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Metabolic Profiling of Strawberry Grape (*Vitis* × *labruscana* cv. 'Isabella') Components by Nuclear Magnetic Resonance (NMR) and Evaluation of Their Antioxidant and Antiproliferative Properties

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Supporting Information

ABSTRACT: In the assessment of the antioxidant properties of edible plants, the widely consumed *Vitis* × *labruscana* cv. 'Isabella', known in Italy as "fragola" (strawberry) grape, was of interest. Phenol and flavonoid contents of the methanolic extracts of peel, pulp, seed, leaf, and stalk components of the plant were determined. The metabolic profile of the extracts was performed by 1D and 2D NMR. Quantitative analysis, obtained in the presence of 0.01% of internal standard trimethylsilyl propionate, evidenced the presence of catechins in both stalk and seed extracts, whereas caffeic acid and quercetin were the main metabolites of the leaf extract. Furthermore, the extracts were tested for their radical scavenging and reducing capacities by measuring their capacity to scavenge $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ and to reduce Fe(III) and Mo(VI) salts. The antioxidant efficacy of the extracts in cell-free systems and their antiproliferative activity toward HepG2 and A549 cells were also evaluated. Seed and stalk components are able to reduce by 39.6 and 40.6%, respectively, the amount of the metabolically active HepG2 cells after only 24 h of exposure.

KEYWORDS: *Vitis* × *labruscana*, 'Isabella' grape, metabolic profiling, NMR analysis, radical scavenging capacity, antioxidant activity, antiproliferative activity

INTRODUCTION

It is well-known that an oxidative stress state is a common denominator in the pathogenesis of several chronic diseases. Even the most modern and complex theories on the role of oxidative stress, in physiological and pathological processes, suggest that a balance between production of oxidizing species and antioxidant defenses is essential to preserve human health and longevity. Furthermore, a growing number of studies underscore the existence of a constant association between diets based mainly on vegetables and a reduced incidence of chronic illnesses such as proliferative, neurodegenerative, and cardiovascular diseases.[1] Thus, the wide range of antioxidant compounds present in fruits and vegetables appears to play an important beneficial role, confirmed by both clinical and epidemiological studies.

It is currently believed that the combination of micronutrients and nonessential phytochemicals, present in fruits, can play a synergistic role in promoting human health. Several systematic analyses of the nutritional composition of edible fruits have been already carried out, and they primarily focus on the content of antioxidants,[2] micronutrients, and, more recently, polyphenolic compounds.[3,4] As a result, the improvement of the nutritional quality of fruits has become an important objective for strategic and cross-combined biotechnological approaches, relevant both for improving the taste-sensory properties of fruits and for boosting productivity, resistance, and adaptation of fruit plants.

In recent decades, targeted searches have been conducted on specific fruits that show an important nutritional value.[5,6] The grape (*Vitis vinifera* L.) is one of the most commonly consumed fruits, in both fresh and processed form. The economic impact of commercial wine production makes the grape the most studied fruit, under agronomic, genomic, and nutritional perspectives. The high health benefits of grapes have been related to a high content of phenolic compounds, many of which show high antioxidant properties in vitro.

Little is known about the phytochemical composition of strawberry grape (*Vitis* × *labruscana* cv. 'Isabella'), a grape with sweet and small berries that was introduced into Europe from America around the end of 1800 when the phylloxera began to spread and compromise the excellence of Italian vineyards. The grape variety, resistant to the aphid, was used as rootstock to save Italian vineyards from attack of the parasite. Italian law 460 permits strawberry grapes to be grown only for use as table grapes or for making wine used for home consumption, with the obligation to grub up the vineyards that exceed the extension request. Recently, an HPLC-DAD-ESI-MS^n study of the most important low molecular weight phenolic compounds in hybrid grape cultivar Isabel (*Vitis labrusca* × *Vitis vinifera*) red table wines (anthocyanins, pyranoanthocyanins, flavonols, and hydroxycinnamic acid derivatives) was performed to look for differences between these and *V. vinifera* red wines.[7] The assessment of anthocyanins content in 'Isabel' grape showed that the pigments were low when compared to other fruits.[8]

In this study, we investigated the phenols and flavonoids content and antioxidant properties of *Vitis* × *labruscana* components

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(peel, pulp, seed, leaf, and stalk). Given the multiple modes of action of antioxidants, several methods have been employed to define the antioxidant capacity of the considered matrices. The antiproliferative activity of *Vitis* × *labruscana* cv. 'Isabella' extracts toward human HepG2 and A549 cell lines was also evaluated. The metabolic profile of the extracts was performed by 1D and 2D NMR.

MATERIALS AND METHODS

Collection and Fractionation of *Vitis* × *labruscana* cv. 'Isabella'. Approximately 1 kg of *Vitis* × *labruscana* cv. 'Isabella' was collected in September 2008 in Garzano (near Caserta, Italy). The grapes were separated into peel (325.0 g), pulp (388.0 g), seed (115.0 g), leaf (76.0 g), and stalk (91.0 g) components, which were stored at −80 °C until further analyses. Three independent samples of each component were suitably reduced to fragments and lyophilized by Flexi-Dry MP (FTS Systems, Vienna, Austria). The obtained freeze-dried powders, placed in Falcon tubes (50 mL), were extracted by sonication (Dr. Hielscher UP 200S, Berlin, Germany) at maximum power for 2 h using pure methanol as extracting solvent and then centrifuged at 3500 rpm for 10 min in aBeckman GS-15R centrifuge (Beckman Coulter, Milano, Italy) fitted with rotor S4180. After removal of the solvent, the three samples of each component had average weights as follows: crude peel, 1.03 ± 0.21 g; pulp, 1.50 ± 0.33 g; seed, 4.35 ± 0.89 g; leaf, 0.42 ± 0.10 g; and stalk, 3.80 ± 0.71 g, methanol extracts.

Determination of Total Phenols. The total phenols amount of the investigated crude extracts was measured according to the Folin−Ciocalteu procedure as reported by Kähkönen et al.[9] Each investigated sample (100 μL; 10 mg/mL) was mixed with 0.5 mL of Folin−Ciocalteu reagent (FCR; Sigma-Aldrich Chemie, Steinheim, Germany) and 4.0 mL of Na_2CO_3 (7.5% w/v). The reaction mixture was stirred at room temperature for 3 h. Then, an aliquot (750 μL) was poured into 750 μL of deionized water. The absorbance was read at 765 nm. The content of total phenols of the samples is expressed as milligram gallic acid equivalents (GAE) per 100 g of fresh material.

Determination of Total Flavonoids. The flavonoid content was measured using a colorimetric assay developed by Zhishen et al.[10] Methanolic extracts were dissolved in 5.0 mL of distilled water. Resulting aqueous solutions were promptly supplemented with 0.3 mL of $NaNO_2$ (5% w/v), after 5 min with 0.6 mL of $AlCl_3$ (10% w/v), and after a further 6 min with 2.0 mL of NaOH (1.0 M). Finally, distilled water was added to a final volume of 10.0 mL. The absorbance of the samples was read at 510 nm. The content of total flavonoids of the samples is expressed as milligram catechin equivalents (CE) per 100 g of fresh material.

Metabolic Profiling of *Vitis* × *labruscana* cv. 'Isabella' Methanol Extracts by NMR. Each investigated methanol extract (40.0 mg) was dissolved in 1.0 mL of a solvent system made up of K_2HPO_4 buffer (pH 6.0, 90 mM) in D_2O (containing the internal standard) and methanol-d_4 (1:1 v/v) and analyzed by NMR. Trimethylsilylpropionic acid sodium salt (TSP, 0.01%, w/v) was used as internal standard. Deuterated solvents and internal standard were purchased from Sigma-Aldrich Chemie.

NMR spectra were recorded at 25 °C on a Varian Mercury Plus 300 Fourier transform NMR at 300.03 MHz for ^{1}H and at 75.45 MHz for ^{13}C. MeOH-d_4 was used as the internal lock. Spectra were calibrated by setting d_4-TSP peak at 0.00 ppm. Data acquisition parameters, for the ^{1}H NMR spectrum, were as follows: 0.16 Hz/point, acquisition time (AQ) = 1.0 s, number of scans (NS) = 512, relaxation delay (RD) = 1.5 s, 90° pulse width (PW) = 6.6 μs, receiver gain = 22, number of data points (NP) = 9014, spectral width = 4506 Hz. Line broadening of 0.3 Hz and zero-filling to 32K were applied prior to Fourier transform. A presaturation sequence was used to suppress the residual H_2O signal.

FIDs were Fourier transformed, and the resulting spectra were manually phased and baseline-corrected using ^{1}H NMR processor (ACDLABS 12.0).

^{1}H−^{1}H correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC) spectra were recorded (see the Supporting Information). COSY spectra were acquired with a 1.0 s relaxation delay and 2514 Hz spectral width in both dimensions. The window function for COSY correlation was sine-bell (SSB = 0). ^{1}H−^{13}C heterocorrelation spectroscopy HSQC and HMBC experiments were obtained with a 1.0 s relaxation delay and 3140 Hz spectral width in F2 and 18116 Hz in F1. Qsine (SSB = 2.0) was used for the window function of the HMBC. The optimized coupling constants were $^1J_{HC}$ = 140 Hz for HSQC and $^nJ_{HC}$ = 8 Hz for HMBC.

The ^{1}H NMR spectra were automatically reduced to the ASCII file and bucketed. Spectral intensities were scaled to total intensity and converted to integrated regions of equal width (0.04 ppm), corresponding to the region at δ 0.10−9.60. The regions at δ 4.65−4.93 and 3.30−3.34 were excluded from the analysis because of the residual solvents signals. Bucketing was performed by ^{1}H NMR processor (ACDLABS 12.0).

Assessment of the Reducing Effectiveness of *Vitis* × *labruscana* cv. 'Isabella' Extracts. The development of distinct methods in terms of substrate, probe, and reaction conditions was helpful to the definition of the samples' antioxidant effectiveness. On the basis of the involved chemical reactions, the assay methods used in the estimation of bioactivity can be distinguished as tests based on reactions involving a hydrogen atom (HAT, hydrogen atom transfer) and tests based on reactions involving a single electron transfer (ET, electron transfer). Methanolic extracts of the examined components from *Vitis* × *labruscana* cv. 'Isabella' were tested in five doses (2.5, 5.0, 15.0, 50.0, and 75.0 μg/mL). For the determination of statistical significance, tests were conducted for three samples of the investigated components of 3three replicate measurements each (in total, 3 × 3 measurements). Recorded activities were compared to a blank arranged in parallel to the samples.

All of the solvents and reagents used in the antioxidant tests were purchased from Sigma-Aldrich Chemie except ABTS, which was from Roche Diagnostics (Roche Diagnostics, Mannheim, Germany).

Determination of 2,2′-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Capability. The determination of DPPH radical scavenging capability[11] was estimated as follows: methanolic extracts from *Vitis* × *labruscana* cv. 'Isabella' were dissolved in 1.0 mL of a methanol solution of DPPH$^\bullet$ (9.4×10^{-5} M) at room temperature. After 10 min of incubation, when the reactions had gone to completion, the absorption at 515 nm was measured by a Shimadzu UV-1700 spectrophotometer in reference to a blank. The results are expressed in terms of the percentage reduction of the initial DPPH radical adsorption by the test samples.

Determination of 2,2′-Azinobis(3-ethylbenzothiazolin-6-sulfonic acid) ABTS Radical Cation Scavenging Capability. The determination of ABTS$^{\bullet+}$ scavenging capacity was estimated.[6] Methanolic extracts from *Vitis* × *labruscana* cv. 'Isabella' were dissolved at room temperature in 1.0 mL of an aqueous solution of ABTS radical cation, previously generated by ABTS (100 μM), H_2O_2 (100 mM), and horseradish peroxidase (10.0 U/mL). The absorption at 734 nm was measured by a Shimadzu UV-1700 spectrophotometer after 10 min of incubation in reference to a blank. The results are expressed in terms of the percentage increase of the initial ABTS$^{\bullet+}$ adsorption by the test samples.

Determination of Mo(VI) Reducing Power. To carry out the quantitative determination of antioxidant capacity,[12] methanolic extracts from *Vitis* × *labruscana* cv. 'Isabella' were dissolved in 1.0 mL of a reagent solution containing sulfuric acid (0.6 M), sodium phosphate

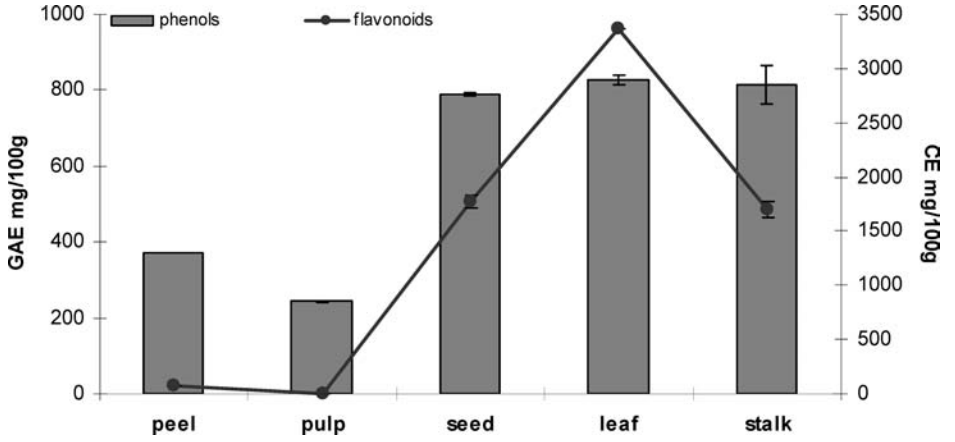

Figure 1. Total phenols and flavonoids content of methanolic extracts of peel, pulp, seed, leaf, and stalk components from *Vitis* × *labruscana* cv. 'Isabella'. Reported values are the mean ± SD of measurements carried out on three independent samples analyzed three times.

(28 mM), and ammonium molybdate (4 mM). The samples were then incubated at 95 °C for 90 min. At the end of the incubation period, the samples were cooled to room temperature, and their absorption was measured at 650 nm by a Shimadzu UV-1700 spectrophotometer against the blank. The increase in absorption in reference to the blank estimates the reducing power.

Determination of Fe(III) Reducing Power. To determine the reducing power,[13] methanolic extracts of *Vitis* × *labruscana* cv. 'Isabella' were dissolved in a mixture containing potassium hexacyanoferrate (1.0 mL, 0.1 M) and trichloroacetic acid (TCA; 1.0 mL, 100.0 mg/mL) in phosphate buffer (0.2 M, pH 6.6). The mixture was incubated at 50 °C for 30 min. Subsequently, ferric chloride (0.5 mL, 1.0 mg/mL) and deionized water (0.5 mL) were added, and the absorbance was read at 700 nm by a Shimadzu UV-1700 spectrophotometer. The increase in absorbance in reference to the blank estimates the reducing power.

Determination of Thiobarbituric Acid Reactive Species (TBARS Method). The determination of thiobarbituric acid (TBA) reactive species was performed by the TBARS.[11] Vegetable fat (olive oil, 500.0 μg) was emulsified with Tween-40 (30.0 mg), previously dissolved in Tris-HCl (1.5 mL, 0.2 M, pH 7.4). The emulsion was stirred for 24 h, then irradiated with UV light (254 nm) at room temperature for 60 min, and finally added to the methanolic extracts of *Vitis* × *labruscana* cv. 'Isabella'. The mixture was again exposed for 60 min at UV light illumination. The TBA reagent (2.0 mL) prepared by dissolving 375 mg of thiobarbituric acid, 30 mg of tannic acid, and 15 g of TCA in an aqueous solution of HCl (100 mL, 0.2 M) was added to the emulsion. The resulting mixture was placed into a boiling water bath for 1 h and then centrifuged using a Beckman GS-15R centrifuge for 3 min at 3500*g*. The absorbance of the supernatant was measured at 532 nm using a Shimadzu UV-1700 spectrophotometer. The decrease in TBARS synthesis in reference to a blank estimates the samples' antioxidant capacity.

Determination of the Effect on Bovine Serum Albumin (BSA) Oxidation. To determine the ability of methanolic extracts of *Vitis* × *labruscana* cv. 'Isabella' to inhibit protein oxidation,[11] reaction mixtures, containing phosphate buffer (20.0 mM, pH 7.2), each test sample, BSA (10.0 mg/mL), $FeCl_3$ (400.0 μM), H_2O_2 (3.0 mM), and ascorbic acid (400 μM), were incubated for 1 h at 37 °C. Dinitrophenylhydrazine (DNPH; 20.0 mM in 2.0 N HCl) and TCA (20% w/v) were then added. The mixture was centrifuged at 1500 rpm for 5 min. The protein was washed three times with EtOH/EtOAc (1:1 v/v, 1.0 mL) and subsequently dissolved in 2.0 mL of guanidine-HCl (6.0 M, pH 6.5). The absorbance of the samples was read at 370 nm by a Shimadzu UV-1700 spectrophotometer.

Determination of the Effect on 2-Deoxyribose Oxidation. To measure the antioxidant effectiveness on 2-deoxyribose,[11] the reaction mixture, containing EDTA (1.0 mM), $FeCl_3$ (1.0 mM), and H_2O_2 (1.0 mM), was incubated at 37 °C for 1 h. After the addition of 2-deoxyribose (28 mM) and ascorbic acid (1.0 mM), the mixture (1.0 mL) was added to samples from *Vitis* × *labruscana* cv. 'Isabella'. Resulting samples were incubated at 37 °C for 24 h. At the end of the incubation period, 1.0 mL of thiobarbituric acid (TBA; 0.5% w/v) and 1.0 mL of TCA (2.5% w/v) were added. The mixtures were then incubated at 90 °C for 3 h, and the absorbance was read at 532 nm versus a blank by a Shimadzu UV-1700 spectrophotometer.

Cell Cultures and Antiproliferative Activity. Human hepatoblastoma cells (HepG2) and lung epithelial cells (A549) were purchased from ICLC (Interlab Cell Line Collection) at Istituto Nazionale per la Ricerca sul Cancro, Genoa (Italy). HepG2 were grown in RPMI (Invitrogen, Paisley, U.K.) containing 10% fetal bovine serum (Invitrogen), 50 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen), at 37 °C in a humidified atmosphere containing 5% CO_2. A549 cells were plated and grown under the same conditions, except that DMEM high glucose (Invitrogen) was used instead of RPMI.

Antiproliferative activity was evaluated by the MTT test, which allows the assessment of cell viability by determining the levels of activity of mitochondrial dehydrogenases toward 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2*H*-tetrazolium bromide (MTT; Sigma-Aldrich Chemie).[14,15] The assay is based on the mitochondrial redox capacity to convert the water-soluble yellow dye MTT into a water-insoluble blue product, called formazan. A549 and HepG2 cells were seeded in 96-multiwell plates at a density of 4.0×10^5 cells/well. After 24 h of incubation, cells were treated with extracts from *Vitis* × *labruscana* cv. 'Isabella' at four doses (5.0, 15.0, 50.0, and 75.0 μg/mL). At 24 and 48 h of incubation, cells were treated with 200 μL of 0.5 mg/mL MTT, dissolved in the culture medium, for 1 h at 37 °C in a 5% CO_2 humidified atmosphere. The MTT solution was then removed, and 100 μL of DMSO was added to solubilize the originated formazan. Finally, the absorbance at 570 nm of each well was determined using a Bio-Rad 680 microplate reader. Cell viability was then expressed as a percentage of mitochondrial redox activity of the cells treated with the extracts compared to the untreated control, and inhibition of cell viability (ICV, %) was calculated using the following formula: [(absorbance of untreated cells − absorbance of treated cells)/absorbance of untreated cells] × 100.

RESULTS AND DISCUSSION

Vitis × *labruscana* cv. 'Isabella' (Vitaceae) was separated in its components (peel, pulp, seed, leaf, and stalk), and each of them,

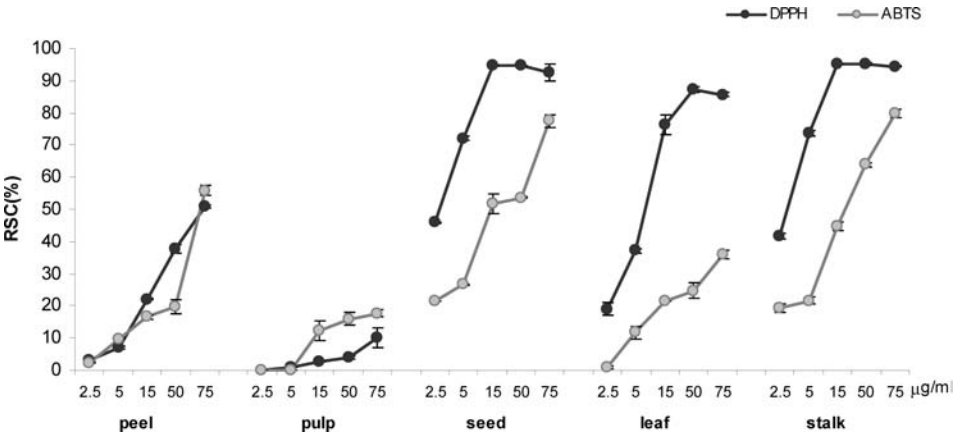

Figure 2. Radical scavenging capacity (RSC, %) of methanolic extracts of peel, pulp, seed, leaf, and stalk components from *Vitis* × *labruscana* cv. 'Isabella' on DPPH radical and ABTS radical cation. Values, reported as percentage versus a blank, are the mean ± SD of measurements carried out on three independent samples analyzed three times.

suitably reduced to fragments, was lyophilized and then extracted by sonication with methanol. The resulting crude methanolic extracts underwent a preliminary chemical screening evaluating their phenol and flavonoid contents as well as their antioxidant capacity.

The assessment of the antioxidant capacity required the use of different tests because of the various action mechanisms of antioxidant compounds. In fact, all of the different methods in vitro cannot fully represent a complex biological system such as food.

The determination of the FCR reducing power, reflecting the content of total phenols and expressed as milligram gallic acid equivalents (GAE) in 100 g of fresh material, showed a marked efficacy and an abundance of phenolic phytochemicals, in seed, leaf, and stalk extracts (Figure 1), in which they reached values of 788.9, 828.0, and 814.6 mg/100 g fw, respectively. Data are in good accordance with those reported for other *Vitis* species,[16] in which phenols are mainly distributed in the peel, stalk, leaf, and seed of grapes rather than their juicy middle section. However, it was observed that the grape total phenol content varied with cultivar, soil composition, climate, geographic origin, cultivation practices, or exposure to diseases such as fungal infections.[17]

The evaluation of total flavonoid content (Figure 1), expressed as catechin equivalents (CE) in milligrams per 100 g of fresh material, highlighted a major presence of these molecules in the leaf component. Comparable flavonoid contents in seed and stalk components were found. Previous research has shown that in grape berries, phenolic compounds are present mainly in peels and seeds. Flavonols and anthocyanins are the most abundant phenolic compounds in peels of red grapes, whereas grape seeds are rich in flavan-3-ols.[18]

In general, antioxidants act against the radicals according to two basic mechanisms: the transfer of a hydrogen atom (HAT) and/or the transfer of a single electron (ET). Antioxidant assays based on a mechanism of mixed HAT and ET actions are the ABTS and DPPH methods. The determination of the effectiveness of radical scavenging components from *Vitis* × *labruscana* cv. 'Isabella' was performed by subjecting increasing amounts of methanolic extracts (2.5, 5.0, 15.0, 50.0, and 75.0 μg/mL) to the two tests (Figure 2). These are characterized by a simple and rapid execution and good reproducibility and consist of specific redox reactions in which the radical acts as an oxidant and probe.

When the DPPH radical scavenging ability was tested, it was observed that the extracts of the seed, leaf, and stalk components exhibited marked reducing activity toward the radical species. The antiradical efficiency became significant after only 10 min of reaction for seed and stalk components by addition of the extract dose of 5.0 μg/mL to the radical methanol solution. In fact, the addition of this extract amount was capable of establishing a visible conversion of the radical DPPH (purple) into its reduced form (yellow), resulting for seed component in a reduction of 72.1% and for stalk component in a reduction of 73.6%. However, the leaf component was able to reduce the radical by 76.3% at 15.0 μg/mL.

Seed and stalk components exerted, similarly, a good reducing capability of the ABTS radical cation generated enzymatically by treating ABTS with hydrogen peroxide and horseradish peroxidase. In fact, seed methanolic extract from *Vitis* × *labruscana* cv. 'Isabella' was able to reduce the radical by 51.9% at 15.0 μg/mL dose; stalk extract produced a net conversion (80%) of the radical cation in the corresponding reduced form at the highest tested dose (75.0 μg/mL).

The use of transition metal iron and molybdenum salts is a valuable method in estimating the antioxidant capacity of the analyzed matrices (Figure 3). In fact, transition metals are particularly important catalysts for the ROS production. The data obtained through analysis of Fe(III) and Mo(VI) reducing power showed the effectiveness of the seed, leaf, and stalk components. The spectrophotometric quantitation of antioxidant capacity by formation of the phosphomolybdenum complex revealed, in particular, that the same matrices were able to induce a comparable reduction of Mo(VI) to Mo(V).

The definition of the antioxidant capacity of analyzed plant matrices (Figure 4) was reached through analysis of the synthesis of TBARS under oxidative stress conditions, using as oxidizable substrate a vegetable fat (olive oil) or the pentose sugar 2-deoxyribose. The ability of extracts to inhibit/prevent oxidative damage to protein molecules was also estimated using BSA as substrate.

It is known that peroxidative processes, being commonplace in living organisms, have their target in biological molecules. Peroxidation of organic substrates results in the formation of a complex variety of degradation products. Analysis of the species produced or consumed during the peroxidative process is therefore

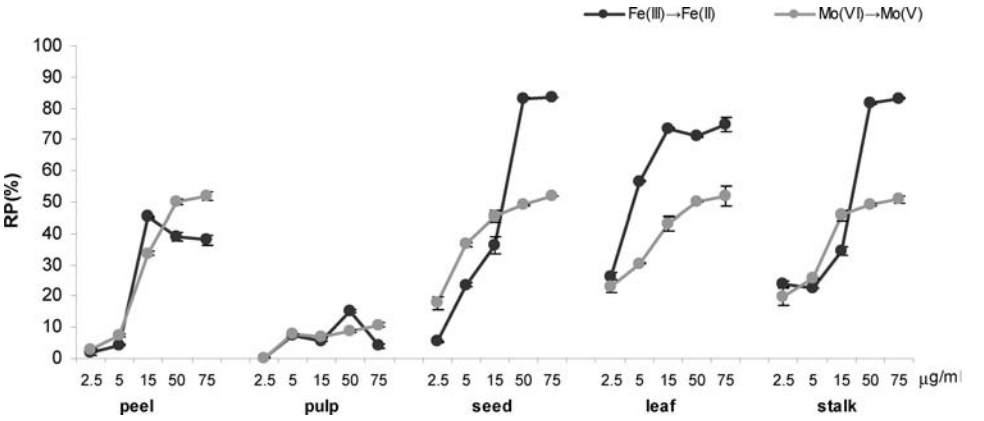

Figure 3. Reducing power (RP, %) of methanolic extracts of peel, pulp, seed, leaf, and stalk components from *Vitis* × *labruscana* cv. 'Isabella' on Fe(III) and Mo(VI). Values, reported as percentage versus a blank, are the mean ± SD of measurements carried out on three independent samples analyzed three times.

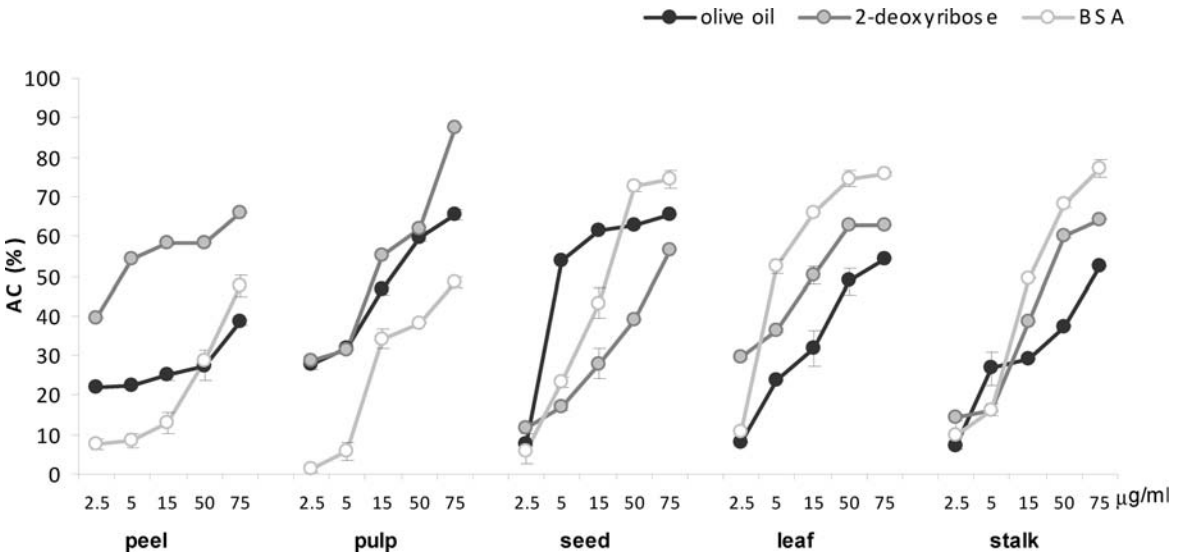

Figure 4. Antioxidant capacity (AC, %) of methanolic extracts from *Vitis* × *labruscana* cv. 'Isabella' on olive oil, 2-deoxyribose, and bovine serum albumin (BSA). Values, reported as percentage versus a blank, are the mean ± SD measurements carried out on three independent samples analyzed three times.

a method of quantitative determination of the peroxidative event itself. The unsaturated hydroxyaldehydes, lipoperoxidation byproducts, can react with the thiobarbituric acid in acid media and at high temperatures, resulting in a typical pink/red product that exhibits a maximum absorption at 532−535 nm. The extracts, added into the reaction medium during the induction of oxidative stress, were found to be effective in preventing the oxidation of the vegetable fat. A particular antioxidant efficacy was showed by leaf and pulp components, which were able to inhibit the lipoperoxidative process by 65.3 and 65.5%, respectively, at the highest tested dose.

Analogously, the TBA−TCA reagent was used to quantify the effect of the analyzed matrices on the oxidation of 2-deoxyribose. In this case, the oxidation process was previously induced by triggering a Fenton reaction. The results of this test underscore again the antioxidant effect of pulp methanolic extract, emphasizing the enormous complexity of the mechanisms of action of antioxidants and the variability of the action/interaction processes. The use of the Fenton reaction, in which Fe^{2+} ions, reacting with the pro-oxidant hydrogen peroxide, form Fe^{3+} ions, hydroxyl anions, and hydroxyl radicals, was also used to promote the oxidation of the protein substrate, BSA. The hydroxyl radical is the oxygen radical with greater toxicity and reactivity. This species lacks any endogenous mechanism of inactivation and is responsible for the initial phase of peroxidative processes that occur in the tissues of the organism. In fact, it is able to react with and damage all of the cellular macromolecules. The interaction of the hydroxyl radical with protein molecules causes fragmentation of the protein into its amino acid components and consequent increase of serum protein carbonyls and oxidation of thiol and hydroxyl free functions. The data obtained are in agreement with those recorded for the estimation of the radical scavenging capacity and reducing power. Seed, leaf, and stalk extracts exhibited a marked antioxidant capacity. The extracts were able to inhibit the protein oxidation by 74.5, 75.8, and 77.1%, respectively.

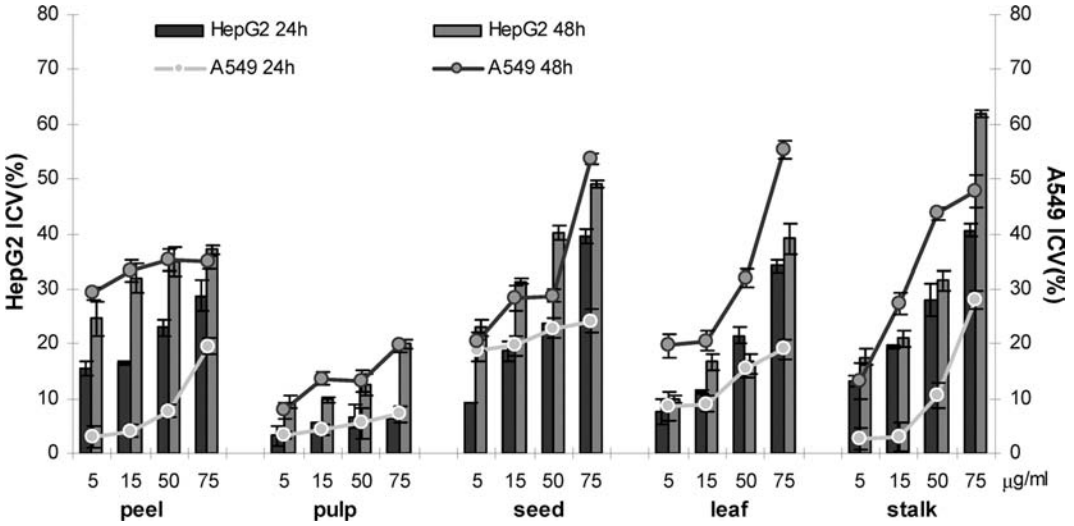

Figure 5. Inhibition of cell viability (ICV, %) of methanolic extracts of peel, pulp, seed, leaf, and stalk components from *Vitis* × *labruscana* cv. 'Isabella' on HepG2 and A549 cells. Values, reported as percentage versus an untreated control, are the mean ± SD of measurements carried out on three independent samples analyzed six times.

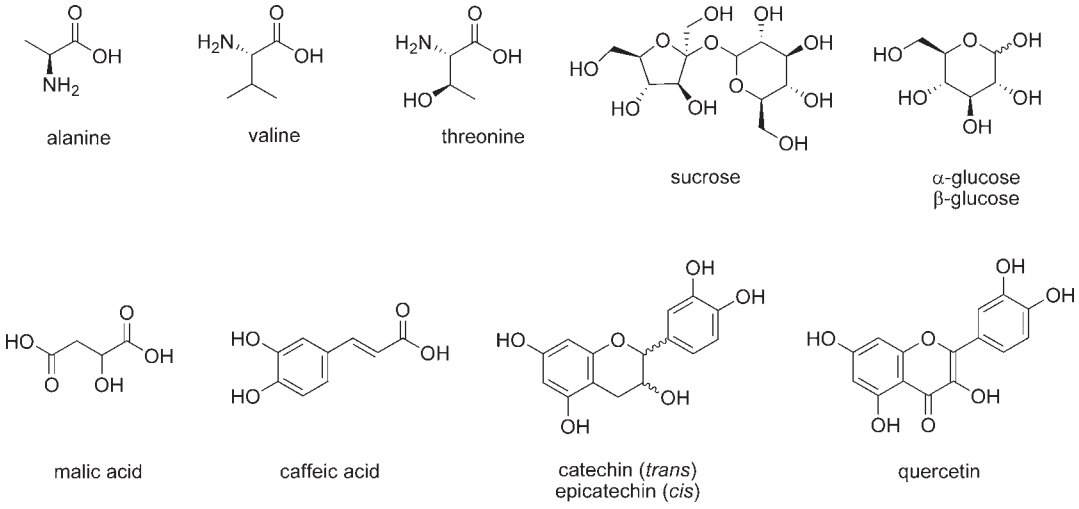

Figure 6. Main primary and secondary metabolites detected from NMR metabolomic analysis of *Vitis* × *labruscana* cv. 'Isabella' extracts.

Cell proliferation was evaluated by determining the viable cells with the MTT assay. This method is based on the intracellular reduction of tetrazolium salts by the mitochondrial enzyme succinate dehydrogenase in crystals of a blue product called formazan. Treatment of HepG2 cells and A549 cells with the extracts for 24 and 48 h reduced cell proliferation in a time- and dose-dependent manner (Figure 5). The results indicate that the antiproliferative effect is exerted in almost comparable ways in the two cell lines. The weak activity recorded for pulp edible component, especially at 24 h of incubation, was accompanied by a marked antiproliferative activity of the seed and stalk components, which were able to reduce by 39.6 and 40.6%, respectively, the amount of the metabolically active HepG2 cells after only 24 h of exposure.

The recognition of the antioxidant and antiproliferative properties of the components of *Vitis* × *labruscana* cv. 'Isabella' can add value to the botanical species widely appreciated on Italian tables.

To quickly identify the main components of the extracts, presumably responsible of the observed biological activities, metabolite profiles were obtained by NMR analysis. The most abundant metabolites (Figure 6) were identified on the basis of the chemical shifts reported in the literature data[19,20] and confirmed by 2D NMR: the homo- and heterocorrelations among protons and carbons, in the HSQC and HMBC experiments, allowed us to characterize unequivocally the prevailing components . The quantitative analyses were obtained by comparing the internal standard area, in the ^{1}H NMR spectrum, with those of unoverlapping signals of each metabolite identified in the mixture. The results are reported in Table 1.

^{1}H NMR spectra of leaf methanolic extract (Figure 7) showed signals belonging to different metabolites. In the region between 1.0 and 2.0 ppm, signals of some amino acids were present as a doublet at δ 1.47 (J = 6.9 Hz), characteristic of alanine, together with a doublet at δ 1.32 (J = 6.9 Hz) belonging to threonine and some overlapped signals attributable to valine, leucine, and

Table 1. ^{1}H Chemical Shifts and Coupling Constants of the Main Metabolites in *Vitis* × *labruscana* cv. 'Isabella' Extracts and Their Concentrations Obtained by Quantitative NMR Analysis

	chemical shifts (δ) and coupling constants (Hz)	peel (mg/g extract)	seed (mg/g extract)	pulp (mg/g extract)	leaf (mg/g extract)	stalk (mg/g extract)
valine	1.04 (d, J = 6.6), 1.02 (d, J = 6.6)	4.5				1.4
threonine	1.32 (d, J = 6.9)	4.7				6.4
alanine	1.47 (d, J = 6.9)	4.0	2.1	6.5		6.5
malic acid	4.31 (dd, J = 8.4 and 4.5), 2.78 (dd, J = 15.3 and 4.5), 2.54 (dd, J = 15.3 and 8.4)	44.7		39.4		
sucrose	5.40 (d, J = 3.6), 4.16 (d, J = 8.7)	35.9	66.6		48.7	86.8
α-glucose	5.18 (d, J = 3.6)	115.6	53.6	135.7	28.6	72.7
β-glucose	4.57 (d, J = 8.1)	145.6	85.9	178.3	36.9	96.7
catechin	6.87 (d, J = 2.5), 6.86 (d, J = 7.8), 6.78 (dd, J = 7.8 and 2.5), 6.03 (d, J = 2.2), 5.94 (d, J = 2.2)		7.5			4.2
epicatechin	7.03 (d, J = 2.0), 6.04 (d, J = 2.1), 6.01 (d, J = 2.1)		7.0			4.0
quercetin	7.70 (d, J = 2.0), 7.57 (dd, J = 8.1 and 2.0), 6.95 (d, J = 8.1), 6.49 (d, J = 1.8), 6.30 (d, J = 1.8)				11.4	
caffeic acid	7.64 (d, J = 16.0), 7.15 (d, J = 2.0), 7.07 (dd, J = 8.0 and 2.0), 6.87 (d, J = 8.0), 6.40 (d, J = 16.0)				5.2	

isoleucine. The presence of malic acid was also clear, due to signals corresponding to H-2 at δ 4.31 (dd, J = 8.4 and 4.5 Hz), H-3 at δ 2.78 (dd, J = 15.3 and 4.5 Hz), and H-3′ at δ 2.54 (dd, J = 15.3 and 8.4 Hz). The chemical shifts of malic acid are slightly different in the peel and pulp spectra; this effect agreed with a previous observation that these values depend on the pH and concentration of the solution.[21] The stalk metabolite profile was similar to that of the seed, but a minor presence of metabolites, particularly amino acids, was observed.

Anomeric protons of sugars were clearly distinguished: α-glucose at δ 5.18 (d, J = 3.6 Hz), β-glucose at δ 4.57 (d, J = 8.1 Hz), and sucrose at δ 5.40 (d, J = 3.6 Hz) and δ 4.16 (d, J = 8.7 Hz). The aromatic region of the spectrum appeared to be quite rich in signals. The main aromatic compounds were doubtless quercetin and caffeic acid. Indeed, there are signals of the 1,2,4-trisubstituted benzene as two doublets at δ 7.15 (J = 2.0 Hz) and δ 6.87 (J = 8.1 Hz) and a doublet of doublet at δ 7.07 (J = 8.1 and 2.0 Hz) and of a *trans* double bond conjugated with the aromatic ring at δ 7.64 (J = 16.0 Hz) and δ 6.40 (J = 16.0 Hz). These signals were diagnostic of a caffeic acid moiety. A two-dimensional experiment confirmed the identification of all the detected metabolites. Quercetin was recognizable on the basis of the signals at δ 7.70 (d, J = 2.0 Hz), δ 7.57 (dd, J = 8.1 and 2.0 Hz), and δ 6.95 (d, J = 8.1 Hz), attributable to H-2′, H-6′, and H-5′, respectively, of the B-ring, and at δ 6.49 (d, J = 1.8 Hz) and δ 6.30 (d, J = 1.8 Hz), attributable to the A-ring protons. The assignment of these metabolites was confirmed by the comparison of these NMR data with those of pure standards registered in the same conditions of the extracts (Table 1). ^{1}H NMR spectra of the seed extract showed the same sugar profile of the leaves. Sucrose is present in small amount in the peel extract but completely lacking in pulp extract.

With regard to amino acids, the signal belonging to alanine was clear, besides other overlapped signals. In the aromatic region, signals relative to catechin and epicatechin were observed. Catechin diagnostic signals were those of the A-ring at δ 5.94 (d, J = 2.2 Hz) and δ 6.03 (d, J = 2.2 Hz), the carbons of which at δ 100.8 and 100.1 correlated, in the HMBC experiment, with the H-4 diastereotopic protons at δ 2.87 and 2.45. The characteristic signals of epicatechin resonated at δ 6.01 (d, J = 2.1 Hz) and δ 6.04 (d, J = 2.1 Hz) and correlated, in the same experiment, with the signals at δ 2.86 and 2.72.

Analysis of the pulp and peel extracts showed no detectable signals in aromatic region. The peel profile showed signals for the same amino acids, sugars, and organic acids found in the leaves, whereas the pulp profile differed only in saccharidic composition: no sucrose was found, the only sugar present was a mixture of α- and β-glucose.

Much is known about the chemical characterization of *V. vinifera*, one of the most important dietary fruits grown worldwide for wine production.[22] A number of chemical constituents of *V. vinifera* have been identified as potent agents having anti-inflammatory and antioxidant actions and capable of promoting protection against several diseases including cancer. The chemical analysis of *V. vinifera* shows the presence of polyphenols such as catechin, epicatechin, gallocatechin, isochlorogenic acid, stilbenes, and anthocyanins having chemopreventive activities.[23] Among them, stilbenes were thought to be the active principle of red wines responsible for their ability to reduce heart disease occurrence. It is also used as a folk medicine for the remedy of various disorders such as rheumoarthritis, ulcers, immunological disorders, and inflammation.

As shown colorimetric tests and metabolic profiling studies, seed, stalk, and leaf components of *Vitis* × *labruscana* cv. 'Isabella' contain a high amount of phenolic and flavonoid compounds, which could be responsible for the strong radical scavenging and antioxidant properties observed. Indeed, the bioactivity of phenolic compounds from grapes being the most notable, several methods were employed to evaluate the antioxidant capacities of natural products extracted from various grapes or different parts of grapes. In different parts of grape, the highest antioxidant capacity was found in grape seeds, followed by peel, and the pulp displayed the lowest antioxidant capacity.[16,24] Accumulating evidence suggests that grape seeds possess a diverse array of actions and may be beneficial in the prevention of inflammatory-mediated disease such as cardiovascular disease and cancer. Grape phenols

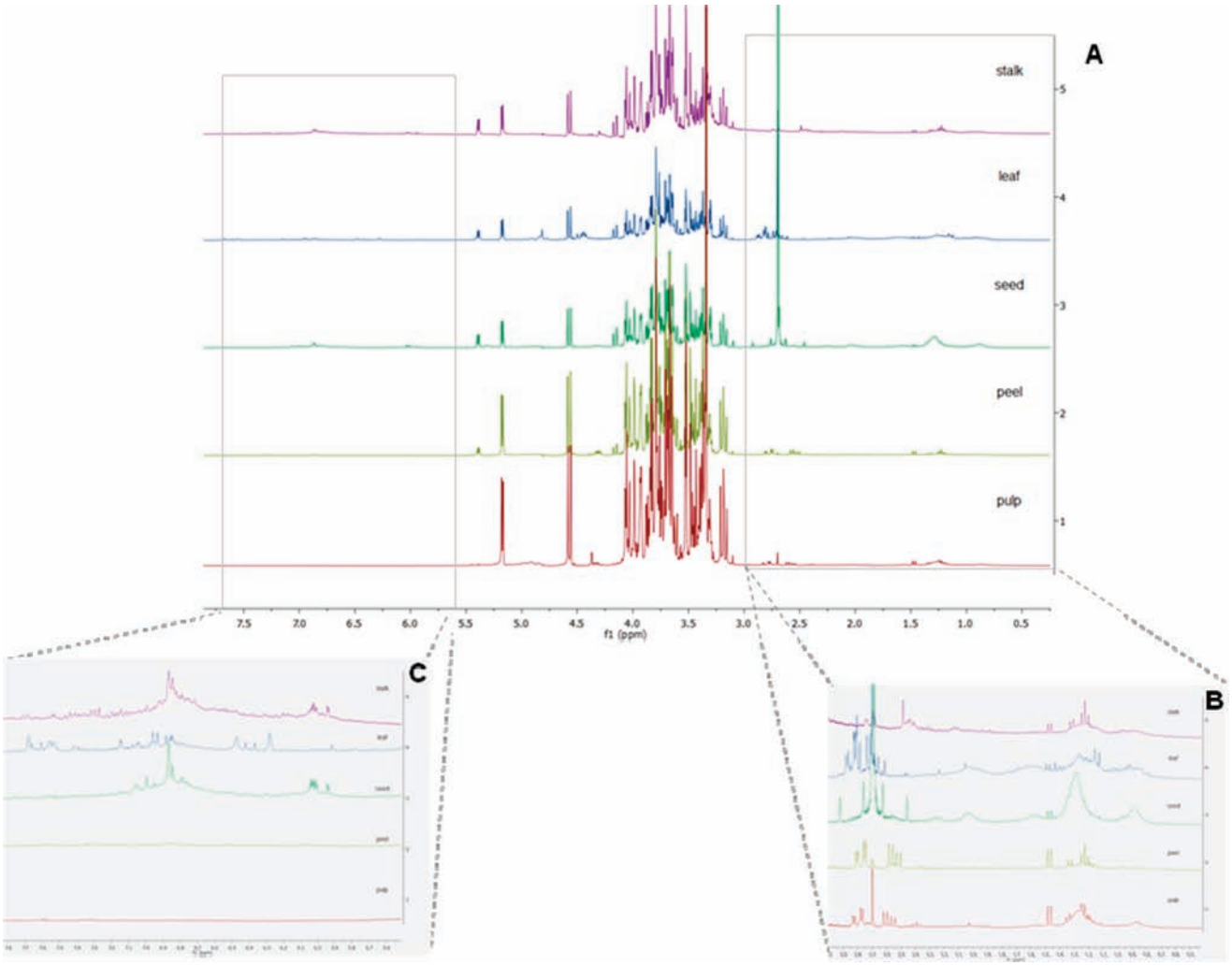

Figure 7. (A) ^{1}H NMR spectra of stalk, leaf, seed, peel, and pulp extracts of *Vitis* × *labruscana* cv. 'Isabella' registered in 50% MeOH-d_4 in D_2O (KH_2PO_4 buffer pH 6.0). (B) Spectral region between 5.5 and 7.8 ppm. (C) Spectral region between 0.4 and 3.0 ppm.

seem to have opposite effects on cells depending on the concentration.[25] Phenolic constituents of grape juice inhibit the promotion stage of DMBA-induced rat mammary tumorigenesis.[26] Grape extract treatment was associated with a modest stage-specific cell cycle arrest. Furthermore, substances such as catechin, quercetin, and resveratrol at nutritional doses prevent the development of atherosclerosis through several indirect mechanisms.[27] *Vitis* × *labruscana* cv. 'Isabella' represents an important source of active and beneficial ingredients. The potent antioxidant activity together with the marked antiproliferative efficacy of the seed and stalk components of *Vitis* × *labruscana* cv. 'Isabella' suggests they be studied phytochemically.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. HSQC and HMBC spectra of leaf extract. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ REFERENCES

(1) Willett, W. C. Diet and health: what should we eat? *Science* **2004**, *254*, 532–537

(2) Fiorentino, A.; D'Abrosca, B.; Pacifico, S.; Mastellone, C.; Scognamiglio, M.; Monaco, P. Identification and assessment of antioxidant capacity of phytochemical from kiwi fruits. *J. Agric. Food Chem.* **2009**, *57*, 4148–4155.

(3) Liu, R. H. Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. *Am. J. Clin. Nutr.* **2003**, *78*, 517S–520S.

(4) D'Abrosca, B.; Pacifico, S.; Cefarelli, G.; Mastellone, C.; Fiorentino, A. 'Limoncella' apple fruit, an Italy apple cultivar: phenol and flavonoid contents and antioxidant activity. *Food Chem.* **2007**, *104*, 1333–1337.

(5) Cefarelli, G.; D'Abrosca, B.; Fiorentino, A.; Izzo, A.; Mastellone, C.; Pacifico, S.; Piscopo, V. Free radical scavenging and antioxidant activities of secondary metabolites from reddened cv. Annurca apple fruits. *J. Agric. Food Chem.* **2006**, *54*, 803–809.

(6) Piccolella, S.; Fiorentino, A.; Pacifico, S.; D'Abrosca, B.; Uzzo, P.; Monaco, P. Antioxidant properties of sour cherries (*Prunus cerasus* L.): role of colorless phytochemicals from the methanolic extract of ripe fruits. *J. Agric. Food Chem.* **2008**, *56*, 1928–1935.

(7) Nixdorf, S. L.; Hermosín-Gutiérrez, I. Brazilian red wines made from the hybrid grape cultivar Isabel: phenolic composition and antioxidant capacity. *Anal. Chim. Acta* **2010**, *659*, 208–215.

(8) Soares, M.; Welter, L.; Kuskoski, E. M.; Gonzaga, L.; Fett, R. Phenolic compounds and antioxidant activity in skin of Niagara and Isabel grapes. *Rev. Bras. Frutic.* **2008**, *30*, 59–64.

(9) Kähkönen, M. P.; Hopia, A. I.; Vuorela, H. J.; Rauha, J. P.; Pihlaja, K.; Kujala, T. S.; Heinonen, M. Antioxidant activity of plant extracts containing phenolic compounds. *J. Agric. Food Chem.* **1999**, *47*, 3954–3962.

(10) Zhishen, J.; Mengcheng, T.; Jianming, W. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chem.* **1999**, *64*, 555–559.

(11) Pacifico, S.; D'Abrosca, B.; Pascarella, M. T.; Letizia, M.; Uzzo, P.; Piscopo, V.; Fiorentino, A. Antioxidant efficacy of iridoid and phenylethanoid glycosides from the medicinal plant *Teucrium chamaedris* in cell-free systems. *Bioorg. Med. Chem.* **2009**, *17*, 6173–6179.

(12) Prieto, P.; Pineda, M.; Aguilar, M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. *Anal. Biochem.* **1999**, *269*, 337–341.

(13) Oyaizu, M. Studies on products of the browning reaction. Antioxidative activities of browning reaction products prepared from glucosamine. *Jap. J. Nutr.* **1986**, *44*, 307–314.

(14) Hansen, M. B.; Nielsen, S. E.; Berg, K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell killing. *J. Immunol. Methods* **1989**, *119*, 203–210.

(15) Pacifico, S.; Scognamiglio, M.; D'Abrosca, B.; Piccolella, S.; Tsafantakis, N.; Gallicchio, M.; Ricci, A.; Fiorentino, A. Spectroscopic characterization and antiproliferative activity on HepG2 human hepatoblastoma cells of flavonoid C-glycosides from *Petrorhagia velutina*. *J. Nat. Prod.* **2010**, *73*, 1973–1978.

(16) Pastrana-Bonilla, E.; Akoh, C. C.; Sellappan, S.; Krewer, G. Phenolic content and antioxidant capacity of muscadine grapes. *J. Agric. Food Chem.* **2003**, *51*, 5497–5503.

(17) Bruno, G.; Sparapano, L. Effects of three esca-associated fungi on *Vitis vinifera* L.: V. Changes in the chemical and biological profile of xylem sap from diseased cv. Sangiovese vines. *Physiol. Mol. Plant Pathol.* **2007**, *71*, 210–229.

(18) Obreque-Slier, E.; Peña-Neira, A.; López-Solís, R.; Zamora-Marín, F.; Ricardo-da Silva, J. M.; Laureano, O. Comparative study of the phenolic composition of seeds and skins from Carménère and Cabernet Sauvignon grape varieties (*Vitis vinifera* L.) during ripening. *J. Agric. Food Chem.* **2010**, *58*, 3591–3599.

(19) Ali, K.; Maltese, F.; Zyprian, E.; Rex, M.; Choi, Y. H.; Verpoorte, R. NMR metabolic fingerprinting based identification of grapevine metabolites associated with downy mildew resistance. *J. Agric. Food Chem.* **2009**, *57*, 9599–9606.

(20) López-Gresa, M. P.; Maltese, F.; Bellés, J. M.; Conejero, V.; Kim, H. K.; Choi, Y. H.; Verpoorte, R. Metabolic response of tomato leaves upon different plant−pathogen interactions. *Phytochem. Anal.* **2010**, *21*, 89–94.

(21) Verpoorte, R.; Choi, Y. H.; Kim, H. K. NMR-based metabolomics at work in phytochemistry. *Phytochem. Rev.* **2007**, *6*, 3–14.

(22) Merillon, M. J.; Fauconneau, B.; Huguel, F. Antioxidant activity of the stilbene astringing, newly extracted from *Vitis vinifera* cell cultures. *Clin. Chem.* **1997**, *43*, 1092–1093.

(23) Fauconneau, B.; Teguo, P.; Huguet, F.; Barrier, L.; Merillon, M. J. Comparative study of radical scavenger and antioxidant properties of phenolic compounds from *Vitis vinifera* cell cultures using *in vitro* test. *Life Sci.* **1997**, *61*, 2103–2110.

(24) Xia, E.-Q.; Deng, G.-F.; Guo, Y.-J.; Li, H.-B. Biological activities of polyphenols from grapes. *Int. J. Mol. Sci.* **2010**, *11*, 622–646.

(25) Ramos, S.; Alia, M.; Bravo, L.; Goya, L. Comparative effects of food-derived polyphenols on the viability and apoptosis of a human hepatoma cell line (HepG2). *J. Agric. Food Chem.* **2005**, *53*, 1271–1280.

(26) Singletary, K. W.; Stansbury, M. J.; Giusti, M.; Breemen, R. B. V.; Wallig, M.; Rimando, A. Inhibition of rat mammary tumorigenesis by concord grape juice constituents. *J. Agric. Food Chem.* **2003**, *51*, 7280–7286.

(27) Auger, C.; Teissedre, P. L.; Gérain, P.; Lequeux, N.; Bornet, A.; Serisier, S.; Besançon, P.; Caporiccio, B.; Cristol, J. P.; Rouanet, J. M. Dietary wine phenolics catechin, quercetin, and resveratrol efficiently protect hypercholesterolemic hamsters against aortic fatty streak accumulation. *J. Agric. Food Chem.* **2005**, *53*, 2015–2021.