, pH 7.8), methionine (13 mM) (Fluka Chemie), riboflavin (2 μM) (Riedel-de Haën, Buchs SG, Schweiz), ethylenediaminetetraacetic acid (100 μM) (Carlo Erba Reagents, Rodano, Milano, Italy), 75 μM nitroblue tetrazolium, and extract solution (100 μL).

To determine the scavenging capacity of each metabolite (0.1 mg/mL), 100 μL of each one was added to reaction mixture (2.9 mL) containing 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 2 μM riboflavin, 100 μM ethylendiaminetetraacetic acid, 75 μM nitroblue tetrazolium. Prepared test tubes were submitted to a fluorescent lamp illumination for 10 min. The increase in absorbance at 560 nm was monitored. The analyses were carried out in triplicate, and the registered activities were compared to those exercised by α-tocopherol and ascorbic acid used as standards in the assay.

NO Scavenging Activity. NO generated from sodium nitroprusside (Fluka Chemie) was measured by the Griess reagent (*18*): 100 μL of water solution of amounts of crude extracts (12.5, 25, 50, 100, 200, 300, and 500 μg in 1.0 mL) and isolated metabolites (0.1 mg/mL) was added to 0.2 mL of 10.0 mM sodium nitroprusside and 1.8 mL phosphate buffer (pH 7.4). The reaction mixture was incubated at 37 °C for 3 h. An aliquot of the reaction mixture (1.0 mL) was removed and diluted with 0.5 mL of Griess reagent (1% sulfanilamide and 0.1% naphthylethylendiamine dihydrochloride in 5% H_3PO_4) (Fluka Chemie). The absorbance of the chromophore formed during the diazotization of nitrite with sulfanilamide and subsequent coupling with naphthylethylendiamine was read at 540 nm and referred to the absorbance of standard solution of sodium nitrite treated in the same way with Griess reagent. The analyses were carried out in triplicate, and the registered activities were compared to those exercised by α-tocopherol and ascorbic acid used as standards.

$ABTS^{\bullet +}$ Scavenging Activity. $ABTS^{\bullet +}$ (*19*) is generated by the interaction of 100 mM ABTS (Roche, Mannheim, Germany), 50 mM H_2O_2 (Sigma, St. Louis, MO), and 4.4 units/mL peroxidase (Sigma). To measure antioxidant capacity, 250 μL of crude extract water solution (12.5, 25, 50, 100, 200, 300, and 500 μg in 1.0 mL) was mixed with an equal volume of ABTS, H_2O_2, horseradish peroxidase, and 1.5 mL of deionized water. After 10 min of incubation, absorbance was measured at 734 nm. The analyses were carried out in triplicate, and the registered activities were compared to those exercised by α-tocopherol and ascorbic acid used as standards in the assay.

Reducing Power. The reducing power of the crude extracts was determined according to the method of Oyaizu (*20*). Extracts in phosphate buffer (2.5 mL, 0.2 M, pH 6.6) were added to potassium ferricyanide (2.5 mL, 10 mg/mL) (Riedel-de Haën, Buchs SG, Switzerland), and the mixture was incubated at 50 °C for 30 min. Trichloroacetic acid (2.5 mL, 100 mg/mL) was added to the mixture. Aliquots of 2.5 mL of reaction mixture were mixed with distilled water (2.5 mL) and ferric chloride (0.5 mL, 1.0 mg/mL), and then the

absorbance was read spectrophotometrically at 700 nm. Higher absorbance of the reaction mixture indicated greater reducing power. The analyses were carried out in triplicate, and the registered activities were compared to those exercised by α-tocopherol and ascorbic acid used as standards in the assay.

Determination of TBARS. End products of polyunsaturated fatty acid oxygenation react with thiobarbituric acid (TBA) to form a red adduct. Determination of thiobarbituric acid reactive substances (TBARS) induced by isolated metabolites was measured according to the method reported by Sroka and Cisowski (*21*). TBA reagent was prepared as follows: for reagent A, 375 mg of thiobarbituric acid (Fluka Chemie) and 30 mg of tannic acid (Riedel-de Haën) were dissolved in 30 mL of hot water. For reagent B, 15 g of trichloracetic acid (Riedel-de Haën) 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. Rapeseed oil (5.2 μL) (Riedel-de Haën) was emulsified with 15.6 mg of Tween-40 (Fluka) initially dissolved in 2.0 mL of 0.2 M Tris-HCl buffer, pH 7.4. 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 1.0 mL of the reaction mixture. The samples were irradiated with UV radiation for 30 min again. After the addition of 2.0 mL of thiobarbituric acid reagent, all test tubes were placed into a boiling water bath for 15 min and then centrifuged using a Beckman GS-15R centrifuge (Beckman, Palo Alto, CA) for 3 min at 1500*g*, and the supernatant was measured at 532 nm. Inhibition of lipid peroxidation was measured as a percentage against a blank containing no test compounds. The analyses were carried out in triplicate, and the registered activities were compared to those exercised by α-tocopherol and ascorbic acid used as standards in the assay.

Determination of Effect on Protein Oxidation. The effects of *P. cerasus* on protein oxidation were determined according to the method of Levine et al. (*22*). The reaction mixture (1.2 mL) in phosphate buffer (20 mM, pH 7.4), containing sample extract (12.5, 25, 50, 100, 200, 300, and 500 μg), bovine serum albumin (20 mg/mL) (Aldrich, Buchs SG, Switzerland), $FeCl_3$ (400 μM), H_2O_2 (3 mM), and ascorbic acid (400 μM) was incubated for 1 h at 37 °C. Dinitrophenyl hydrazine (1.0 mL, 20 mM in 2 N HCl) (Fluka) was added to the reaction mixture. One milliliter of trichloroacetic acid (20% w/v) was added to the mixture and centrifuged at 650*g* for 10 min. The protein was washed first three times with 2.0 mL of EtOH/EtOAc (1:1) solution and then was dissolved in 2.0 mL of 6 M guanidine−HCl (pH 6.5). The absorbance of the sample was read at 370 nm. The analyses were carried out in triplicate, and the registered activities were compared to those exercised by α-tocopherol and ascorbic acid used as standards in the assay.

Determination of the Effects on Oxidation of Deoxyribose. The determination was carried out as described by Halliwell et al. (*23*). The reaction mixture (1.4 mL) in phosphate buffer (20 mM, pH 7.4), which contained extracts (0.2 mL), deoxyribose (6 mM) (Fluka), H_2O_2 (3 mM), $FeCl_3$ (400 μM), ethylendiaminetetraacetic acid (400 μM), and ascorbic acid (400 μM), was incubated at 37 °C for 1 h. The extent of deoxyribose degradation was tested by using the thiobarbituric acid method. One milliliter of thiobarbituric acid (1%, w/v) and 1.0 mL of trichloroacetic acid (2.8%, w/v) were added to the mixture, which was heated in a water bath at 90 °C for 20 min. The absorbance of the mixture was read spectrophotometrically at 532 nm. The analyses were carried out in triplicate, and the registered activities were compared to those exercised by α-tocopherol and ascorbic acid used as standards in the assay.

RESULTS AND DISCUSSION

The stoned fruits of *P. cerasus* were infused in MeOH, EtOAc, and hexane, each one for 7 days. The crude organic extracts, obtained after the removal of the solvents, were submitted to a preliminary biological screening aiming at the evaluation of fruit antioxidant capacity. Several electron transfer capacity assays, each one involving a specific redox reaction with the oxidant (also as the probe for monitoring the reaction) as an indicator of the reaction end point, were performed. In **Figure 1** the results of four different radical scavenging capacity

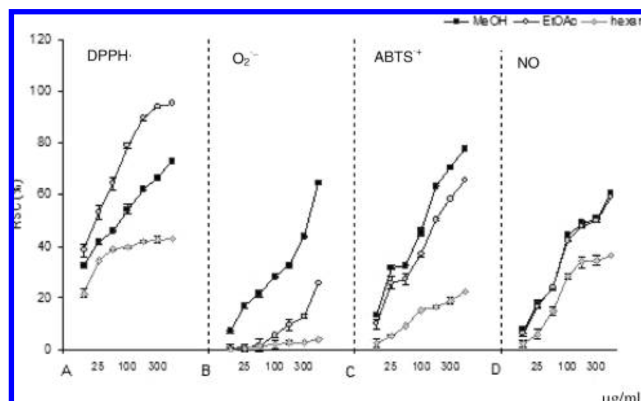

Figure 1. *P. cerasus* crude extract radical scavenging capacity (RSC) on $DPPH^•$ (**A**), $ABTS^{•+}$ (**B**), $O_2^{•-}$ (**C**), and NO (**D**). Values are reported as percentage versus blank ± SD.

assays, using, respectively, $DPPH^•$, $ABTS^{•+}$, $O_2^{•-}$, $NO^•$ as probe, on aliquots of three investigated crude extracts are reported.

Aliquots of each extract (12.5, 25, 50, 100, 200, 300, and 500 μg) were added to a methanolic solution of the free radical $DPPH^•$, one of a few stable and commercially available organic nitrogen radicals. The more polar MeOH and EtOAc extracts had an interesting reducing power on DPPH radical because they were able to reduce it by 32.6 and 38.4% after 30 min of incubation at the lowest tested dose, respectively. The scavenging capacity of the EtOAc crude extract increased massively with the amount of the samples and became equal to 95.3% at 500 μg. Methanolic extract registered a similar but slighter effect. The scavenging capacity analysis of the organic and aqueous fractions from methanolic extract led us to hypothesize the saccharidic components inhibiting effect. In fact, the EtOAc fraction from the MeOH extract scavenged the DPPH radical for 46% at 12.5 μg (**Table 1**). By plotting scavenging activity against the concentration of the each extract, the IC_{50} value was determined. The results evidenced an IC_{50} value of 0.47 μg/mL for the methanolic extract organic fraction. The surprising data gained peculiar value when compared with the standard antioxidants ascorbic acid and α-tocopherol ($IC_{50} = 4.3$ and 15.1 μg/mL, respectively).

Analogously, alcoholic extract reduced strongly the ABTS cation radical generated treating 2,2′-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid with hydrogen peroxide and horseradish peroxidase. The registered capacity ranged from 17.0% (at 12.5 μg) to 64.4% (at 500 μg) When its organic fraction scavenging capacity was tested, it determined a considerable radical reduction. In fact, a 50 μg dose already inhibited the oxidant probe for 65.0%, determining a sudden color change of the assay media. Organic extracts registered no significant scavenging efficiency.

The MeOH extract is responsible for accentuated dose–response capacities on anion superoxide radical and nitric oxide radical. From an evaluation of the inhibiting capacity the photochemical reduction of nitroblue tetrazolium in the riboflavin−light UV system, the extract showed an average inhibiting power of 70% at the highest tested doses (200–500 μg). The NO scavenging assay data evidenced that MeOH and EtOAc crude extracts exercised similar activities (60% at 500 μg). From a comparison of the scavenging capacity registered data, the methanolic extract was determined to be the more effective antiradical agent source.

Table 1. Radical Scavenging Capacity (Percent of Control ± SD) of Organic Fraction (OF) and Aqueous Fraction (AF) from MeOH Extract on $DPPH^{•}$, $ABTS^{•+}$, $O_2^{•-}$, and NO

	$DPPH^{•}$		$ABTS^{•+}$		$O_2^{•-}$		NO	
$\mu g\ mL^{-1}$	OF	AF	OF	AF	OF	AF	OF	AF
12.5	47.2 ± 1.7	23.9 ± 1.2	44.2 ± 0.9	25.6 ± 1.8	37.2 ± 1.7	13.9 ± 1.2	7.9 ± 1.7	11.3 ± 2.2
25	56.5 ± 0.8	30.6 ± 0.7	42.3 ± 1.5	29.7 ± 1.2	46.5 ± 0.8	20.6 ± 0.7	17.3 ± 2.4	17.3 ± 2.1
50	65.5 ± 1.3	38.2 ± 1.6	64.7 ± 0.7	31.7 ± 1.2	55.5 ± 1.3	28.2 ± 1.6	35.5 ± 1.1	21.1 ± 1.0
100	76.3 ± 1.8	50.8 ± 1.5	71.7 ± 1.0	45.6 ± 1.2	66.3 ± 1.8	40.8 ± 1.5	35.3 ± 2.0	42.8 ± 1.1
200	89.4 ± 0.6	53.4 ± 0.9	80.5 ± 1.4	52.3 ± 0.8	79.4 ± 0.6	43.4 ± 0.9	38.4 ± 1.6	46.3 ± 0.6
300	93.9 ± 0.6	58.6 ± 1.2	91.3 ± 0.5	55.9 ± 1.7	83.7 ± 0.6	48.6 ± 1.2	39.9 ± 1.3	47.7 ± 0.8
500	96.4 ± 0.7	63.6 ± 1.5	95.0 ± 0.6	57.0 ± 1.3	86.4 ± 0.7	53.6 ± 1.5	41.5 ± 0.9	54.2 ± 1.1

Table 2. *P. cerasus* Crude Extracts Reducing Power

$\mu g\ mL^{-1}$	MeOH (% of control ± SD)	EtOAc (% of control ± SD)	hexane (% of control ± SD)
12.5	6.8 ± 0.9	20.3 ± 2.1	4.9 ± 1.1
25	14.4 ± 1.3	30.4 ± 1.4	9.1 ± 1.5
50	35.6 ± 1.4	38.2 ± 0.4	14.8 ± 1.2
100	44.4 ± 0.8	47.6 ± 1.6	21.1 ± 2.0
200	55.3 ± 0.9	63.7 ± 0.8	27.9 ± 1.3
300	70.9 ± 0.7	74.0 ± 1.8	31.6 ± 1.6
500	79.8 ± 1.5	80.7 ± 1.1	39.4 ± 1.7

The reductive ability of each extract was also measured through an electron transfer reaction ($Fe^{3+}-Fe^{2+}$) using a ferric salt as oxidant agent. The most polar extracts exercised a massive and dose-increasing reducing power (**Table 2**).

The potential antioxidative efficiency was also investigated by measuring the amount of thiobarbituric acid reactive species produced in several reactions having as oxidizable substrates biological components or their moieties. From an evaluation of lipoperoxidation inhibition induced in a rapeseed oil−Tween 40 system, massive activity of the methanolic extract was observed. The registered curve evidenced that the extract exercised the strongest antioxidant power at a 100 μg dose. An amount increasing capacity was determined for organic extracts.

The TBA−TCA reagent was also used to quantify the effect of the samples on oxidation of deoxyribose and BSA. In both tests the oxidation of the substrate was induced by a Fenton reaction. The deoxyribose oxidation was equally reduced from MeOH and EtOAc extracts (**Figure 2**). Analogously, the addition of the same samples showed a significant decrease of the protein carbonyl content.

A preliminary analysis of total phenol, flavonoid, and anthocyanin contents was spectrophotometrically carried out.

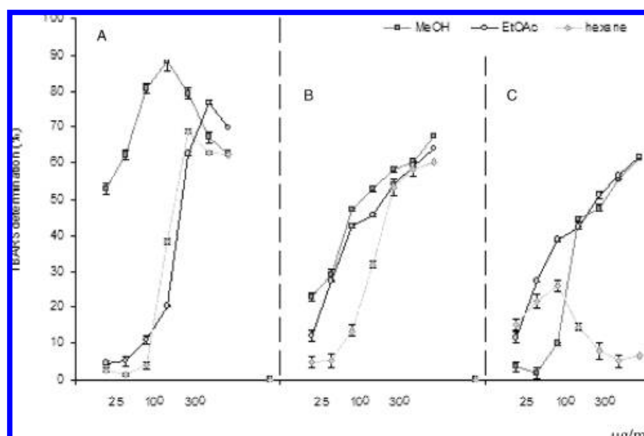

Figure 2. *P. cerasus* crude extract TBARS determination on rapeseed oil (**A**), BSA protein (**B**), and deoxyribose (**C**). Values are reported as percentage versus blank ± SD.

The amount of phenolic substances was equal to 141.9 mg of gallic acid equivalents in 100 g of fresh product. Comparison of these data to the apple phenol content (about 75.0–80.0 mg) (*8*) emphasized the cherry phenol wealth. Total flavonoid content was estimated to be equal to 3.9 mg of catechin equivalents in 100 g of fresh product, whereas the amount of anthocyanin substances was 1.1 mg of kouromanin in 100 g of fresh product.

The biological screening data put the basis of *P. cerasus* fruits phytochemical investigation on methanolic extract. Therefore, the aqueous and organic fractions of the more antioxidative methanolic extract were the object of extractive and chromatographic analyses. Twenty compounds (**Figure 3**) were isolated and identified on the basis of their spectroscopic and spectrometric features. Glycosides **1–4** and the glucosylated alkaloid **9** were isolated from the aqueous fraction (fraction B) and the other ones from the ethyl acetate fraction (fraction A).

Compounds **1**, **2**, and **9** were classified as glucosylated benzylic alcohols and their derivatives (*24–26*). Compounds **3** and **4** were isolated for the first time in *P. cerasus*. Compound **3** showed a molecular formula of $C_{20}H_{30}O_{13}$ according to the NMR data and ESI-MS spectrum. In the ^{1}H NMR spectrum were present two aromatic signals of a AA′BB′ spin system at δ 7.19 and 6.75, a carbinol methine at δ 4.54, and two anomeric protons as two doublets at δ 4.20 and 4.18. The DQ-COSY and TOCSY experiments showed correlations between the proton at δ 4.54 and a diastereotopic methylene as two signals at δ 3.81 and 3.30. Both of these signals were found to be bonded to the carbon at δ 72.5 in the HSQC experiment. The ^{13}C NMR spectrum showed signals of a *p*-hydroxyphenyl moiety as a carbon-bearing oxygen at δ 158.5, two methines at δ 129.3 and 116.1, a quaternary carbon at δ 133.5, two anomeric carbons at δ 105.0 and 104.6, and coincident signals of the C-2′ to C-6′ of the sugar moieties, besides a methine and a methylene carbinol at δ 76.2 and 72.5, respectively. The values of the saccharide carbons were in good agreement with those of the glucopyranose. In the HMBC experiment were present correlations between the aromatic protons at δ 7.19 with the carbons at δ 158.5 (C-4′) and 76.2 (C-1), which was bonded at the proton at δ 4.54. The latter was heterocorrelated with the carbons at δ 133.5 (C-1′), 129.3 (C-2′/C-6′), and 72.5 (C-2), which was bonded to the protons at δ 3.81 and 3.30. These data suggested the presence of 1-(4-hydroxyphenyl)-1,2-ethanediol. The correlations in the HMBC between the anomeric carbons and the methylene protons at δ 3.81 and 3.30 and the methine proton at δ 4.54 suggested the linkage between a sugar moiety and the C-1 and C-2 carbons. The constant coupling value of the anomeric proton indicated the presence of β anomer for the glucoses.

Compound **4** showed the same molecular formula, $C_{20}H_{30}O_{13}$, in accordance with the ^{13}C NMR and ESI-MS spectra, which showed the $[M + Na]^{+}$ pseudomolecular peak at *m/z* 501. The ^{1}H NMR spectrum showed three aromatic protons as two

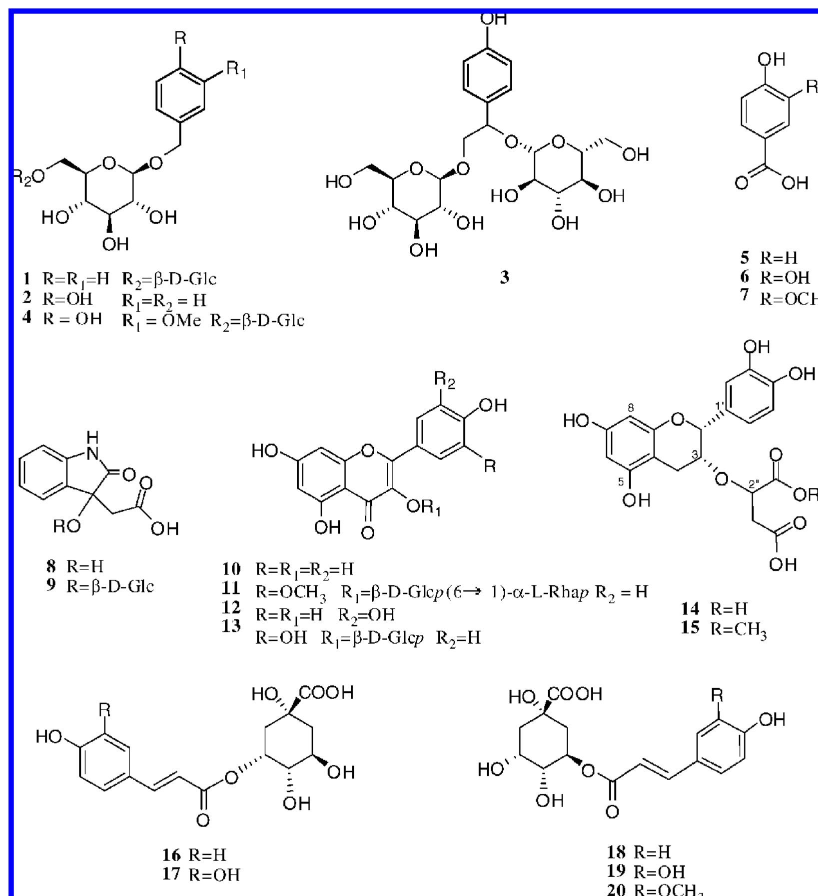

Figure 3. Structures of secondary metabolites from *P. cerasus*.

doublets at δ 7.00 and 6.75 and a double-doublet at δ 6.82, a methine as doublet at δ 4.55, a methoxyl at δ 3.82, and two coincident anomeric protons at δ 4.28. Within the overlapped sugar proton, on the basis of the 2D NMR experiments, were identifiable four methylene protons of two hexose moieties as two double-doublets at δ 4.06 and 3.65 bonded to the carbon at δ 64.0 and two double-doublets at δ 3.84 and 3.59 bonded to the carbon at δ 62.6. The carbon at δ 64.0 was correlated, in the HMBC experiment, with the anomeric signal at δ 4.28, which was also correlated with the doublet at δ 4.55 bonded to the methylene carbon at δ 77.8. The latter was correlated with the aromatic doublets at δ 6.75 and 7.00. These data were in good accordance with the presence of (4-hydroxy-3-methoxyphenyl)methanol-1-*O*-*β*-D-gentiobioside.

Metabolites **5–7** were identified by comparison with standard molecules as derivatives of the benzoic acid and precisely as *p*-hydroxybenzoic, protocatechuic, and vanillic acids. Compound **8** is a hydroxyindolic derivative of the acetic acid named 2-(3-hydroxy-2-oxoindolin-3-yl)acetic acid (*27*). Metabolites **10−13** are flavonoids, known as kaempferol, isorhamnetin-3-*O*-*β*-rutinoside (*28*), quercetin, and quercetin-3-*O*-*β*-glucopyranoside.

Compound **14** was isolated for the first time in *P. cerasus* and identified as epicatechin-3-malate. It showed a molecular formula of $C_{19}H_{18}O_{10}$ according to the ^{13}C NMR spectrum, and the ESI-MS analysis showed the pseudomolecular ion at *m*/*z* 429. The ^{1}H NMR spectrum showed, in the aromatic region, three signals of a 1,2,4-trisubstituted ring as two doublets at δ 6.96 and 6.75 and a double-doublet at δ 6.80 and two meta-coupled signals as two doublets at δ 5.93 and 5.90. In the upfield region of the spectrum were evident three methines as a broad singlet at δ 4.80, a double-doublet at δ 4.48, and a multiplet at δ 4.17, besides four methylene protons as four double-doublets at δ 2.86, 2.80, 2.74, and 2.64. Applications of two-dimensional correlation spectroscopy were needed for structural elucidation: COSY and TOCSY experiments allowed allocation of the spin systems of the methylene protons, which were connected to the corresponding ^{13}C nucleus by the HSQC experiment. In this way the double-doublets at δ 2.86 and 2.74 were bonded to the carbons at δ 40.0, whereas the double-doublets at δ 2.80 and 2.64 were bonded to the carbon at δ 29.2. The ^{13}C NMR spectrum showed 19 carbon signals identified, on the basis of the DEPT experiment, as 2 methylenes, 9 methines, and 9 tetrasubstituted carbons. The HSQC−TOCSY experiment allowed the identification of all the spin systems in the molecule. The carbons at δ 79.8, 67.4, and 40.0 showed correlations with the protons at δ 4.80, 4.17, 2.86, and 2.74. These data suggested the presence of the epicatechin, but the NMR signals indicated the presence of a further moiety bonded to the molecule. The HMBC experiment showed correlation between both the carboxyl groups at δ 174.1 and 177.1 with the methylene protons at δ 2.80 and 2.64 and the methine at δ 4.48 bonded to the carbon at δ 68.4. The latter showed cross peaks with the protons at δ 4.17 and 4.80. These data suggested the presence of a malic acid moiety bonded to the epicatechin through an ethereal bridge among the 3–2″ carbons.

Compound **15** showed a molecular formula of $C_{20}H_{20}O_{10}$ in accordance with the NMR data and the ESI-MS spectrum that showed the pseudomolecular peak at *m*/*z* 443. The ^{1}H and ^{13}C NMR data were identical with those shown by the previous compound with the exception of the presence of a methyl at δ 3.77 correlated with the carboxyl at δ 169.9, which was correlated to the protons at δ 4.46. These data

Table 3. Radical Scavenging Activity of Phytochemicals **1**−**18** from *P. cerasus*

compound	DPPH (% of control ± SD)	$O_2^{\bullet-}$ (% of control ± SD)	NO (% of control ± SD)
1	3.3 ± 0.2	33.0 ± 0.3	51.9 ± 1.1
2	3.8 ± 0.5	15.7 ± 0.7	49.0 ± 0.6
3	3.5 ± 0.7	15.9 ± 1.1	43.0 ± 0.3
4	4.2 ± 0.5	21.3 ± 0.9	49.1 ± 0.4
5	35.7 ± 0.3	44.7 ± 1.3	42.4 ± 0.5
6	46.7 ± 0.2	34.0 ± 0.8	78.5 ± 0.7
7	34.3 ± 0.3	52.8 ± 1.1	76.1 ± 1.3
8	8.5 ± 0.2	5.8 ± 0.5	51.2 ± 0.8
9	5.3 ± 0.5	2.8 ± 0.2	46.2 ± 1.2
10	49.7 ± 0.3	60.5 ± 0.7	67.8 ± 0.9
11	28.3 ± 1.6	31.3 ± 1.3	35.7 ± 1.3
12	77.1 ± 0.5	69.2 ± 1.3	72.3 ± 1.5
13	42.1 ± 1.1	37.4 ± 1.9	65.0 ± 0.9
14	58.1 ± 0.4	67.3 ± 0.7	68.8 ± 0.5
15	50.2 ± 0.2	44.3 ± 0.9	69.3 ± 0.6
16	29.4 ± 0.7	45.8 ± 1.2	72.1 ± 1.1
17	33.5 ± 0.9	52.8 ± 1.3	78.9 ± 0.9
18	35.5 ± 1.2	51.8 ± 0.7	80.1 ± 1.4
AA[a]	29.8 ± 1.5	31.8 ± 1.3	51.8 ± 0.7
T[b]	22.4 ± 0.6	34.9 ± 0.7	52.8 ± 1.3

[a] Ascorbic acid. [b] α-Tocopherol (vitamin E).

were in good accordance with the presence of a derivative of compound **14** bringing a methyl group to the C-1 carbon of the malate moiety.

Metabolites **16–20** are quinic acid derivatives (*29–32*). In particular, **16** and **18** are, respectively, neochlorogenic and chlorogenic acids.

To assess the antioxidant capacity of isolated metabolites, all of the compounds were tested for their scavenging activities on DPPH radical, anion superoxide radical, and nitric oxide. The standards used in all of the methods were the known natural antioxidants α-tocopherol (T) and ascorbic acid (AA). The results are reported in **Table 3**. Flavonoids **12**−**15** are, not surprisingly, more antioxidative compounds. In fact, it was widely demonstrated that phenolics and flavonoids are bioactive compounds contributing to the health benefits of fruits. The capacity of flavonoids to act as antioxidants depends upon their molecular structure. Watanabe (*33*) compared the antioxidant capacities of the flavanols catechin and epicatechin with quercetin and its glycoside derivatives, showing similar effects although the flavanols have a C2−C3 saturated bond and no 4-oxo functional group.

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