Food & Function

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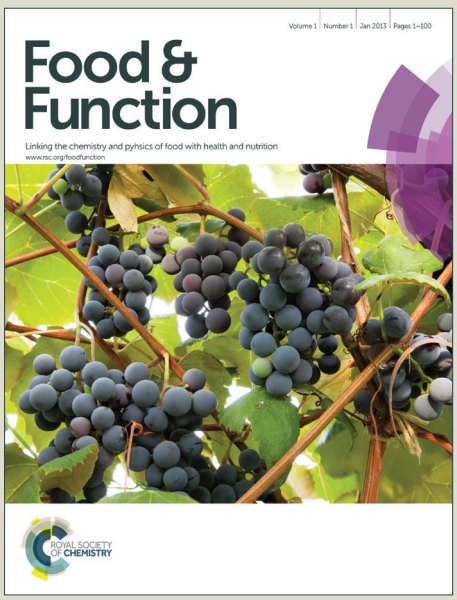

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METABOLIC AND BIOLOGICAL PROFILE OF AUTOCHTHONOUS *VITIS VINIFERA* L. ECOTYPES

Impei Stefania, Gismondi Angelo, Canuti Lorena and Canini Antonella*

Dept. of Biology, University of Rome "Tor Vergata", Via della Ricerca Scientifica 1, 00133 – Rome, Italy.

* Corresponding author: Prof. Antonella Canini, Head of the Dept. of Biology of the University of Rome "Tor Vergata", canini@uniroma2.it, tel. +39 06 7259 4333; fax. +39 06 20 23 500.

Abstract

Vitis vinifera L. is a plant species rich in phenolic compounds that are usually associated to the healthy benefits of wine and grape consumption in the diet. Anthocyanins, catechins, flavonol, phenolic acids and stilbenes are key molecular constituents of the *Vitis* berries, affecting the quality of grape products. Purpose of this work was the identification of the metabolic profile of 37 genetically certified *V. vinifera* Latial accessions. In particular, qualitative and quantitative analysis of specific secondary metabolites and total phenolic and tannin contents were performed by LC-MS and spectrophotometric analysis. In addition, since plant molecules are well-known for their free radical scavenging properties, the antioxidant effects of the sample extracts was evaluated through two different antiradical assays: DPPH and FRAP tests. Finally, a preliminary screening on the antiproliferative activity of each specimen on HCT-116 human colorectal cancer cells was investigated. All the results showed a great variety and amount of phenolic compounds in all accessions; moreover, we observed a significant correlation in the extracts between metabolite concentration and bioactivity. Besides, some samples presented extraordinary biological effects, such as reduction of tumor cell growth not associated with cytotoxiticy, suggesting them like possible future adjuvant for cancer therapy. In conclusion, the present research increased the scientific knowledge about Italian autochthonous vine ecotypes in order to valorize them and support their reintroduction in the local economic system.

Keywords :

grape skins; grape seeds; phenolic compounds; anthocyanins; cancer cells; LC-MS.

Introduction

Vitis genus is made up of about 60 grapevine species but, among them, *V. vinifera* L. is the most widely cultivated in Europe. In Italy, viticulture is particularly rich of numerous autochthonous ecotypes and grapevine biodiversity is completing part of the territory, tradition and history. These events are the result of both natural and human-induced selections that, through the centuries, has led to a strong relationship between cultivars and environments.[1-3] In fact, grape organoleptic and nutraceutical features are highly associated and connected to the geographical area where vines grow up.[4] This phenomenon is due to evolutionary mechanisms by which plants developed specific biosynthetic pathways, able to produce secondary metabolites, in order to protect vegetal tissues from biotic and abiotic environmental stresses and to promote their propagation.[5] For these reasons, the metabolic profile can be used as biochemical marker for the characterization and the identification of grapevine varieties. Moreover, different scientific studies reported how the spectrum of specific phenolic substances could be employed as fingerprint to determine the authenticity and the correspondence between grapes and wines.[6-8] In *V. vinifera* berries, phenolic compounds are the most abundant among the secondary metabolites. These molecules are characterized by a basal structure of an aromatic ring with one or more hydroxyl groups and they are classified into several classes (i.e. simple phenols, phenolic acids, coumarins), according to their additional chemical subunits and number, type and orientation of their substituents. In particular, plant phenolic substances presenting more than one benzenic ring are usually known as polyphenols (i.e. flavonoids, stilbenes, lignans, tannins).[9-11]

Phenolic metabolites are distributed everywhere in grapes. In particular, anthocyanins, a flavonoid subclass, are responsible for the red, purple and blue color of black and rose grape skins. In *V. vinifera* ecotypes, the principal anthocyanins, present as glycosides or acyl-glycosilated forms, are delphinidin, cyanidin, petunidin, peonidin and malvidin. On the other hand, the skin of white grapevine berries is rich of phenolic acids (i.e. gallic acid) and flavonols (i.e. quercetin). Finally, grape seeds are full of tannins, responsible for the bitterness and the astringent sensation of wines, while the pulps, well-stocked in sugars, contain the lowest levels of secondary metabolites.[12-15]

In literature, several works demonstrated that phenolic compounds play an important role in regulating the oxidative status of plant cells.[16,17] In fact, the free-radical scavenging activity of these molecules was clearly confirmed and even associated with specific structural elements: i) number, position and chemistry of the substituents, especially hydroxyl groups; ii) presence of double bonds; iii) methylations; iv) glycosilation; v) degree of polymerization.[18] For these reasons, a lot of research teams also investigated the antiradical effect of secondary metabolites, extracted from food and non-edible plants, directly on mammalian *in vitro* and *in vivo* systems. Moreover, these studies

proved the healthy properties of these extracts on human health and in preventing of different diseases, including cancer, diabetes and atherosclerosis.[19-24] Among them, wine gained a special interest, since the antioxidant power of some of its constituents, such as the proanthocyanidins, showed to be 20 times greater than the vitamin E and 50 times greater than the vitamin C.[13] In particular, the resveratrol, a wine stilbene, was investigated as potential antineoplastic molecule and for the treatment of human cardiovascular pathologies.[25,26]

This work represents the last step of a national project whose object was the study, the valorization and the conservation of autochthonous grapevines, in order to increase the knowledge about local ecotypes and to preserve plant biodiversity from the extinction, essentially due to the globalization, and the agronomic cultural heritage. Therefore, principal aim of the present research was the biochemical characterization of *V. vinifera* accessions previously identified as well-known or hypothetical new autochthonous varieties.[27] In particular, to generate a specific metabolic profile for each grape specimen (sampled in Autumn in hilly areas of Frosinone district), total phenolic content and wine specific anthocyanins, flavonols, phenolic acids, tannins and stilbenes were detected and quantified by LC-MS and spectrophotometrical analysis.

Since the *V. vinifera* samples analyzed were typical autochthonous cultivars or peculiar new ecotypes, naturally selected by the evolution during the time, additional purpose of this study was also the identification of possible correlations between sample biochemical composition and their bioactivity. Therefore, the antioxidant properties of each berry extract was evaluated, by two different *in vitro* antiradical assays, and a preliminary screening on their antiproliferative effect, on HTC-116 human colorectal cancer cells, was investigated.

2 Material and Methods

2.1 Sample collection and preparation

Red and white *V. vinifera* berries were sampled in Autumn starting from 37 grapevine accessions previously genetically identified by our research team.[27] Each vegetal sample was collected from different individuals of each biotype, without symptoms of pathologies and grown in different vineyards located in Frosinone district (Latium, Central Italy). The growing conditions of all the accessions were similar since they were sampled in the same geographical area, in the same annual period and at the same maturity *status*. The extraction of secondary metabolites was performed according to Hollecker *et al.*[28] method with some modifications. Grapes were carefully cleaned with H_2Odd and manually processed, in order to isolate skins and seeds that were finely powdered in liquid nitrogen by mortar and pestle. Two g of pulverized skins, or seeds, were resuspended with 20 mL of ethanol (0.1% HCl, v/v) and subjected for 2 h, in the dark, under stirring after flushing with nitrogen. The final concentration of the extracts was 100 mg mL^{-1}. The solutions were purified with 0.45 μm cellulose acetate filters (Albet-Jacs) before their analysis. After the extraction, samples were stored at -80 °C until their analysis.

2.2 Determination of total phenolic content

Phenolic content in skins and seeds of sampled grapes was estimated using a modified spectrophotometric Folin-Ciocalteu method.[29,30] Briefly, 100 μL of skin or seed extract was mixed with 500 μL of 2 N Folin-Ciocalteu reagent (Sigma-Aldrich, Italy). After 5 min, 1 mL of 20% Na_2CO_3 solution was added to the mixture and adjusted to 10 mL of volume with distilled water. The reaction was kept in the dark for 30 min and then the absorbance was read at 725 nm using a Cary 50 Bio Uv-Visible Spectrophotometer (Varian). Pure gallic acid was used to extrapolate a standard curve (100, 200, 300 and 400 mg L^{-1}; $R^2 = 0.9970$) in order to calculate the correspondent concentration of the phenolics in each specimen. Results were expressed, as suggested by Di Marco *et al.*[17], in mg of gallic acid equivalent g^{-1} of sample fresh weight (mg GAE g^{-1} SFW).

2.3 Determination of tannins

The content of tannins was spectrophotometrically determined using the method described by Weidner *et al.*[31] with appropriate modifications. This assay is based on the capacity of tannins to precipitate proteins; in fact, it measures the amount of protein-tannin complexes generated by the interaction between samples and a solution of bovine serum albumin (BSA). One mL of 1 mg mL^{-1} BSA, dissolved in a solution of 0.2 M acetate buffer pH 4.9 and 0.17 M NaCl, was added to a volume of 500 μL containing 10 mg mL^{-1} of seed extract in ethanol. The sample was incubated for

15 min with slow agitation and then centrifuged for 5 min at 20817 g. The pellet was washed and centrifuged for 3 times with acetate buffer. Finally, the sediment was dissolved in 875 µL of resuspension buffer (5% SDS, 5% TEA pH 4.9) and incubated for 10 minutes before the reading of the absorbance at 510 nm. Then, 125 µL of ferric chloride reagent (0.01 M $FeCl_3$ in 0.01 M HCl) were added and, after other 10 minutes, the absorbance was read again at 510 nm. Catechin was used to calculate a standard curve (0, 50, 100, 150, 200, 250 and 300 mg L^{-1}; $R^2 = 0.98$) in order to extrapolate the correspondent concentration of the tannins in each specimen. Results were expressed as mg of catechin equivalent g^{-1} of sample fresh weight (mg CE g^{-1} SFW).

2.4 Detection of plant secondary metabolites by LC-MS

Chromatographic analyses were performed using an LC system with LC-20AD pump, CBM-20A controller, SIL-20a HT auto-sampler and diode array SPD–M20A (Shimadzu, Tokyo, Japan) associated with mass spectrometer. Mass spectrometry was carried out using a LC-MS 2020 single quadrupole mass spectrometer (Shimadzu). The instrument was operated using a electro-spray ionization (ESI) source in positive and negative ion modes. Data acquisition was performed using LAB-SOLUTION software equipped to LC-MS (Shimadzu). For all the analyses, mass spectrometer parameters were: capillary voltage 3.0 kV, interface voltage 4.5 kV, heat block 200 °C, DL temperature 250 °C, nebulising gas 1.5 L min^{-1} and drying gas flow 15 L min^{-1} (N_2).

2.4.1 Anthocyanin analysis

Anthocyanin identification and quantification were carried out on the skin extracts of red grapes. They were performed using the procedure described by Downey and Rochfort[32], with appropriate modifications. Metabolite separation was achieved by using Kinetex column 7.5 mm x 2.1 mm x 4.6 µm (Phenomenex, USA) associated with column guard. It was carried out with a mobile phase consisting of 10% formic acid in MS grade water (v/v) (phase A) and 10% formic acid in methanol (phase B) at a constant flow rate of 0.3 mL min^{-1}. Gradient elution was conducted as follows: 0 min 82% A 18% B; 20 min 71% A 29% B; 22 min 68% A 32% B; 24 min 59% A 41% B; for 5 min 59% A 41% B linear; 32 min 50% A 50% B; for 8 min 50% A 50% B linear; and back to initial condition 82% A 18% B for 45 min, followed by 10 min for re-equilibration. The total run time, including cleaning and equilibration steps, was 50 min. Injection volume was 10 µL and column temperature was set at 30 °C. Anthocyanin identification was carried out by detecting in the extracts the presence of their ion precursor $[M+H^+]$ and their different aglycone forms, corresponding to the loss of glycosides and/or phenolic acids that could be linked to the various anthocyanidins. The concentration of the identified anthocyanins was expressed in malvidin 3-*O*-glucoside (Sigma-

Aldrich, Milan, Italy) equivalents (ME) and reported as mg of ME kg^{-1} of fresh skin weight (mg ME kg^{-1} SFW).

2.4.2 Flavonol analysis

Rutin, quercetin and myricetin were identified in skin extracts of white grapes using the method described by Castillo-Muñoz *et al.*[7] with appropriate modifications. The mobile phases were 5% formic acid in MS grade water (v/v) (phase A) and 5% formic acid in methanol (phase B), at a constant flow rate of 0.3 mL min^{-1}. The chromatographic separation was obtained using a Kinetex column 7.5 mm x 2.1 mm x 2.1 µm (Phenomenex, USA) associated with column guard. Gradient elution was set up as follows: 0 min 85% A 15% B; 3 min 65% A 35% B; 9 min 25% A 75% B; and back to initial conditions 85% A 15% B for 11 min. Injection volume was 100 µL, column temperature was set up at 30 °C and negative ion mode [m/z $M\text{-}H^-$] was used for detection of the different flavonols. Rutin, quercetin and myricetin were identified by comparing their mass spectra and retention times with the adequate standards (Sigma-Aldrich, Milan, Italy) and were expressed as mg of respective secondary metabolite (SM) kg^{-1} of fresh skin weight (mg SM kg^{-1} SFW).

2.4.3 Phenolic acid and stilbene analysis

Phenolic acids and stilbenes were identified in the skin extracts of white and red grapes by using the method described by Hollecker *et al.*[28] with some modifications. The column used for the separations of the plant molecules was a Kinetex Biphenyl Column 100 mm x 2.1 mm x 2.6 µm (Phenomenex, USA) including a guard column. Column temperature was set at 30 °C. Fractions were eluted with a gradient of 0.1% acetic acid (v/v) - 1 mM ammonium acetate (w/v) in MS grade water (solvent A) and 0.1% acetic acid (v/v) - 1 mM ammonium acetate (w/v) in MS grade methanol (solvent B), according to the following gradient program: 0 min 95% A 5% B; 18 min 70% A 30% B; for 12 min 70% A 30% B linear; 45 min 5% A 95% B; for 2 min 5% A 95% B linear; 49 min 95% A 5% B; followed by 10 min for re-equilibration. Injection volume was 100 µL and negative ion mode [m/z $M\text{-}H^-$] was used for detection of the different ionized molecules. All identified phenolic acids were expressed as gallic acid (Sigma-Aldrich, Milan, Italy) equivalents kg^{-1} of fresh skin weight (mg GAE kg^{-1} SFW), while stilbenes were reported as resveratrol glucoside (Sigma-Aldrich, Milan, Italy) equivalents kg^{-1} of fresh skin weight (mg RGE kg^{-1} SFW).

2.5 DPPH free radical-scavenging activity

Radical scavenging activity of each extract against 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) was estimated according to the procedure described by Brand-Williams *et al.*[33] with appropriate

modifications. One hundred µL of skin or seed extract was mixed with 1.9 mL of DPPH radical solution in methanol (6×10^{-5} M) and 1 mL of sodium acetate Buffer (0.1 M pH 5.5). The mixture was vigorously shaken and then incubated for 30 min in the dark. A control sample (A_{Blank}), just containing sodium acetate buffer and the same volume of sample extract, was also carried out to measure the maximum DPPH absorbance value. The reduction of DPPH radical, after addition of the plant extract, was determined by measuring the absorbance of the mixture at 515 nm (Cary 50 Bio Uv-Visible Spectrophotometer, Varian) and applying by the following formula, % IC = ([A_{Blank} – A_E]/A_{Blank}) × 100, where IC is the Inhibitory Concentration of radical scavenging activity, A_{Blank} is absorbance of the control sample and A_E is absorbance of the grape extract in presence of DPPH molecule. Data were expressed as IC50 which corresponds to mg of sample fresh weight able to quench 50% of the initial $DPPH^{\bullet}$ radicals (mg SFW), as reported in Gismondi *et al.*.[34]

2.6 Ferric reducing antioxidant power assay (FRAP) assay

FRAP assay is a colorimetric method based on the reduction of the Fe^{3+} TPTZ (2,4,6-tripyridyl-S-triazine) colorless complex in the corresponding Fe^{2+} TPTZ colored complex in presence of antioxidant molecules. It was performed according to Benzie and Strain[35] method after adequate modifications, as reported in Gismondi *et al.*.[36] Fresh FRAP reagent was prepared by mixing 10 volumes of 300 mM acetate buffer (pH 3.6), 1 volume of 10 mM TPTZ solution in 40 mM HCl and 1 volume of 20 mM ferric chloride ($FeCl_3$ $6H_2O$). Briefly, 200 µL of skin or seed extracts (0.025 g mL^{-1}) was mixed with 200 µL of ethanol and 1 mL of FRAP reagent. Then, the reaction mixture was incubated at 37 °C for 10 min and its absorbance was read at 593 nm. Results were expressed as µmol ascorbic acid equivalents mg^{-1} of sample fresh weight (µmol AAE mg^{-1} SFW).

2.7 Cell cultures, treatments and proliferation assays

HCT116 human colon cancer cells were grown and propagated as reported in Gismondi *et al.*.[37] For cellular tests, 200 µL of *V. vinifera* seed extracts, obtained as previously described, were completely dried under vacuum at room temperature (Concentrator plus, Eppendorf) to eliminate the extraction solvent, that could be toxic for cells, and finally resuspended with 200 µL of phosphate-buffered saline (PBS), in order to maintain the same concentration of the original mixture. Cell treatments were performed by adding 8 µL of sample (corresponding to the plant extract obtained from 800 µg of fresh weight sample) mL^{-1} of culture medium for 24 and 48 hours. Other concentrations (2-4-6-10 and 12 µL) of treatment were also investigated even if they did not show very interesting results as described in Results and Discussion section. Control cells (CNT) were exposed, for the same times, to an equal volume of pure PBS. Proliferation curves and cytotoxicity percentage were

measured by counting alive and dead cells, with a Neubauer modified chamber, after Trypan blue staining (1%, w/v).

2.8 Statistics

For each sample, the extractions and the analyses were repeated, at least, three times and the results were reported as means ± standard deviation (sd) of the independent measurements. The significance of the analyses was calculated by one-way ANOVA test, using PAST software (p values <0.05 were considered significant).

3 Results and Discussion

V. vinifera species was widely studied by the scientific community because of its numerous beneficial effects on human health. Several works also showed how polyphenols were the principal plant components connected to the benefits of wine and grape consumption in the diet.[38,39] Moreover, anthocyanins, catechins, flavonol, phenolic acids and stilbenes, whose concentrations are highly influenced by the *habitat* of the territory of production, were recognized as the specific molecular constituents closely related to the quality of grape products.[4]

Object of the present work was the characterization of 37 autochthonous grapevine accessions, by the determination of their metabolic profile and the evaluation of their antioxidant and biological properties, in order to valorize and launch them in the local and national economy. Moreover, since the same specimens were also genetically certified in a previous study, [27] the present research represented the final step of a national project (see Acknowledgment) about the description and the preservation of a part of the Italian grapevine biodiversity (Tab. 1).

Firstly, the total phenolic content was determined in sample skins and seeds by Folin-Ciocalteau colorimetric method. In the skins (Fig. 1A), the phenolic concentrations varied between 3.31 mg GAE g^{-1} of SFW (detected in the V4 sample) and 33.4 mg g^{-1} (V2). Exceptionally, the sample V34 revealed an extraordinary level of these molecules (181.1 mg g^{-1}). According to these data, the grape skins could be divided into 3 principal groups: samples showing a phenolic amount included between 0 mg g^{-1} and 10 mg g^{-1} (light grey bars); skins with phenolic metabolites in concentration >10 mg g^{-1} and <20 mg g^{-1} (dark grey bars); extracts with values greater than 20 mg g^{-1} (black bars). The concentration of these molecules was higher in red berry skins than in white ones. Among the red varieties, V34 and V23 accessions presented the highest and the lowest concentrations of phenolic compounds and, respectively, of 181 mg g^{-1} and 9 mg g^{-1}. On the other hand, V26 and V4 berry skins, belonging to white ecotypes, possessed the highest (25.50 mg g^{-1}) and the lowest (3.31 mg g^{-1}) levels of phenolic metabolites. With respect to the skins, these secondary metabolites were present with higher levels in seeds (Fig. 1B). In particular, in these plant districts, the concentration of phenolic substances ranged between 11.36 mg g^{-1} (V9) and 259.60 mg g^{-1} (V25). Also in this case, all the samples were clustered in 3 groups: seeds with values between 0 mg g^{-1} and 90 mg g^{-1} (light grey bars); specimens having a metabolite content between 90 mg g^{-1} and 150 mg g^{-1} (dark grey bars); phenolic levels present in concentration greater than 150 mg g^{-1} (black bars). These data were in agreement with results previously described.[40] In the seeds, the amount of phenolic compounds was not always higher in red cultivars with respect to the white ones. Indeed, for example, V17 and V33 white varieties showed a phenolic concentration (respectively, 176.10 and 171.30 mg g^{-1}) comparable to that detected in V19 and V34 red samples (respectively, 179.40 and

183.60 mg g^{-1}). These results were consistent with the data reported by Ky *et al.*[41]; however, the V34 variety surprisingly showed a total phenolic content three times higher than Alicante, the well-known prestigious grapevine cultivar. Moreover, the 54% of seed samples described in the present study presented a quantity of phenolic compounds that exceeded the values reported in the work of Ky *et al.*.[41] Finally, V19, V25, V33 and V34 specimens were the only accessions that contemporary showed the best content of phenolic compounds both in their skins and seeds.

In *V. vinifera* seeds, the catechins and their relative polymers, the tannins, are the most abundant secondary metabolites that, during vinification, are responsible of the typical sensory properties of the wines.[42] A protein precipitation assay was used to determine the presence of both condensed and hydrolyzed tannins in seed sample extracts. The results, expressed as mg CE $g^{-1}$of SFW, were reported in Figure 1C. Tannin concentration in the samples varied between 1.67 mg g^{-1} (V31) and 51.05 mg g^{-1} (V12). The obtained values can be divided in 3 clusters: the first one including samples with tannin values between 0 mg g^{-1} and 10 mg g^{-1} (light grey bars); the second group with concentrations between 10 mg g^{-1} and 30 mg g^{-1} (dark grey bars); varieties with seeds presenting amounts of tannins greater than 30 mg g^{-1} (black bars). All these results were consistent with the data reported by Weidner *et al.*.[42]

In general, the significant level variations of the phenolic concentrations observed in seeds and skins of the different accessions could be strongly associated to various endogenous and exogenous factors, such as climate, berry size, grapevine genetics and nutrients.[40]

In Tables 2, 3 and 4 were reported the biochemical profiles of all the samples, as amount of each secondary metabolite detected and investigated by LC-MS analysis.

Significant quantitative and qualitative differences were identified by comparing the anthocyanin spectra of the various red grape skin extracts (Tab. 2), supporting previous literature data that described how anthocyanin content in *Vitis* was strictly related to the cultivar type and the weather conditions of its habitat.[43] Chromatographic analysis showed that the higher amount of anthocyanins was present in the accession V22 (11469.39 mg ME kg^{-1} SFW), while the lowest content was revealed in V15 sample (1534 mg kg^{-1}). These data were consistent with other scientific works; in particular, some of the extracts studied in the present work (V22, V25 and V34) also showed a total anthocyanin content higher or comparable with the respective value measures in Aglianico berry skins, an Italian grapevine cultivar rich in anthocyanin.[38] Six samples showed an anthocyanin level between 4666 mg and 6434 mg kg^{-1} (V1, V2, V12, V16, V19 and V23), whereas the remaining 5 accessions indicated a value minor or equal to 3000 mg kg^{-1} (V6, V10, V15, V25 and V31). Malvidin was the only anthocyanin detectable in the aglicone-form in all red skin extracts. On the other hand, among the malvidin alternative glicosidic forms, malvidin-3-*O*-

glucoside, malvidin-3-*O*-(6-*O*-acetyl)-glucoside, malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside and malvidin-3-*O*-(6-*O*-*p*-coumaryl)-glucoside were the most abundant, detectable and variable in the samples, as also reported by Chiou *et al*..[44] Delphinidine and petunidine molecules were both present in 2 alternative glicosidic forms: delphinidine-3-*O*-glucoside, delphinidine-3-*O*-(6-*O*-acetyl)-glucoside, petunidine-3-*O*-(6-*O*-acetyl)-glucoside and petunidine-3-*O*-(6-*O*-*p*-coumaryl)-glucoside). Moreover, three peonidin and three cyanidin glicosidic species were also identified in the samples. In general, the most copious anthocyanins were the malvidins, followed, respectively, by peonidins, petunidines, delphinidin and cyaniding.

The quantification of phenolic acids in red berry skins was reported in Table 2. In particular, caftaric acid, *t*-ferulic-tartaric acid, two epimeric forms of 5-*O*-feruroil-quinic acid, gallic acid and ellargic acid were investigated. These secondary metabolites were detected in traces only in 6 samples (V1, V2, V15, V16, V19 and V22) and their total content ranged from 0.09 to 0.19 mg GAE kg^{-1} SFW. The higher amount was found in V2 accession. Gallic acid, ellargic acid and 5-*O*-feruroil-quinic acids were absent in all extracts, while caftaric acid and *t*-ferulic-tartaric acid were identified in very low concentrations, as reported by Nile *et al*..[10]

The stilbenes analyzed in the skin extracts of the red cultivars were measured and shown in Table 2. The interest about the presence of these compounds in the samples was principally aimed by the great interest of the stilbenes in preventive pharmacological applications.[45,46] Moreover, only a few number of *Vitis* species is able to produce stilbenes: in particular, the biosynthesis of these compounds is activated as plant defense reply to pathogens, such as fungi or bacteria.[47] In this study, resveratrol aglicone forms (*cis* and *trans* isomers), resveratrol dimer, *t*-resveratrol-glucoside and stilbenoid piacetannol, an analog of the resveratrol with well-known anti-inflammatory, anti-proliferative and anti-adipogenic properties, [46,48] were investigated. All the specimens resulted positive to stilbenes whose total concentration ranged between 0.46 and 873.26 mg RGE kg^{-1} SFW. Resveratrol dimer was the most abundant species in almost all the extracts, while piacetannol was detectable only in two accessions: V2 (high doses) and V16 (in traces).

In Table 3 and 4 were reported the data corresponding to the content of secondary metabolites revealed in white berry samples, by LC-MS analysis. In particular, phenolic acids, stilbenes and flavonols were investigated.

The total phenolic acid content detected in the different grape skin extracts varied between 0.98 (V26) to 83.08 (V7) mg GAE kg^{-1} SFW, in accordance with literature data.[49] The highest concentrations of this molecular class were identified in V7 (83.08 mg kg^{-1}), V4 (66.69 mg kg^{-1}), V3 (52 mg kg^{-1}) and V17 (49.86 mg kg^{-1}) accessions. In particular, hydroxycinamates caftaric and ferulic tartaric acids were the principal phenolic acids traced in the extracts.

In the same samples, stilbene levels ranged between 0.21 (V26) and 20.50 (V17) mg RGE kg^{-1} SFW. The resveratrol was mainly present in its dimeric form. On the other hand, the piacetannol, when observable, was detected at concentrations higher than resveratrol. As reported in literature,[28,49] the quantity of resveratrol and other stilbenoids measured in the white grape accessions (Table 3 and 4) was lower than the amount estimated in red grapes (Table 2).

The flavonols, an important subclass of the flavonoids, are plant molecules essentially involved in UV protection and in the determination of the color of white grape skins.[7] In particular, quercetin, myricetin and rutin (quercetin 3-*O*- rutinoside) amount was monitored and quantified in the present specimens, since they are the most abundant flavonols detectable in the skins of the white grapes. Their values ranged from 0.75 (V35) to 24.24 (V3) mg SM kg^{-1} of SFW. The major concentrations of total flavonols were identified in V3 (24.24 mg kg^{-1}), V4 (18.57 mg kg^{-1}), V20 (18.44 mg kg^{-1}), V9 (18.03 mg kg^{-1}), V5 (17.05 mg kg^{-1}), V11 (16.48 mg kg^{-1}) and V7 (15.99 mg kg^{-1}) samples. Rutin was the predominant flavonoid almost in all samples, followed by quercetin and myricetin.

Generally, the grapes contain high levels of bioactive compounds that exert important effects on human health thanks to their antioxidant power and inhibitory effect of LDL oxidation. In fact, they play the principal role in the "*French Paradox*". This phenomenon consists in a very low incidence of cardiovascular pathologies in presence of diet rich in saturated lipids and cholesterol, due to the high presence in diet of red wines.[50] Two antioxidant assays, DPPH and FRAP, based on different biochemical principles,[33,35] were carried out on skins and seeds of all the berry samples, in order to determine the antiradical potentiality of each accession. Results were shown, respectively, in Figure 2A and B. Briefly, in both graphs, on the x-axis (1/DPPH) were reported the reciprocal of the mg of plant matrix (SFW) able to reduce of 50% (IC50) the radicalic $DPPH^{\bullet}$ solution (6×10^{-5} M); low IC50 values corresponded to high antioxidant properties. On y-axis FRAP assay results were indicated: the antioxidant power was expressed as μmol AAE mg^{-1} of matrix (SFW) and, in this case, high values indicated elevated free radical scavenging activity. In Figure 2A, skin extracts showing the highest DPPH and FRAP measurements were reported in the left upper section of the graph (V12, V19, V2, V25, V30, V15, V22, V10, V34 and V36). As demonstrated by the previous chromatographic analysis, the same accessions also contained the greater concentrations of anthocyanins and stilbenes among all the grapes, confirming the essential role of these secondary metabolites in the determination of plant antioxidant properties.[51,52] Data obtained from these samples showed the best correlation between DPPH and FRAP assays. Numerous other samples exhibited a very high reductive potential by DPPH test and high and moderate values in FRAP assay (V1, V16, V6, V23, V33, V31, V28, V32, V8, V17, V5, V9, V11, V4, V7, V3 and V37).

Finally, low antioxidant capacities were revealed, through both the antiradical tests, only for 10 accessions (V21, V27, V26, V20, V14, V18, V29, V13, V35 and V24).

In Figure 2B were reported the data obtained by analyzing the antioxidant properties of seed extracts. Generally, in all the grape samples, the antiradical power was higher in seeds than in skins (Fig. 2A); in fact, for example, the lowest FRAP value obtained for the seed extract of V9 accession (Fig. 2B) corresponded to high values identified in numerous skin samples (i.e. V25, V30, Fig. 2A). Similarly, the lowest DPPH value obtained in seed extracts (V12, Fig. 2B) could be easily comparable to high level of antioxidant capacity in DPPH test performed on skin extracts (Fig. 2A).

For the seed extracts (Fig. 2B), the accessions that showed the maximum DPPH and FRAP antioxidant effect were reported in the left upper section of the panel (V22, V34, V25, V37, V17, V32, V28, V24, V33, V18, V30, V35, V16, V26, V27 and V29). Samples that exhibited a very high DPPH antioxidant potential associated to high or moderate FRAP free scavenging properties were V21,V20, V11, V10, V14, V15, V23, V36, V6, V19, V13, V2, V4, V3, V1 and V5. Also for seed extracts, a strong and positive correlation between total phenolic content and antioxidant effect could be individuated.

Among the various biological activities of *V. vinifera* extracts, the antitumoral function[53] surely is one of the most important. Interestingly, S-harma *et al.*[54] also suggested the possibility of a synergistic use of grape seed extracts with doxorubicin, a well-known drug, in order to improve chemotherapeutical breast cancer treatment. According to these evidences, in the present research, preliminary studies were performed with the aim to determine the bioactivity of the grape extracts, obtained from the 37 studied accessions, on human cancer cells. However, the principal concern of the application of plant products as preventing agents or for medical purposes, in relation to human diseases, is represented by the low bioavailability of the natural molecules. In fact, the absorption and the distribution of plant compounds in the organism depends on different factors (i.e. genetics, physiology) and only about 1-60% of the total molecules ingested by food can be effectively detected in blood or body tissues after meals.[55] For example, the intake efficiency of anthocyanins, that are very abundant in grape red berries, was estimated to be lower than 4% of the initial available amount.[56] For these reasons, we planned to investigate the antiproliferative effects of the grape extracts, usually introduced by diet (i.e. as fruit or wine), on HCT-116 human colorectal cancer cells, since, in the human body, they are directly exposed and in contact with food substances, partially overtaking the limits of the bioavailability. The principal molecular mechanism that plant molecules generally determine on animal cells is the regulation of their redox state. Oxidative and reducing reactions are essential and highly equilibrated events for cell survival and when their balance is altered the cellular instability is inevitable.[57,58] In fact, a lot of studies reported

how neoplastic tissues, with respect to normal ones, generally present an overproduction of Reactive Oxygen Species (ROS) that induce them to an uncontrolled proliferation.[59,60] On the other hand, these reports suggest the application of plant compounds, because of their well-known antioxidant properties, as potential agents able to rescue the disequilibrium present in tumor cells, in order to restore the correct redox conditions and a regular cell cycle.[61] All the present *in vitro* experiments were carried out by treating cells with the seed extracts of the different samples since they showed, as indicated in the previous results, the best antioxidant power, with respect to grape skin specimens. For each seed extract, as described in Material and Method section, different concentrations of treatment were tested on HCT-116 cells but, in this section, because of the large amount of obtained data, only the most interesting ones were described (8 µL of extract per mL of medium) (Supplemental Material 1). In fact, lower doses did not induce significant or remarkable alterations of cell proliferation rate, while higher concentrations excessively amplified the effects observed with the selected dose. In Figure 3 were reported all the proliferation curves of HCT-116 cells after treatment, for 24 and 48 hours, with the various seed extracts of red and white grapes, with respect to the control cells (CNT). The same results were also showed in detail in Table 5, as percentage of proliferation rate of each treatment, for the respective times, normalized for the control that was considered as unit (100%). Moreover, in order to understand if the treatments could be able to modulate the cell proliferation by activating the cell death, the cytotoxicity of all the extracts was measured, after 24 and 48 hours of cell exposure to the plant molecules. Relative results were shown in Table 5 as percentage of dead cells, also evaluating the basal level of physiological apoptosis (4% at 24 h and 5.13% at 48 h) detected in the control (CNT). According to the variegate results obtained in these cellular experiments, the seed extracts could be clustered in 3 principal groups. The first one included the samples that did not caused significant changes in cell proliferation or that promoted the cell growth (V1, V7, V12, V20, V21, V23, V30 and V31). This phenomenon could be probably justified by the presence in these samples of molecules unable to alter cell mechanisms or high levels of sugars that provided nutrients and *stimuli* of growth to cancer cells. The second cluster was made up by the extracts that induced a moderate (V8, V9, V25, V27 and V29) or strong (V10, V13, V16, V17, V19, V22, V24, V26, V28, V34, V35 and V37) inhibition of HTC-116 cell growth associated, respectively, to a little (normalized value 6-15%) or elevated (normalized value 16-58%) percentage of cell toxicity. This group was composed of the major part of specimens and its general antiproliferative effect could be strictly connected to the activation in the cells of death pathways, as demonstrated by the Trypan-blue assay. In fact, a great number of published data reported the ability of grape extracts to induce apoptosis.[62-65] The final group, on the contrary, consisted of all the remaining extracts provoking a weak (V5, V11, V14,

V15, V18 and V36) or pronounced (V2, V3, V4, V6, V32 and V33) reduction of cell growth not correlated to cytotoxicity (max value 5%). These extracts represented the most intriguing samples because they were able to decrease cell proliferation through molecular mechanisms that did not include the apoptosis, for example cell cycle arrest or differentiation, as suggested by other authors.[66-69]Among them, some samples (i.e. Lecinaro V6, Capolongo V36, Maturano Bianco V5, Pampanaro V14) represented autochthonous varieties, genetically certified by our research team,[27] that were recently reevaluated and also introduced in the *Vitis* National List. Another theory that might explain the previous last results would be the possibility that the seed extracts were able to modify the oxidized cell compartment towards a more reduced cytoplasmic environment.[70] However, other investigations are absolutely necessary to deeper these preliminary hypotheses. The present observations could support the idea of a potential use of these last extracts as possible *in vivo* antitumoral drugs, or natural adjuvants to the actual chemotherapics; in fact, because of their antineoplastic activity not associated to toxicity would represent an excellent tool able to block tumor progression without affecting the vitality of healthy tissues present around the malignant cell masses. In order to connect the bioactivity of the seed extracts with their content in total polyphenols (Fig. 1 B), we observed that, on the average, the cytotoxic samples were those that contained the major concentrations of phenolic compounds, while the antiproliferative non-toxic and the inactive specimens presented, respectively, median and low levels of these compounds. However, the FRAP and DPPH assays (Fig. 2) corroborated the previous observations. In particular, the toxic extracts were essentially the most antiradical; however, it is well known in literature that high concentration of secondary metabolites, or extremely antioxidant compounds, would act as pro-oxidant factors in *in vitro* cellular tests.[55,71] On the other hand, the samples able to reduce cell growth without causing cell death showed a general intermediate DPPH and FRAP antioxidant value. Finally, it is impossible to state which type of molecule present into an extract can exert a specific biological activity because, as well documented,[72] it is the whole phytocomplex that determines the properties of an extract and not just some of its components.

4 Conclusions

The present research was aimed to characterize 37 autochthonous *Vitis* accessions through the determination of their metabolic profiles. Additionally, notable antioxidant effects and peculiar biological activities were demonstrated for these samples. Therefore, this study and its data: i) increase the scientific knowledge about the local ancient ecotypes; ii) support the importance to preserve of their biological diversity; iii) favor the reintroduction of these grape cultivars in the economic system, thanks to the present valorization.

Acknowledgement

The present research, financially supported and promoted by the "Regione Lazio", is a part of the project "Progettazione Integrata di Filiera" (PIF, cod.RL058, Misura 124). The authors thank the Agricultural Farms of Frosinone district which contributed to this work by providing the grapevine samples.

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Figure captions

Figure 1. Total phenolic compounds, expressed as mg of gallic acid equivalent g^{-1} of sample fresh weight (mg GAE g^{-1} SFW), were measured in skin (A) and seed (B) extracts of each sample.
The amount of total tannins, expressed as mg of catechin equivalent g^{-1} of sample fresh weight (mg CE g^{-1} SFW), were measured in seed extracts of each sample (C). In each graph (A, B and C), the samples were clustered in three main groups, according to their high (black bars), moderate (dark grey bars) or low (light grey bars) concentration of respective metabolites. All the results were reported as mean ± standard deviation of three independent measurements ($p<0.01$).

Figure 2. The results of DPPH and FRAP antioxidant assays on skin (A) and seed (B) extracts of each sample were shown. In order to correlate the two tests, on the x-axis (1/DPPH) were reported the reciprocal of the mg of plant matrix (SFW) responsible for the reduction of 50% (IC50) of a radical $DPPH^{\bullet}$ solution (6×10^{-5} M), while on the y-axis were indicated the results of FRAP assay, expressed as μmol of ascorbic acid equivalent mg^{-1} of sample fresh weight (μmol AAE mg^{-1} SFW). In both graphs (A and B), each sample was represented by a colored circle, according to its high (black circles), moderate (dark grey circles) or low (light grey circles) level of antioxidant power, obtained by correlation between DPPH and FRAP analyses. Light grey circles, in the graph A, corresponded to V26, V20, V14, V27, V18, V13, V21, V29, V35 and V24. All the results were reported as mean ± standard deviation of three independent measurements ($p<0.05$).

Figure 3. The proliferation curves of HCT116 human colon cancer cells after treatment, for 24 and 48 hours, with the seed extracts obtained from white (A and B panel) and red (C and D panel) grape samples were shown. In all graphs, growth levels of control cells were also indicated (CNT). All the data were expressed as mean ± standard deviation of three independent measurements ($p<0.01$).

Supplemental material 1 caption

The proliferation curves of HCT116 human colon cancer cells after treatment, for 24 and 48 hours, with the seed extract V13 at different concentrations (2, 4, 6, 8, 10 and 12 μL/Ml) were shown. In the graph, growth levels of control cells were also indicated (CNT). All the data were expressed as mean ± standard deviation of three independent measurements ($p<0.01$).

Table captions

Table 1. For each accession, sample code, berry color (r: red; w: white), traditional name and effective identity, revealed by microsatellite analysis (Gismondi et al., 2014), were reported; (n.p.: new genetic profiles).

Table 2. Qualitative and quantitative data about the secondary metabolites present in the skins of red berry accessions (V1, V2, V6, V10, V12, V15, V16, V19, V22, V23, V25, V30, V31 and V34) were shown. In particular, anthocyanins, phenolic acids and stilbenes were analyzed by LC-MS; the retention time (t_R) and the monitored ions, in negative [MH^-(M/Z)] or positive [MH^+(M/Z)] mode, were reported. MAL: malvidin; PEO: peonidin; CIA: cyanidin; DEL: delphinidin; PET: petunidin. The total amount of the various molecular classes was also indicated (in italics) for each sample.

[a] Quantified as mg of malvidin-3-*O*-glucoside equivalent kg^{-1} of fresh skin weight.

[b] Quantified as mg of gallic acid equivalent kg^{-1} of fresh skin weight.

[c] Quantified as mg of resveratrol glucoside equivalent kg^{-1} of fresh skin weight.

Table 3. Qualitative and quantitative data about the secondary metabolites present in the skins of white berry accessions (V3, V4, V5, V7, V8, V9, V11, V13, V14, V17, V18 and V20) were shown. In particular, phenolic acids, stilbenes and flavonols were analyzed by HPLC-MS; the retention time (t_R) and the monitored ions, in negative [MH^-(M/Z)] mode, were reported. The total amount of the various molecular classes was also indicated (in italics) for each sample.

[b] Quantified as mg of gallic acid equivalent kg^{-1} of fresh skin weight.

[c] Quantified as mg of resveratrol glucoside equivalent kg^{-1} of fresh skin weight.

Table 4. Qualitative and quantitative data about the secondary metabolites present in the skins of white berry accessions (V21, V24, V26, V27, V28, V29, V32, V33, V35, V36 and V37) were shown. In particular, phenolic acids, stilbenes and flavonols were analyzed by LC-MS; the retention time (t_R) and the monitored ions, in negative [MH^-(M/Z)] mode, were reported. The total amount of the various molecular classes was also indicated (in italics) for each sample.

[b] Quantified as mg of gallic acid equivalent kg^{-1} of fresh skin weight.

[c] Quantified as mg of resveratrol glucoside equivalent kg^{-1} of fresh skin weight.

Table 5. HCT116 human colon cancer cells were treated, for 24 and 48 h, with the sample seed extracts. The bioactive effects of the plant extracts, identified with their respective sample codes,

were evaluated by counting dead and alive cells, after Trypan blue staining. On the left, the percentage of proliferation rate was reported for each treatment, after 24 and 48 h, with respect to the control (CNT) that was considered as unit (100%). On the right, the percentage of cell death was indicated for each treatment, after 24 and 48 h, also reporting the basal level of physiological apoptosis detected in the control cells (CNT). All the present results were the mean value of four independent measurements ($p<0.05$).

TABLES

Table 1

Sample code	Berry color	Traditional cultivar name	Effective name
V1	r	Abbuoto 110	Cabernet Sauvignon
V2	r	Abbuoto 1103	Merlot
V3	w	Bombino 140	n.p.
V4	w	Gialep 1103	n.p.
V5	w	Maturano 1103	Maturano Bianco
V6	r	Zampa di Piccione/ Cesanese	Lecinaro
V7	w	Bianco Tenero 1103	n.p.
V8	w	Pandastro	n.p.
V9	w	Passerina	n.p.
V10	r	Olivella	n.p.
V11	w	Pampanaro	Pampanaro
V12	r	Cerasola	Olivella Nera di Selvi
V13	w	Maturano Bianco C	Maturano Bianco
V14	w	Pampanaro C	Pampanaro
V15	r	Lecinaro C	n.p.
V16	r	Maturano Nero C	Merlot
V17	w	Capolongo C	Capolongo
V18	w	Mecella C	Mecella
V19	b	Uva Giulia S. Annarita	n.p.
V20	w	Maturano Bianco S.Annarita	Maturano Bianco
V21	w	Uva da Tavola	Zimavacca
V22	r	Vitigno α	Pedicello Rosso
V23	r	Cabernet Sauvignon	Cabernet Sauvignon
V24	w	Chardonnay	Chardonnay
V25	r	Cabernet	Carmenere
V26	w	Maturano Gab	Maturano Bianco
V27	w	Mostoso o Pampanaro	Pampanaro
V28	w	Tustarella	n.p.
V29	w	Mustosa	Pampanaro
V30	r	Scrocchiarella	Varousset Noir
V31	r	Barbera Paesana	Jacquez
V32	w	Tostarella	n.p.
V33	w	Uva Rosa	n.p..
V34	r	Uva Americana	n.p.
V35	w	Uva Mecella	Mecella
V36	w	Tostarello Bianco	Capolongo
V37	w	Verdacchio	n.p.

Table 2

ANTHOCYANINS	t_R	MH^+(M/Z)	V1	V2	V6	V10	V12	V15	V16	V19	V22	V23	V25	V30	V31	V34
MAL[a]	39.34	331	424.65	514.31	0.00	331.57	634.77	290.28	745.31	416.63	379.70	757.74	0.00	608.70	868.62	545.12
MAL-3-*O*-glc	13.49	493	1133.77	754.98	895.44	282.65	1009.23	321.60	1873.27	1579.17	0.00	1310.53	2152.71	200.91	421.28	3520.81
MAL-3-*O*-(6-*O*-acetyl)-glc[a]	27.00	535	1636.69	1008.02	245.73	550.96	1155.14	14.83	1738.19	598.57	326.03	2250.84	1872.96	92.69	645.58	1985.71
MAL-3-*O*-(6-*O*-caffeoyl)-glc[a]	28.46	655	188.92	100.13	0.00	86.26	40.17	7.29	184.69	302.27	6634.96	120.04	398.52	8.77	58.35	472.54
MAL-3-*O*-(6-*O*-*p*-coumaryl)-glc[a]	34.16	639	689.58	934.32	1204.25	1473.78	1181.92	59.28	499.96	1969.66	330.77	167.96	1874.77	100.18	299.78	1898.83
PEO-3-*O*-glc[a]	12.06	463	147.82	267.38	201.40	0.00	238.52	557.03	492.14	185.66	286.85	140.44	225.27	48.42	109.27	372.09
PEO-3-*O*-(6-*O*-acetyl)-glc[a]	24.33	505	136.90	198.23	10.40	0.00	155.66	0.00	315.85	39.34	11.42	172.67	169.29	16.09	125.97	152.98
PEO-3-*O*-(6-*O*-*p*-coumaryl)-glc[a]	33.63	609	115.58	232.73	226.78	66.11	285.41	76.80	146.86	194.04	12.70	22.91	233.11	22.53	59.03	174.48
CIA-3-5-diglc[a]	26.26	611	12.63	91.40	0.00	0.00	113.73	35.14	52.52	113.77	0.00	11.71	282.80	171.45	22.29	194.76
CIA-3-*O*-(6-*O*-acetyl)-glc[a]	19.11	491	0.00	23.40	0.00	0.00	6.23	5.83	0.00	0.00	828.39	4.68	12.92	13.71	65.29	14.89
CIA3-*O*-(6-*O*-*p*-coumaryl)-diglc[a]	28.30	595	5.60	40.33	35.32	3.18	21.80	88.28	20.16	8.18	114.93	1.97	26.52	36.31	13.27	28.90
DEL-3-*O*-glc[a]	15.93	465	58.01	66.10	52.78	0.00	28.27	0.00	47.30	6.69	2068.03	4.22	0.00	21.66	0.00	0.00
DEL-3-*O*-(6-*O*-acetyl)-glc[a]	15.00	507	5.53	23.37	0.00	0.00	20.60	0.63	24.59	7.33	271.76	33.07	96.47	42.15	0.00	36.96
PET-3-*O*-(6-*O*-acetyl)-glc[a]	21.40	521	42.37	85.52	4.37	0.00	71.67	4.08	142.21	34.95	75.83	104.57	245.51	69.71	23.23	207.22
PET-3-*O*-(6-*O*-*p*-coumaryl)-glc[a]	30.35	625	68.05	223.51	84.72	211.03	165.94	73.37	151.47	435.93	128.02	21.09	509.75	180.05	60.16	676.91
TOTAL ANTHOCYANINS			*4666.10*	*4563.73*	*2961.19*	*3005.56*	*5129.05*	*1534.43*	*6434.50*	*5892.20*	*11469.39*	*5124.44*	*8100.61*	*1633.33*	*2772.12*	*10282.22*
PHENOLIC ACIDS	**t_R**	**MH^-(M/Z)**	**V1**	**V2**	**V6**	**V10**	**V12**	**V15**	**V16**	**V19**	**V22**	**V23**	**V25**	**V30**	**V31**	**V34**
CAFTARIC ACID[b]	41.28	311	0.04	0.05	0.00	0.00	0.00	0.02	0.04	0.06	0.05	0.00	0.00	0.00	0.00	0.00
trans- FERULIC TARTARIC ACID[b]	41.71	325	0.07	0.09	0.00	0.00	0.00	0.07	0.06	0.12	0.08	0.00	0.00	0.00	0.00	0.00
5-*O*- FERUROYL QUINC ACID (ep. 1)[b]	36.66	367	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5-*O*- FERUROYL QUINC ACID (ep. 1b)[b]	44.87	367	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
GALLIC ACID[b]	0.95	169	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ELLARGIC ACID[b]	31.10	301	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TOTAL PHENOLIC ACIDS			*0.11*	*0.19*	*0.00*	*0.00*	*0.00*	*0.09*	*0.10*	*0.18*	*0.14*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*
STILBENES	**t_R**	**MH^-(M/Z)**	**V1**	**V2**	**V6**	**V10**	**V12**	**V15**	**V16**	**V19**	**V22**	**V23**	**V25**	**V30**	**V31**	**V34**
RESVERATROL[c]	43.35	227	0.54	0.55	0.46	0.63	0.37	0.39	0.47	0.67	0.93	0.59	0.49	0.59	0.99	0.45
RESVERATROL-*dimer*[c]	44.45	455	0.12	236.89	0.10	443.54	0.09	0.11	0.08	872.58	445.48	0.09	0.13	411.49	509.78	0.12
trans-RESVERATROL-glc	15.78	389	0.02	0.01	0.00	0.01	0.00	0.01	0.01	0.02	0.02	0.01	0.00	0.00	0.01	0.01
PIACETANNOL[c]	1.76	243	0.00	6.42	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TOTAL STILBENES			*0.68*	*243.87*	*0.56*	*444.18*	*0.46*	*0.51*	*0.57*	*873.26*	*446.43*	*0.69*	*0.62*	*412.08*	*510.79*	*0.58*

Table 3

PHENOLIC ACIDS	t_R	MH^-(M/Z)	V3	V4	V5	V7	V8	V9	V11	V13	V14	V17	V18	V20
CAFTARIC ACID[b]	41.28	311	15.88	19.81	8.83	26.96	3.16	1.32	4.63	2.07	17.66	14.04	2.78	3.36
trans- FERULIC TARTARIC ACID[b]	41.71	325	26.63	34.98	18.51	43.99	5.48	3.45	9.94	4.66	23.99	27.68	6.28	6.85
5-*O*- FERUROYL QUINC ACID (ep. 1)[b]	36.66	367	0.15	0.10	0.13	0.59	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00
5-*O*- FERUROYL QUINC ACID (ep. 1b)[b]	44.87	367	6.08	9.89	3.33	11.54	0.95	3.54	5.80	3.46	7.98	8.10	4.15	3.29
GALLIC ACID[b]	0.95	169	0.02	0.02	0.03	0.01	0.01	0.01	0.01	0.05	0.04	0.04	0.04	0.03
ELLARGIC ACID[b]	31.10	301	3.25	1.88	0.93	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TOTAL PHENOLIC ACIDS			*52.01*	*66.69*	*31.76*	*83.08*	*9.60*	*8.33*	*20.40*	*10.24*	*49.67*	*49.86*	*13.26*	*13.53*
STILBENES	**t_R**	**MH^-(M/Z)**	**V3**	**V4**	**V5**	**V7**	**V8**	**V9**	**V11**	**V13**	**V14**	**V17**	**V18**	**V20**
RESVERATROL[c]	43.35	227	0.05	0.05	0.04	0.04	0.14	0.03	0.36	0.03	0.03	0.06	0.15	0.02
RESVERATROL-*dimer*[c]	44.45	455	4.69	3.56	2.23	3.98	2.24	3.22	3.12	2.81	2.64	6.98	5.94	3.35
trans-RESVERATROL-glc	15.78	389	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
PIACETANNOL[c]	1.76	243	13.50	5.00	14.55	0.00	0.00	0.00	0.00	5.04	6.11	13.45	8.68	0.00
TOTAL STILBENES			*18.24*	*8.61*	*8.58*	*4.02*	*2.39*	*3.26*	*3.48*	*15.27*	*8.78*	*20.50*	*14.77*	*3.37*
FLAVONOLS	**t_R**	**MH^-(M/Z)**	**V3**	**V4**	**V5**	**V7**	**V8**	**V9**	**V11**	**V13**	**V14**	**V17**	**V18**	**V20**
RUTIN	7.45	609	22.86	16.38	15.70	14.49	3.26	17.78	13.64	6.25	0.63	2.94	5.26	18.09
QUERCETIN	9.33	301	1.25	2.17	1.28	1.48	3.87	0.23	2.74	0.36	0.14	0.23	0.14	0.33
MYRICETIN	8.13	317	0.13	0.03	0.08	0.02	0.09	0.02	0.10	0.02	0.02	0.02	0.01	0.02
TOTAL FLAVONOLS			*24.24*	*18.57*	*17.05*	*15.99*	*7.22*	*18.03*	*16.48*	*6.62*	*0.78*	*3.19*	*5.40*	*18.44*

Table 4

PHENOLIC ACIDS	t_R	MH^-(M/Z)	V21	V24	V26	V27	V28	V29	V32	V33	V35	V36	V37
CAFTARIC ACID[b]	41.28	311	1.64	0.80	0.29	1.31	1.37	0.33	1.69	3.18	1.87	1.21	2.00
trans- FERULIC TARTARIC ACID[b]	41.71	325	4.75	1.58	0.48	2.54	3.31	0.42	3.39	6.09	3.63	1.93	4.63
5-*O*- FERUROYL QUINC ACID (ep. 1)[b]	36.66	367	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5-*O*- FERUROYL QUINC ACID (ep. 1b)[b]	44.87	367	2.88	1.67	0.21	2.39	1.95	0.46	2.95	3.31	3.56	1.13	2.79
GALLIC ACID[b]	0.95	169	0.04	0.00	0.00	0.01	0.02	0.00	0.01	0.11	0.01	0.01	0.01
ELLARGIC ACID[b]	31.10	301	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TOTAL PHENOLIC ACIDS			*9.31*	*4.06*	*0.98*	*6.25*	*6.64*	*1.21*	*8.04*	*12.68*	*9.06*	*4.28*	*9.43*
STILBENES	**t_R**	**MH^-(M/Z)**	**V21**	**V24**	**V26**	**V27**	**V28**	**V29**	**V32**	**V33**	**V35**	**V36**	**V37**
RESVERATROL[c]	43.35	227	0.02	0.01	0.03	0.02	0.07	0.01	0.05	0.01	0.03	0.01	0.19
RESVERATROL-*dimer*[c]	44.45	455	2.48	0.82	0.19	1.76	5.21	0.24	7.31	6.35	3.43	1.83	5.34
trans-RESVERATROL-glc	15.78	389	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
PIACETANNOL[c]	1.76	243	3.81	0.26	0.00	1.03	4.48	0.19	3.73	6.46	2.00	0.63	2.27
TOTAL STILBENES			*6.32*	*1.09*	*0.21*	*2.80*	*9.76*	*0.44*	*11.09*	*12.82*	*5.46*	*2.46*	*7.80*
FLAVONOLS	**t_R**	**MH^-(M/Z)**	**V21**	**V24**	**V26**	**V27**	**V28**	**V29**	**V32**	**V33**	**V35**	**V36**	**V37**
RUTIN	7.45	609	2.57	2.64	3.92	0.81	10.00	1.41	1.11	11.51	0.46	10.46	13.32
QUERCETIN	9.33	301	0.13	0.29	0.28	0.18	0.27	0.26	0.00	2.18	0.28	0.24	0.25
MYRICETIN	8.13	317	0.00	0.02	0.04	0.01	0.02	0.00	0.00	0.08	0.01	0.03	0.01
TOTAL FLAVONOLS			*2.70*	*2.94*	*4.24*	*1.00*	*10.28*	*1.67*	*1.11*	*13.78*	*0.75*	*10.72*	*13.57*

Table 5

Sample code	Proliferation rate (%)		Cell death (%)	
	24 h	48 h	24 h	48 h
CNT	100	100	4.00	5.13
V1	146.1	91.8	8.00	13.00
V2	123.4	43.3	0.20	7.86
V3	105.8	54.8	7.11	2.96
V4	118.2	36.2	4.20	3.36
V5	77.6	81.4	9.27	4.06
V6	84.6	54.8	2.83	9.73
V7	189.8	87.6	0.00	8.00
V8	113.8	79.1	3.00	13.00
V9	70.5	65.4	12.61	11.70
V10	100.6	31.5	11.00	29.00
V11	112.9	73.4	3.75	5.37
V12	119.2	161.1	0.30	2.00
V13	75.9	37.8	21.00	23.00
V14	88.2	83.1	5.08	2.93
V15	79.4	87.6	7.57	4.48
V16	70.2	25.9	23.00	23.00
V17	115.7	43.4	9.00	24.00
V18	49.4	90.2	7.68	5.01
V19	98.7	23.8	8.00	34.00
V20	67.8	160.7	0.50	1.00
V21	93.1	128.5	4.00	3.00
V22	64.5	14.7	23.00	34.00
V23	140.3	145.9	0.40	2.00
V24	70.2	23.8	9.00	34.00
V25	125.2	71.4	7.00	15.00
V26	57.5	91.3	44.00	30.00
V27	67.8	106.5	15.00	13.00
V28	69.8	42.1	31.00	22.00
V29	78.1	61.3	19.00	15.00
V30	117.1	89.1	0.30	2.00
V31	112.9	118.5	2.26	4.90
V32	84.6	57.5	5.56	9.98
V33	119.5	39.2	0.70	6.00
V34	54.6	100	26.29	4.93
V35	78.7	42.1	51.00	63.00
V36	85.6	90.4	0.20	1.20
V37	33.4	7.1	46.93	22.11

FIGURES

Figure 1

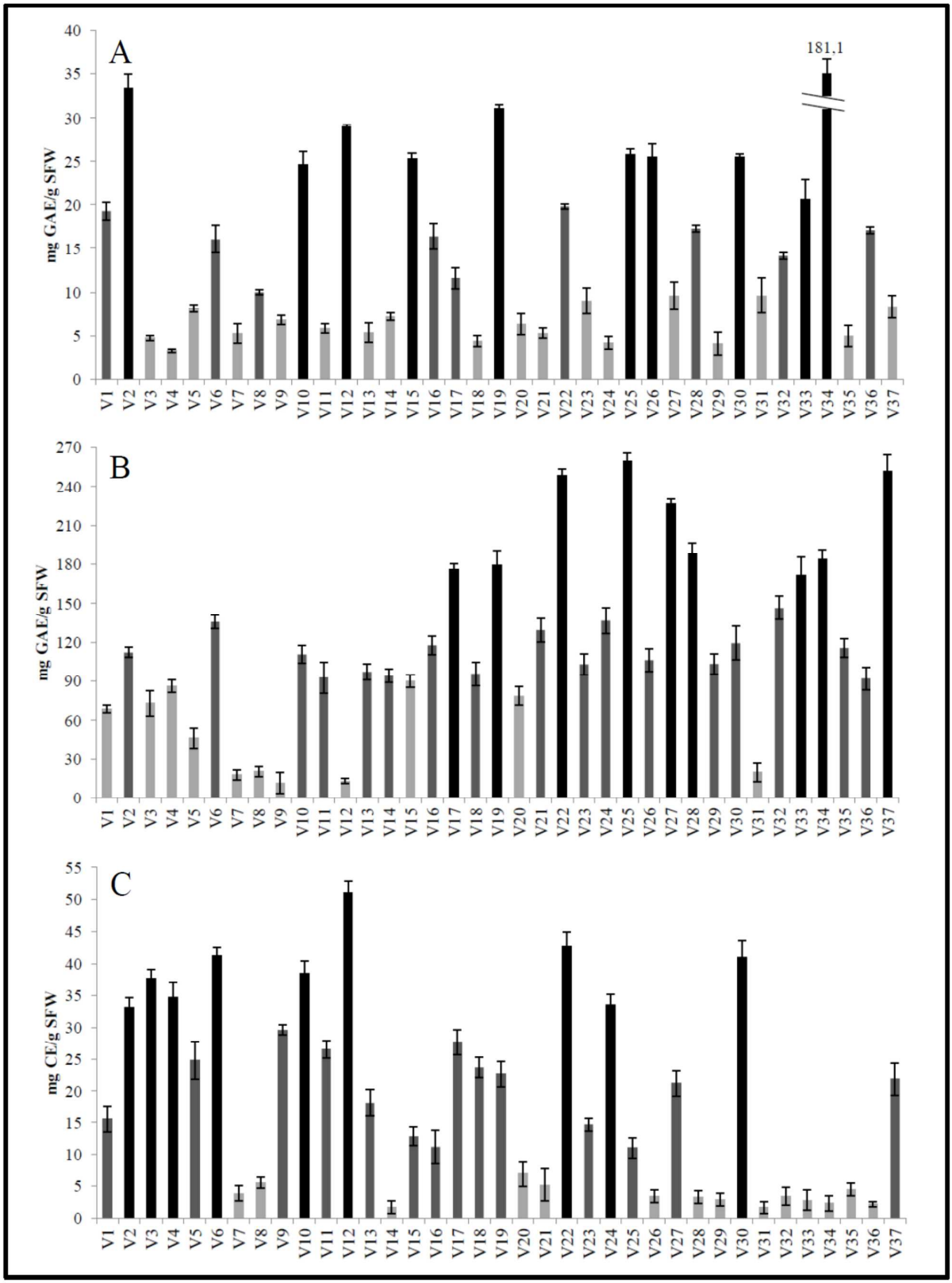

Figure 2

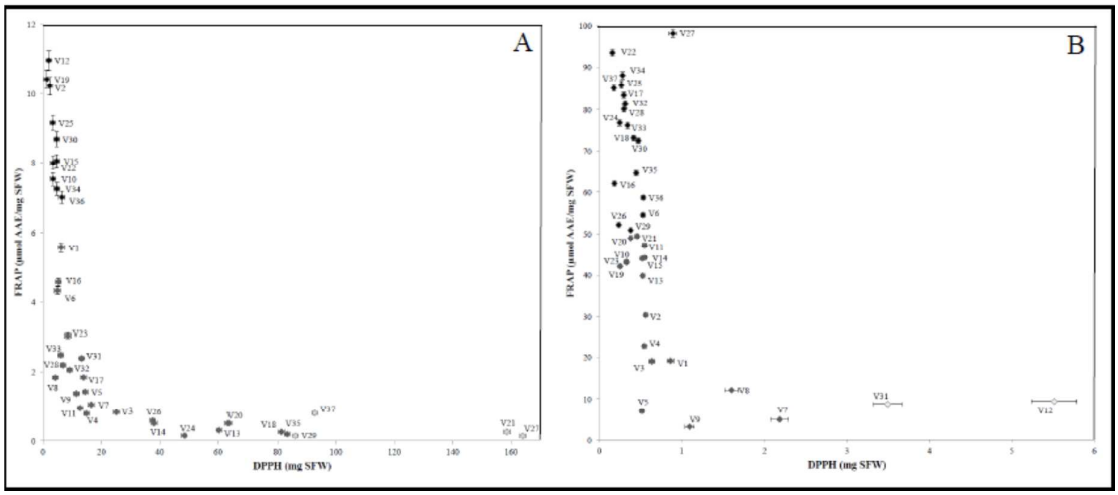

Figure 3