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Antiradical activity of phenolic metabolites extracted from grapes of white and red *Vitis vinifera* L. cultivars

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Summary

A diet rich in plant foods is strongly recommended for its beneficial effect on human health. In fact, plant secondary metabolites may exert various biological activities on mammalian cells. Among them, phenolics are excellent natural antioxidants able to rescue cell redox unbalance responsible for the onset of different pathologies. For these reasons, the present work was focused on the study of grape extracts obtained from eight different Italian *Vitis vinifera* cultivars, quite rare in Italian viticulture and not yet completely chemically characterized. For each preparation, total simple phenolic, flavonoidic and anthocyaninic content was measured through spectrophotometrical assays, while detailed biochemical profile was revealed by LC-MS analyses. In order to valorize the products of these varieties and increase our knowledge about their potential healthy role, the antioxidant power of the samples was evaluated by two different *in vitro* antiradical tests: DPPH and FRAP. Moreover, free radical scavenging properties of eleven grape pure compounds were investigated, with the aim to: a) compare their real antiradical property with the theoretical one; b) identify which one of them possessed the best bioactivity; c) understand how they might singularly contribute to the nutraceutical effect of the whole grapevine phytocomplex.

K e y w o r d s : antioxidant; plant molecules; biochemical profile; grapevines; food quality.

Practical application

Biochemical profile and antioxidant activity of eight white and red Italian *V. vinifera* cultivars was analysed out in order to valorize the nutraceutical power of their products, already existing in commerce. The antiradical power of pure plant molecules, responsible for grape and wine beneficial effect on human health, was theoretically and experimentally investigated to clarify the role of these compounds in grapevine phytocomplex and respect to free radicals generated in mammalian cells.

Introduction

During the *millennia*, plants developed some peculiar devices in order to adapt themselves to the Earth's life (Bohnert *et al.* 1995). Among them, surely, the skill to produce secondary metabolites can be considered the most important plant evolutionary feature (Wink 2003).

Secondary metabolites are natural compounds whose synthesis occurs only in plant cells by specific metabolic pathways (*i.e.* Shikimic acid pathway). These molecules can be easily grouped in classes, according to their chemical structures. The three principal clusters of secondary metabolites are phenolics, containing the phenol as elementary building block, alkaloids, that are characterized by the presence of nitrogen heterocyclic rings, and terpenoids, whose basal unit is represented by the isoprene (Croteau *et al.* 2000).

The sessile nature of the plant organisms essentially forced them to produce the secondary metabolites as key components of survival mechanisms against terrestrial adverse conditions and mobile predators and parasites. In fact, these compounds are able to protect plants from biotic and abiotic environmental stresses and to promote their reproduction and propagation (Bourgaud *et al.* 2001, Edreva *et al.* 2008). In particular, the antioxidant activity is one of the principal functions carried out by the secondary metabolites in plant tissues (Agati *et al.* 2012).

Animal and plant cell homeostasis and stability is finely regulated by balanced oxidative and reducing processes. Consequently, the alteration of this *equilibrium*, a pathological condition known as oxidative stress, can determine modification of cellular functions and induction of cell structural damage and death. Therefore, the amount of reactive species (RS) and antiradical compounds is always monitored and maintained constant in living organisms (Devasagayam *et al.* 2004, Ahmad 2012).

The previous observations support how RS are necessary for cells and do not represent dangerous agents in adequate concentrations. As reported in literature, indeed, they carry out important cell functions: for example, they represent important intracellular second messengers and are also the real executors of macrophage antimicrobial activity (Mittler 2002, Fang 2004). On the other hand, stress,

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smoke, toxic substances, metabolic defects, specific genetic alterations, etc. were demonstrated to be responsible for the increase of RS production in animal cells with consequent tissue degeneration. In fact, RS are extremely unstable molecules that, lacking of an electron, rapidly react with different cellular targets, to turn off their necessity of charge, and consequently transform them in new active radicals. Thus, these events trigger chain reactions that terminate producing various irreversible damages in cells (Devasagayam *et al.* 2004). For these reasons, a diet rich in fruits and vegetables is highly recommended to prevent the onset of different pathologies and to rescue *redox* unbalances (Pandey and Rizvi 2009). Similarly, plants subjected to environmental critical conditions (*i.e.* pollution, UV radiations, drought) were observed to up-regulate their antioxidant defenses, both enzymatic (*i.e.* catalase, superoxide dismutase) and metabolic (*i.e.* flavonoids), in order to reduce RS levels and recover their original healthy state (Korkina 2007, Di Marco *et al.* 2014, Giovannini *et al.* 2016).

The free radical scavenging activity of the plant metabolites is mainly due to four factors: a) amount, typology and position of the chemical groups conjugated to the phenol rings; b) steric obstacle of the reducing molecule; c) interaction capacity between radical and antioxidant; d) reaction mechanism. The phenols represent the most antiradical class of the plant secondary metabolites and, certainly, they also are the best ones among all the natural compounds. In fact, the radical phenols that generate after reduction of RS are exceptionally less reactive and more metabolizable than the other classical radicals, interrupting the propagation of the oxidative chain and its relative consequences. This phenomenon is principally due to the resonance effect: the capacity of an aromatic ring to distribute a radical charge among its various carbon atoms. In fact, the continuous delocalization of the radical in this special chemical structure causes dissipation and reduction of its reactivity (Rice-Evans *et al.* 1997, Wright *et al.* 2001, Decker 2008, Dai and Mumper 2010). In general, more stable is a radical phenol and more antioxidant will be the molecule that contains it. The stability of the radical phenols depends on its substituent groups and their capacity to soften the reactive charge by increase of resonance structures (*i.e.* additional double bonds and aromatic rings), mesomeric effect (electron delocalization and redistribution, $Mes^{+/-}$) and inductive power (electronegativity of the atoms, $Ind^{+/-}$) (Hepworth *et al.* 2002).

Since the Roman Age, the grapevine has been one of the most important and diffused crops in the world because of its economic, nutritive and cultural value (Zohary *et al.* 2012). From 2009 to 2015, on average, the Italian wine production represented the 17.5 % of the world total product, suggesting how the *Vitis vinifera* L. species covers a central role in the financial system of this country (Castriota 2015, OIV 2015). Nowadays, in Italy, only about 500 varieties of grapevines have been genetically identified and registered at national level, while many others are still unknown or ambiguous. Therefore, scientific papers which describe new vine varieties or characterize chemistry, genetics and bioactivity of those not yet studied are fundamental.

According to all these observations, principal object of the present research was the study of *in vitro* antioxidant properties of different plant secondary metabolites detectable in *Vitis vinifera* L. berries. Our interest in this type of research was stimulated by the great amount of literature data that associates the bioactivity of grapevine products (*i.e.* antineoplastic, neuroprotective, cardioprotective effects) to the free radical scavenging function of their compounds without clarifying the potential reducing power of each one of them (Leifert and Abeywardena 2008, Garcia-Alonso *et al.* 2009, Pandey and Rizvi 2009, Dai and Mumper 2010, Flamini *et al.* 2013a, Ongaratti *et al.* 2014). Therefore, the phenolic profile of 4 red ('Sangiovese', 'Bonamico', 'Montepulciano' and 'Albarossa') and 4 white ('Malvasia Bianca di Candia', 'Bellone', 'Malvasia del Lazio' and 'Trebbiano Giallo') grapevine cultivars, genetically well-known but quite rare in Italian viticulture, was determined by spectrophotometrical measures and LC-MS analyses and the antiradical activity of their extracts was evaluated by two different assays (DPPH and FRAP). In literature, indeed, only a partial phenolic composition of 'Sangiovese' and 'Montepulciano' berries or wines is reported (Gambelli and Santaroni 2004, Sagrantini *et al.* 2012, Filippetti *et al.* 2013). Moreover, antiradical tests were also performed on the pure molecules identified in the grape samples, in order to discriminate which one of them was theoretically and effectively characterized by the best antioxidant activity and how they could singularly contribute, both endogenously in plants and in animal cell (*i.e.* by diet), to the protective antiradical functions of the grape phytocomplex.

Material and Methods

Sample collection: *Vitis vinifera* L. plants, belonging to 'Sangiovese' (S), 'Bonamico' (B), 'Montepulciano' (M), 'Albarossa' (A), 'Malvasia Bianca di Candia' (MBC), 'Bellone' (BL), 'Malvasia del Lazio' (ML) and 'Trebbiano Giallo' (TG) Italian cultivars, were cultivated and grown in the Botanical Gardens of Rome "Tor Vergata". The genetic identity of these varieties was confirmed by microsatellite analysis, according to the methods widely described in our previous work (Gismondi *et al.* 2014). For each variety, in summer 2015, a total of twelve mature bunches (identified measuring their sugar content between 15-20 %, using a AYHF-refractometer Brix grapes ATC), without symptoms of pathology, were harvested from four different adult grapevine plants. Samples were rapidly processed in order to avoid the degradation of their secondary metabolites.

Extraction of secondary metabolites: Grapes were firstly weighed and then homogeneously crushed and positioned into a stove, at 45 °C for 48 hours, to allow the water to evaporate. The residual epicarps and pulps were finely powdered with liquid nitrogen by mortar and pestle. Then, as suggested by Wang and Weller (2006), the plant material was subjected to Soxhlet extraction. Briefly, 50 g of sample was added to 200 mL of solvent for 24 hours at 70 °C. In particular, the extraction solvent was ethyl-acetate:methanol:water (60:30:10, v/v/v) and ethanol:water:hydrochloric acid (70:30:1, v/v/v), respectively, for white and red berry samples, as reported in Baydar *et al.* (2004) and Mattivi *et al.* (2006). The extract was concen-

trated, dissolving the solvent, using a rotary evaporator (Buchi Rotavapor EL 130, Brinkman Instruments). Finally, the pellet was resuspended in 10 mL of acidified methanol (pH 3.5, HCl).

Determination of simple phenol, flavonoid and anthocyanin content: The phenolic content of the grape samples was measured, according to the Folin-Ciocalteu method (Singleton and Rossi 1965) opportunely modified as reported in Gismondi *et al.* (2013). Briefly, 1 mL of extract was incubated, for 1 hour in the dark, with 1 mL of 2 N Folin-Ciocalteu reagent (Sigma-Aldrich, Italy), 10 mL of 7 % (w/v) Na_2CO_3 and 13 mL of ddH_2O. Then, the absorbance of the solution was read at 725 nm using a Cary 50 Bio UV-visible spectrophotometer (Varian). The amount of flavonoids was estimated following the method described in Chang *et al.* (2002). Five hundred µL of extract were incubated, for 30 minutes in the dark, with 100 µL of 10% $AlCl_3$, 100 µL of 1M CH_3CO_2K, 1.5 mL of methanol and 2.8 mL of ddH_2O. Then, the absorbance of the solution was spectrophotometrically read at 415 nm. Finally, the anthocyanin concentration was calculated using the pH-differential method described in Wrolstad (1993). Briefly, 200 µL of extract were opportunely diluted (1:10) in two buffer solutions: potassium chloride buffer (0.025 M, pH 1.0) and sodium acetate buffer (0.4 M, pH 4.5). The two samples were incubated for 15 min in the dark and, then, spectrophotometrically analyzed at 520 nm and 700 nm. The final absorbance and the concentration of monomeric anthocyanins were obtained according to the formulas reported in Stanciu *et al.* (2010). Pure gallic acid, quercetin and malvidine-3-*O*-glucoside standards were used to create calibration curves (according to the methods previously described) that, in that order, were applied for the quantification of total phenols, flavonoids and anthocyans in the samples. Results were expressed, respectively, in µg of gallic acid equivalent (µg GAE), µg of quercetin equivalent (µg QE) and µg of malvidine-3-*O*-glucoside equivalent (µg M3GE) per g of sample fresh weight (g SFW).

Liquid Chromatography-Mass Spectrometry (LC-MS) analysis: The phenolic profiles of the samples were carried out using an LC system associated with a LC-20AD pump, a CBM-20A controller, a SIL-20a HT auto-sampler, a diode array SPD-M20A, a LC-mass spectrometer 2020 single quadrupole, an electro-spray ionization (ESI) source in positive and negative ion modes and a LABSOLUTION software for the data acquisition (Shimadzu, Tokyo, Japan). The detection of phenolic acids (gallic acid, *p*-coumaric acid, caffeic acid and chlorogenic acid), stilbenes (*trans*-resveratrol), flavonoids (myricetin, quercetin and kaempferol) and anthocyanins (malvidin-3-*O*-glucoside, cyanidin-3,5-*O*-diglucoside and peonidin-3-*O*-glucoside) were performed following the exact conditions and the same parameters (column, gradient elution, mobile phases, injection volume, etc.) reported in Impei *et al.* (2015). The identification of each plant molecule was carried out comparing their mass spectra and retention times with those obtained by the respective standards (Sigma-Aldrich, Milan, Italy) and published in literature (Sun *et al.* 2007, Flamini *et al.* 2013b). The concentration of these compounds was also obtained creating appropriate calibration curves with the same pure standards. Data were expressed as µg of the respective standard equivalent per g of fresh skin weight (µg SE/g SFW).

In vitro antiradical assays: DPPH and FRAP: The 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) test was performed according to the spectrophotometrical method described by Brand-Williams *et al.* (1995) and adequately modified as reported in Impei *et al.* (2015). The radical scavenging activity was expressed as $1/IC50_{DPPH}$ value, that is the reciprocal of the milligrams of plant sample fresh weight (1/mg SFW) necessary to reduce the 50 % of a 60 µM $DPPH^{\bullet}$ radical solution. The Fe^{3+} 2,4,6-tripyridyl-S-triazine (FRAP) assay was carried out following the technical procedure showed in Benzie and Strain (1999) and the relative modifications reported in Gismondi *et al.* (2012). For this assay, the antioxidant power was measured as µmol of ascorbic acid equivalents per mg of sample fresh weight (µmol AAE/mg SFW), according to a calibration curve adequately created with pure ascorbic acid. DPPH and FRAP tests were also carried out in presence of standard samples containing pure secondary metabolites (gallic acid, *p*-coumaric acid, caffeic acid, chlorogenic acid, *trans*-resveratrol, myricetin, quercetin, kaempferol, malvidin-3-*O*-glucoside, cyanidin-3,5-*O*-diglucoside and peonidin-3-*O*-glucoside; Sigma-Aldrich, Milan, Italy) at different concentrations (0-300 µM). In these last cases, results were expressed, respectively, as percentage amount of radical $DPPH^{\bullet}$ and oxidized Fe^{3+} that, compared to the original radical solution (considered as unit, 100 %), still remained in the preparation after the reaction occurred.

Statistics: All the experiments were performed in triplicate and the data were expressed as means ± standard deviation (sd) of the three independent repetitions. Significance was calculated by one-way ANOVA test (p values < 0.05 were considered significant).

Results and Discussion

First of all, the amount of simple phenols and flavonoids was measured in all samples by spectrophotometric evaluation. As indicated in Fig. 1A, the levels of simple phenolic compounds varied between 1314 and 1403 µg GAE/g SFW for MBC, TG, S, B, M and A cultivars, while they were strongly lower in BL (774 µg GAE/g SFW) and ML extracts (867 µg GAE/g SFW). A similar trend was obtained in the flavonoidic analysis (Fig. 1B). This class of secondary metabolites was estimated to be of 1052, 580, 650, 1024, 1122, 1008, 1112 and 920 µg QE/g SFW, respectively, for MBC, BL, ML, TG, S, B, M and A berry preparations. Considering that our extracts were prepared starting from whole pericarps, including seeds, the obtained data can be considered consistent with those described in other works (Rockenbach *et al.* 2011, Impei *et al.* 2015). In conclusion, we observed a constant phenolic concentration in all samples, except that BL and ML which approximately presented a halved content of these molecules. On the other hand, the anthocyanins were detected only in red berry varieties (Fig. 1C), as scientifically supported by Boss *et al.* (1996). In particular, B, M and A extracts revealed, in

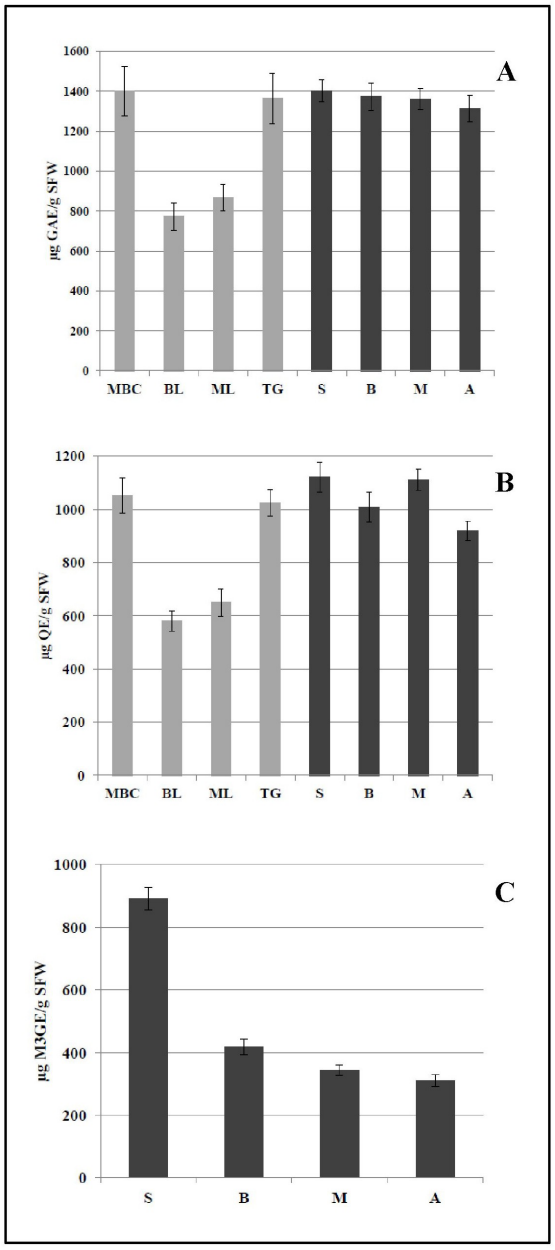

Fig. 1: Spectrophotometrical analyses of plant secondary metabolites. The total amount of phenolic (**A panel**), flavonoidic (**B panel**) and anthocyaninic (**C panel**) compounds were measured in the grape extracts. Data were reported as µg of standard equivalent (GAE, QE and M3GE) per g of sample fresh weight (SFW). In all panels, the light grey bars indicate white varieties, while the dark grey columns represent the red ones ($p < 0.01$ *vs.* S sample).

that order, 419, 344 and 311 µg M3GE/g SFW, while the S sample extraordinarily outdid the others (892 µg M3GE/g SFW). These concentrations are excellent if compared to those identified by Liang *et al.* (2008) in skin pure extracts of different *Vitis* species.

In order to determine the biochemical profile of the samples, each extract was subjected to LC-MS analysis. The molecular spectra of plant secondary metabolites detected in the various *V. vinifera* cultivars were reported in the Table. Among the phenolic acids, gallic acid was the most abundant molecule in all white varieties, with remarkable peaks in MBC (117.22 µg SE/g SFW) and TG (87.03 µg SE/g SFW), while it was present in lower concentration in the red ones, ranging between 7.13 and 14.82 µg SE/g SFW. On the contrary, an opposite distribution in white and red samples was shown by chlorogenic, *p*-coumaric and caffeic acids. However, the amount of these last compounds was not so prominent, varying from 1.46 to 12.52 µg SE/g SFW among the different grape preparations. In general, *p*-coumaric acid was discovered to be the less copious phenolic acid, even reaching the not detected level in MBC, BL, S and B extracts. It should be also noted how *p*-coumaric/caffeic acids and chlorogenic/caffeic acids were not identified in BL and ML samples, respectively, probably due to their low concentrations (under LC-MS detection threshold). Moreover, our results indicated a wide presence of flavonoids in the extracts. In particular, myricetin concentration varied between 3.62 and 28.3 µg SE/g SFW, while kaempferol level was detectable in the range 3.33-31.09 µg SE/g SFW. However, quercetin was the most appreciable flavonoidic compound revealed in all samples, amounting to 73.59, 77.54 and 100.86 µg SE/g SFW in MBC, S and M, respectively. In conclusion, MBC and TG were the richest samples of phenolic acids and flavonoids among the white grapevines analyzed in the current study, while S and M among the red ones. On the other hand, as expected and also previously demonstrated by our spectrophotometrical test, the anthocyanins were found only in the berries of red *V. vinifera* varieties. Malvidin-3-*O*-glucoside was the anthocyanin that mainly characterized our extracts: indeed, its levels ranged from 118.48 to 483.74 µg SE/g SFW. Cyanidin-3,5-*O*-diglucoside was principally identified in S (13.76 µg SE/g SFW) and M (5.2 µg SE/g SFW) samples, while peonidin-3-*O*-glucoside was just detectable in traces. Finally, among the stilbenes, we focused our attention on *trans*-resveratrol. It was revealed in all samples, even if its concentration was higher in red extracts with respect to white ones. All these qualitative and quantitative data are quite in accordance with the scientific literature reporting similar analyses on different *V. vinifera* varieties (Cantos *et al.* 2002, Chira *et al.* 2008, Impei *et al.* 2015), considering that our samples were produced starting from the whole *V. vinifera* berries, as occurs during vinification process, and not just privileging grape skins or seeds. The direct comparison between our results and those described in Gambelli and Santaroni (2004), Sagrantini *et al.* (2012) and Filippetti *et al.* (2013), the only works that have characterized two of our varieties ('Sangiovese' and 'Montepulciano', as previously mentioned), revealed that the grapes analyzed in the current research were surely more abundant in secondary metabolites with respect to the relative samples reported in these other studies. This phenomenon can be easily explained by the fact that grape metabolic profile is deeply determined from the environmental features where vines grow (De la Cerda-Carrasco *et al.* 2015). With great surprise, our samples resulted to possess a higher, or even similar, malvidin-3-*O*-glucoside content with respect to extracts obtained from four famous wine grape cultivars: 'Vranec', 'Cabernet Sauvignon', 'Merlot' and 'Pinot Noir' (Dimitrovska *et al.* 2011). On the contrary, the other detected anthocyanins were strongly lower in concentration if compared to those same reference varieties. Interestingly, we found that the level of gallic acid identified in the

Table

Metabolic profile of grape extracts from white and red *Vitis vinifera* varieties

Plant secondary metabolites	Chemical features		*Vitis vinifera* ecotypes							
			White cultivars					Red cultivars		
	tR	[M-H]	MBC	BL	ML	TG	S	B	M	A
Phenolic acids (μg/g SFW)										
Gallic acid	5.95	169^{-}	117.22 ± 3.22	39.33 ± 1.08	38.01 ± 1.05	87.03 ± 2.39	14.82 ± 0.61	7.13 ± 0.29	10.15 ± 0.5	10.16 ± 0.46
Chlorogenic acid	21.4	353^{-}	2.54 ± 0.09	4.62 ± 0.16	n. d.	1.46 ± 0.05	12.52 ± 0.63	8.67 ± 0.52	13.64 ± 0.68	2.34 ± 0.08
p-Coumaric acid	34.5	163^{-}	n.d.	n.d.	2.68 ± 0.11	1.65 ± 0.06	n.d.	n.d.	3.80 ± 0.11	3.24 ± 0.15
Caffeic acid	23.9	179^{-}	2.4 ± 0.05	n.d.	n.d.	2.12 ± 0.05	6.39 ± 0.19	9.19 ± 0.46	7.23 ± 0.26	5.72 ± 0.3
MEAN TOTAL			*122.16*	*43.95*	*40.69*	*92.26*	*33.73*	*24.99*	*34.82*	*21.46*
Flavonoids (μg/g SFW)										
Myricetin	46.5	317^{-}	28.3 ± 0.45	4.34 ± 0.55	3.62 ± 0.62	22.55 ± 0.5	14.94 ± 0.67	10.78 ± 0.46	9.21 ± 0.45	8.59 ± 0.43
Quercetin	50.57	301^{-}	73.59 ± 1.3	10.52 ± 0.19	8.18 ± 0.09	39.21 ± 0.69	77.54 ± 3.87	14.33 ± 0.83	100.86 ± 6.05	11.18 ± 0.53
Kaempferol	53.0	285^{-}	13.07 ± 0.38	9.17 ± 0.27	3.33 ± 0.1	5.26 ± 0.15	9.49 ± 0.59	14.56 ± 0.19	31.09 ± 1.63	4.61 ± 0.2
MEAN TOTAL			*114.96*	*24.03*	*15.13*	*67.02*	*101.97*	*39.67*	*141.16*	*24.38*
Anthocyanins (μg/g SFW)										
Malvidin-3-*O*-glucoside	13.49	493^{+}	n.d.	n.d.	n.d.	n.d.	483.74 ± 26.9	233.40 ± 12.83	118.48 ± 5.92	149.98 ± 6.75
Cyanidin-3,5-*O*-diglucoside	26.26	611^{+}	n.d.	n.d.	n.d.	n.d.	13.76 ± 0.82	1.79 ± 0.07	5.20 ± 0.31	0.73 ± 0.0
Peonidin-3-*O*-glucoside	12.06	463^{+}	n.d.	n.d.	n.d.	n.d.	0.23 ± 0.01	0.13 ± 0.004	0.16 ± 0.004	0.40 ± 0.02
MEAN TOTAL			*0.0*	*0.0*	*0.0*	*0.0*	*497.73*	*265.32*	*123.84*	*151.11*
Stilbenes (μg/g SFW)										
trans-Resveratrol	44.4	227^{-}	0.41 ± 0.03	0.24 ± 0.04	0.11 ± 0.03	0.33 ± 0.02	0.79 ± 0.03	0.59 ± 0.02	0.56 ± 0.04	1.02 ± 0.07

white varieties was totally comparable, or superior, to 'Chardonnay' and 'Merlot' samples (Yilmaz and Toledo 2004). These biochemical evidences valorize the grapevine cultivars described in the present work and consequently may promote diffusion, application and demand of their products.

Several scientific works reported how the diet consumption of wine and grape may favor the introduction in the human body of a large amount of plant secondary metabolites with healthy effects. In fact, grapevine phytocomplex was demonstrated to possess a great bioactivity on both *in vitro* and *in vivo* experimental systems (Leifert and Abeywardena 2008, Rodrigo *et al.* 2011). Therefore, the different classes of phenolic molecules and their relative concentrations strongly affect quality and beneficial properties of the grape products. However, among all the possible biological functions carried out by *V. vinifera* compounds, the free radical scavenging one probably is the most important with respect to human health. Indeed, the necessity to contrast, soften and rescue the oxidative stress, induced by RS in tissues, is a vital and undelayable function that mammalian cells largely accomplish by food antioxidants, such as plant phenolics. For all these reasons, we also decided to measure the antiradical activity of our samples by two different assays: DPPH and FRAP tests (Fig. 2). In general, the red grape extracts revealed a greater antiradical power compared to the white ones, probably due to their elevated concentration of anthocyanins. The best antioxidant activity was performed by the S sample ($1/IC50_{DPPH}$: 1.88 mg SFW; FRAP: 4.67 μmol AAE/mg SFW), strongly rich in malvidin-3-*O*-glucoside, followed, in that order, by M, B and A. In fact, the M variety, although the least abundant of the red cultivars in total anthocyanins, presented the highest level of flavonoids (Table). Among the white extracts, as expected according to their content of phenolic acids and flavonoids, MBC and TG were the most antiradical samples, while ML revealed a lower free scavenging activity ($1/IC50_{DPPH}$: 0.53 mg SFW; FRAP: 1.82 μmol AAE/mg SFW). In particular, MBC was the only white variety that showed an antioxidant power quite comparable to that obtained with red varieties. These results were in accordance with other scientific data and associated a good antioxidant activity to the present extracts (Impei *et al.* 2015).

Several works report the contribution of total phenols to the biological activities

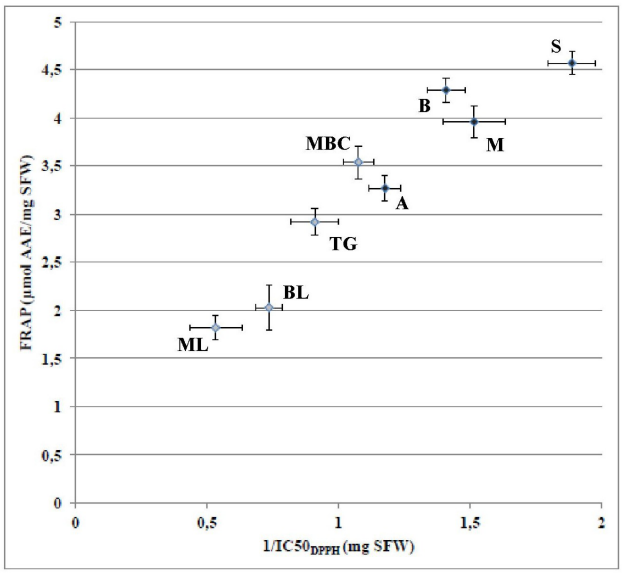

Fig. 2: Antioxidant activity of grape extracts. The free radical scavenging propertiy of the grape samples was expressed in the current graph as the combination of the data obtained in FRAP (y-axis; µmol AAE per mg SFW) and DPPH (x-axis; $1/IC50_{DPPH}$) assays. White variety samples were represented as light grey circles, while the red ones were shown as dark grey circles ($p < 0.05$ *vs.* S sample).

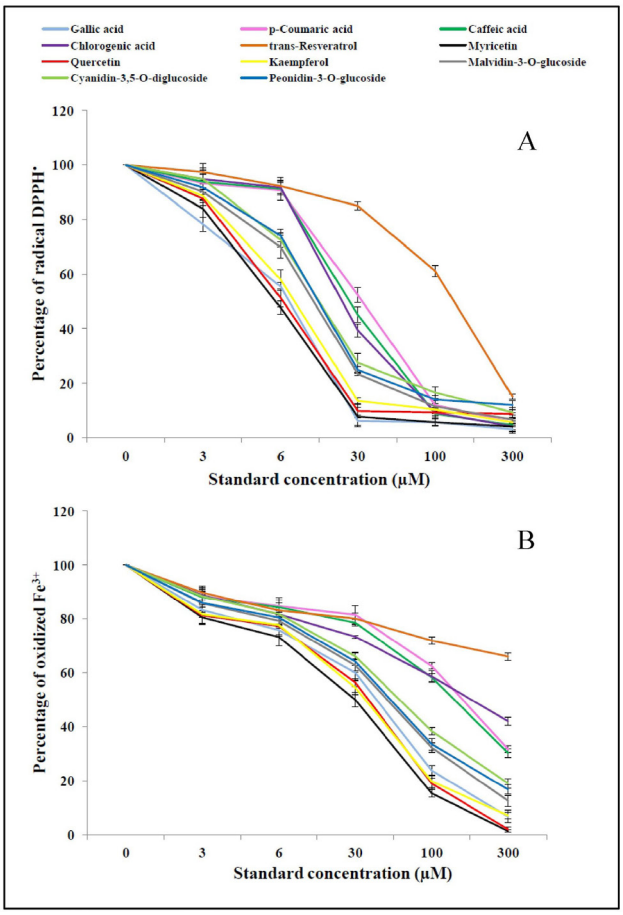

Fig. 3: Antioxidant activity of grape pure metabolites. The antiradical property of grape pure metabolites was evaluated by DPPH (**A panel**) and FRAP (**B panel**) assays. Graphs expressed the antioxidant power of each plant standard (0-300 µM) as percentage reduction of the reactive species (DPPH· and Fe^{+3}) present in the original radical solution, considered as unit (100 %) ($p < 0.05$ *vs.* myricetin).

(*i.e.* antioxidant property) of grape and wine introduced by diet (Garcia-Alonso *et al.* 2009, Dai and Mumper 2010, Rodrigo *et al.* 2011, Ongaratti *et al.* 2014), flying over the specific influence exerted by each one of their single components. For this reason, we also decided to perform DPPH and FRAP tests directly on the pure secondary metabolites previously detected in *V. vinifera* samples, to individuate the most antioxidant ones and produce new insights about their antiradical role in the grape phytocomplex. A similar approach was rarely carried out in literature; however, the few articles describing it analyzed plant molecules not evaluated in the current study or used different antiradical assays (Soobrattee *et al.* 2005, Cheng *et al.* 2006, Mishra *et al.* 2012, Hajimehdipoor *et al.* 2014, Koroleva *et al.* 2014). The percentage reduction of DPPH and FRAP radical solutions was calculated after incubation with various concentration (0-300 µM) of grapevine pure compounds, with respect to their initial concentration of oxidant species (DPPH· and Fe^{+3}) considered as unit (100 %, concentration 0) (Fig. 3). Both experiments showed very analogous outputs, indicating how they may be considered exchangeable between themselves and not differently informative. In general, in both cases, the antioxidant activity of the standards was dose-dependent. However, while DPPH assay (Fig. 3A) was able to better distinguish the antiradical power of the metabolites when used in low doses (0-30 µM), FRAP test (Fig. 3B) resulted to be more discriminating in presence of high concentrations (100-300 µM) of plant molecules. In the two analyses, four different clusters of standards could be identified according to their similar free radical scavenging properties. The best antioxidant group included myricetin, gallic acid, quercetin and kaempferol. The second class, rather antiradical as the first one, was made up of malvidin-3-*O*-glucoside, cyanidin-3,5-*O*-diglucoside and peonidin-3-*O*-glucoside. Caffeic, *p*-coumaric and chlorogenic acids composed the third cluster, showing a lower bioactivity than the others. Finally, unexpected in relation to its well-known healthy potential, *trans*-resveratrol presented the worst antioxidant property among all. These results are extremely coherent with those obtained in DPPH and FRAP assays performed on the grapevine extracts (Fig. 2); the antioxidant properties of these samples highly reflected their specific content in secondary metabolites. In particular, according to the data shown in Fig. 3, we were able to: i) associate the great antiradical power of all the red varieties to their anthocyanin concentration; ii) justify the best free radical scavenging activity of S and M samples, among the red cultivars, with their higher level of flavonoids; iii) explain the major bioactivity of MBC and TG varieties, among the white ones, thanks to their abundance in flavonoids and gallic acid.

In the last part of our study, we wanted to verify if the antioxidant properties of pure standards extrapolated from *in vitro* experiments (Fig. 3) could be in accordance with those predicted for the same molecules by theoretical chemistry. For this purpose, we classified the plant compounds for their antiradical capacity, just on the basis of the molecular structure (Soderberg 2016). Flavonoids were considered

the best antiradical molecules thanks to their aromatic rings; the minimal differences among them were essentially connected to presence and distribution of hydroxyl groups able to stabilize radical species with a Mes^{+} effect. Anthocyanins, possessing multiple resonance structures as myrcitin, quercetin and kaempferol, were reputed good antioxidants but the positive charge located on the oxygen atom of the heterocyclic ring, determining Ind^{-} phenomena, reduced its free radical scavenging power with respect to flavonoids. Moreover, among them, the methoxyl group of the malvidin-3-*O*-glucoside and the hydroxyl one of the cyanidin-3,5-*O*-diglucoside allowed us to further distinguish their antioxidant properties. Among the remaining compounds, *trans*-resveratrol was the most antiradical, presenting the higher number of resonance systems, followed by chlorogenic and caffeic acids, in that order, thanks to the *Ind* effect of the ester and the carboxyl acid present on their structures, respectively. *p*-Coumaric acid, lacking of an hydroxyl group at the *meta* position with respect to chlorogenic and caffeic acid, possessed a lower reducing activity. Finally, gallic acid, due to the absence of specific structures able to delocalize radical forms, appeared to be the last antiradical metabolite. According to these observations, the theoretical list of the antioxidants was edited as follows: myrcitin > quercetin > kaempferol > malvidin-3-*O*-glucoside > peonidin-3-*O*-glucoside > cyanidin-3,5-*O*-diglucoside > *trans*-resveratrol > chlorogenic acid > caffeic acid > *p*-coumaric acid > gallic acid. As evident, we surprisingly found that in some cases (*i.e.* flavonoids, anthocyanins) the experimental data corroborated the suppositions based on the chemical principles, while in other situations (*i.e.* gallic acid, *trans*-resveratrol) the *in vitro* tests did not absolutely matched with the predicted results, suggesting how it is difficult to prefigure and guess the antioxidant power of plant metabolites without performing specific laboratory tests. In fact, in addition to the chemical features, several factors may influence the reducing activity of a molecule, such as its steric obstacle, reaction mechanism and concentration.

Conclusion

Chemical characterization and determination of the antioxidant power of extracts derived from eight Italian *V. vinifera* cultivars, never studied before, were carried out, to valorize the products of these varieties and to increase our knowledge about their potential biological effects. Moreover, grape pure molecules were investigated for their theoretical and real free radical scavenging properties, with the aim to identify which one of them possessed the best reducing activity and how they could singularly contribute to the antiradical effect of the whole grapevine phytocomplex, essential for preservation and defense of plant and human health.

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AG and AC designed the study, interpreted the results and wrote the paper; AG, GDM and LC performed the experiments. The authors state no conflict of interest about the present work.

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