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Original Article

Catechin, epicatechin, quercetin, rutin and resveratrol in red grape: Content, *in vitro* antioxidant activity and interactions

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Abbreviations:
3-NT, 3-nitro-tyrosine
DM, dry matter
DPPH•, 2,2-diphenil-1-picridazil
GAE, gallic acid equivalent
LDL, low density lipoprotein
ME, malvidin-3-*O*-glu equivalents
$ONOO^-$, peroxynitrite

ABSTRACT

The extracts obtained from skin and seeds of 10 native Tuscan and international *Vitis vinifera* varieties were evaluated for their antioxidant activity, total phenolic and anthocyanin content and subjected to HPLC–UV analysis to quantify the content of five phenolic constituents of biological interest: catechin and epicatechin in seeds and quercetin, rutin and resveratrol in skin extracts. The antioxidant activity of the extracts and pure compounds was assessed by means of two different *in vitro* tests: scavenging of the stable DPPH• radical and of authentic peroxynitrite ($ONOO^-$). All the extracts showed significant antiradical capacity: Merlot skin was most active towards both radicals. All the five phenols investigated possessed strong antiradical activity. Quercetin, catechin and epicatechin showed maximum activity (respectively, $IC_{50(DPPH•)}$ 5.5, 6.7 and 6.8 μM, $IC_{50(ONOO^-)}$ 48.8, 55.7 and 56.7 μM). Potential antiradical interactive effects among the five compounds were also investigated and results indicated possible synergy between quercetin, rutin and resveratrol towards $ONOO^-$. The effect was additive for catechin and epicatechin.

1. Introduction

The consumption of fruits plays an important role as an health-protecting factor. This beneficial effect is mainly associated with the antioxidant activity of the phenolic compounds which are largely present in fruits and in beverages made from them. These effects are due to the properties of antioxidants to act as reducing agents by donating hydrogen, by quenching singlet oxygen, by acting as chelators and by trapping free radicals. These highly reactive molecules are present in biological systems and may oxidize nucleic acids, proteins, lipids, which may initiate degenerative diseases such as cancer, hearth disease, dermal disorders and aging (Cook and Samman, 1996; Harborne and Williams, 2000; Heim et al., 2002). The antioxidants can reduce this risk. Fruits are one of the most important sources of antioxidants such as vitamins and phenolic phytochemicals. The antioxidant activity of dietary polyphenols is considered to be much greater than that of the essential vitamins, therefore contributing significantly to the health benefits of fruits (Tsao and Yang, 2003).

In the past few years there has been an increasing interest in determining relevant dietary sources of antioxidant phenolics. Grape (*Vitis vinifera*) is among the fruits with the highest content of these compounds. A large amount of different phenolic compounds is present in skin, pulp and seeds, and they also undergo partial extraction during winemaking processes (Revilla and Ryan, 2000). Phenolics are divided into two groups: flavonoid (anthocyanins, flavan-3-ols, flavonols) and non-flavonoid compounds (hydroxybenzoic and hydroxycinnamic acids, stilbenes). Every family of polyphenols is directly responsible for the special characteristics of specific grapes varieties and the resulting wine. Anthocyanins are important polyphenols in the red grape skin,

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while flavan-3-ols are the major polyphenols in the seeds (Bourzeix et al., 1986; Cheynier and Rigaud, 1986; Makris et al., 2006).

In the polyphenolic pool of red grape skin and seeds, there are some secondary compounds important for their antioxidant activity: catechin and epicatechin (flavan-3-ols), quercetin and its glycoside rutin (flavonols), and *trans*-resveratrol (stilbene). They are proven to be potent antioxidants and to have important biological, pharmacological and medicinal properties. All five compounds are shown to protect human low density lipoprotein (LDL) against oxidation more efficiently than α-tocopherol on a molar basis, acting as cardio protective agents (Frankel et al., 1993; Frankel et al., 1995; Meyer et al., 1998; Yilmaz and Toledo, 2004). Resveratrol and quercetin inhibit human platelet aggregation *in vitro* and exhibit potential anticancer properties, the first one by inducing cell differentiation (Frémont, 2000), the second one, as its glycoside, by inhibiting protein-tyrosine kinase (Sakkiadi et al., 2001). Finally resveratrol is able to mediate anti-inflammatory processes mainly inhibiting the expression of cyclooxygenase-1 and 2 (COX-1 and 2) and hydroperoxidase functions. It also shows estrogenic properties towards different cell lines (Frémont, 2000; King et al., 2006).

Several methods have been proposed to evaluate the antioxidant activity of vegetal extracts and pure compounds and it is accepted that this effect depends on the environmental conditions and procedures. *In vitro* assays for the free radical scavenging capacity are commonly based on the inactivation of stable synthetic radicals, such as the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$), first envisaged by Blois (Blois, 1958). Another interesting approach is the study of the peroxynitrite ($ONOO^{-}$) scavenging capacity. Peroxynitrite is nowadays considered one of the most relevant radical generator involved in pathophysiological and toxicological processes. This anion is a product of the reaction between nitric oxide and superoxide and is a potent and versatile oxidant. Its importance in biological systems is based on its powerful ability to react with almost all classes of biomolecules. In fact, while it is relatively stable under basic conditions, at physiological conditions it forms two radicals ($NO_2^{\bullet}$ and $OH^{\bullet}$) that induce lipid peroxidation, disruption of cellular structures, inactivation of enzymes and ion channels through protein oxidation and nitration, and DNA damages (Virág et al., 2003). All these actions contribute to the onset and maintenance of pathologies such as atherosclerosis, neurodegenerative diseases (Torreilles et al., 1999) and cardiovascular disorders (Wattanapitayakul et al., 2000). Scavengers of these deleterious radicals and compounds able to prevent the consequences of their reactivity can contribute to the maintenance of health or healing processes (Heijnen et al., 2001; Chericoni et al., 2005).

Although the literature abounds with reports about phenolic composition and antiradical activity of wine or grape seed samples, there are very few papers that report data about grape seeds and skin of the same sample. Moreover, native Tuscan *V. vinifera* varieties have been occasionally studied and there have been no reports so far about their phenolic content and antiradical potential, especially in comparison to well-known international and Italian varieties.

The present study was undertaken to provide information related to the free radical scavenging capacity of red grapes by using different methods and to relate the results to their phenolic profile. Native Tuscan, international and Italian red grape grown in the same location were analyzed for their free radical scavenging activity by using two different *in vitro* models: the $DPPH^{\bullet}$ assay and the inhibition of the tyrosine nitration by peroxynitrite. To our knowledge, this is the first time that grape extracts are tested to determine their ability to prevent *in vitro* peroxynitrite-induced formation of 3-nitro-tyrosine (3-NT), a biomarker of the oxidative stress (Althaus et al., 2000). Moreover, while it is well demonstrated that several purified phenolic compounds individually exert significant antioxidant activity in various *in vitro* assays, very few papers are available about any interactions between phenolic compounds (Meyer et al., 1998; Guarnieri et al., 2007). In recent papers (Lotito and Frei, 2004; Guarnieri et al., 2007), there is an increasing interest in understanding how phenolic compounds can interact and in providing new and better knowledge of the antioxidant effect of complex phytochemical mixtures such as grape extracts.

Therefore in the present work we investigated the antiradical activity of five biologically and pharmacologically active phenolic compounds present in grape seeds and skin extracts, focusing on the interactions that can be involved in the activity of mixtures of these phytochemicals.

2. Materials and methods

2.1. Chemicals

Gallic acid, catechin, epicatechin, resveratrol, rutin, quercetin, malvidin-3-*O*-glu, trolox, Folin–Ciocalteau reagent, tyrosine, 3-NT, 2,2-diphenil-1-picridazil ($DPPH^{\bullet}$ 90%) and HPLC grade methanol, acetic acid, ethanol were purchased from Sigma Aldrich (Milan, Italy). All chemicals were of analytical or higher grade and the aqueous solutions were prepared by using ultra-pure water purified by Milli-Q System (Millipore, Milan, Italy).

2.2. Plant material

Ten *V. vinifera* genotypes were chosen among seven grape varieties: three native Tuscan (Colorino del Valdarno COL N6 clone, Canaiolo Nero N8 clone, Foglia Tonda FT BRO1 clone), two international (Merlot R12 clone, Cabernet Sauvignon R5 clone) and five clones of two Italian varieties (Montepulciano AP MP1 clone and Sangiovese ISV RC1, F9 A5 48, ISV 2 and AP SG 1 clones). The investigation was performed on mature vines of the collection fields of the Experimental Institute of Viticulture in Arezzo (Italy). Grape samples were collected in triplicate during the 2005 harvest season. Berry fresh and dry weight, berry skin dry weight, seeds per berry and seed dry weight were estimated on hundred berries of each replicate. Dry weight was measured after oven-drying the samples at 60 °C until constant weight.

2.3. Preparation of grape skin and seed extracts

After harvest, berries were snipped from the cluster. The skin and the seeds from 40 berries were manually separated from pulp and extracted with ethanol:water:hydrochloric acid 0.12 M (70:29:1 v/v/v) for 4 h. The extracts were centrifuged for 10 min at 3300 rpm (2500 g), using a Sigma 302K Centrifuge from Bicasa (Milan, Italy), with a swing-out rotor. The precipitate was extracted again with the same solvent and made up to a final volume of 200 mL for skin and 100 mL for seeds. Extracts were stored at −20 °C and analyzed within a month after extraction.

Extracts were purified through a Sep-Pak Plus C-18 cartridges from Waters (Milan, Italy) before analyses. The cartridge was first conditioned with 4 mL of methanol, followed by 4 mL of water, and then loaded with 1 mL of extract appropriately diluted with 19 mL of Milli-Q water in order to reduce the ethanolic content. The loaded cartridge was first washed with 4 mL of water and then the compounds were eluted with 2 mL of methanol. This solution was finally injected into an HPLC system (described in section HPLC) after filtering through a 0.45 μm cellulose filter (Millipore).

2.4. Total phenolics

The amount of total polyphenols in the extracts was determined according to the Folin–Ciocalteau method (Singleton and Rossi, 1965). Gallic acid was employed as a calibration standard and results were expressed as gallic acid equivalents (GAE) (mg GAE/100 g of seeds or skin dry matter (DM)). The absorbance was measured using a UV–vis spectrophotometer (Lambda 25, PerkinElmer, Italy) at the wavelength of 750 nm.

2.5. Total anthocyanins

Total anthocyanin contents of the samples were determined using the method of Rigo et al. (2000). Briefly, extracts were appropriately diluted with ethanol:water:hydrochloric acid 0.12 M (70:29:1 v/v/v) and the absorbance was measured by using a UV–vis spectrophotometer (Lambda 25, PerkinElmer, Italy) at the wavelength of 540 nm. Malvidin-3-*O*-glu was employed as a calibration standard and results were expressed as malvidin-3-*O*-glu equivalents (ME) (mg ME/100 g of skin DM).

2.6. Synthesis of peroxynitrite

Peroxynitrite was synthesized according to the method of Beckman et al. (1994). A solution composed of 0.6 M HCl and 0.7 M H_2O_2 was mixed with 0.6 M $NaNO_2$ on ice and the reaction quenched with ice-cold 1.2 M NaOH. Residual H_2O_2 was removed by mixing with granular MnO_2 prewashed with 1.2 M NaOH. The yellowish stock solution was filtered and then stored at −80 °C. The concentration of the stock solution was evaluated immediately before use by measuring the absorbance at 302 nm ($\varepsilon = 1670\,M^{-1}\,cm^{-1}$).

2.7. Antioxidant activity. Inhibition of tyrosine nitration

This method is based on the determination of the 3-NT quantity formed from the reaction between free tyrosine and peroxynitrite at the physiological pH by reversed-phase HPLC–UV analysis. The reaction was started adding, under vigorous vortexing, 5–40 μL of peroxynitrite (reaching a 1 mM final concentration) to the test solutions containing: grape extracts or pure compounds, 2 mM tyrosine and 50 mM HCO_3^- dissolved in 0.2 M phosphate buffer (pH 7.4). Test compounds were dissolved in ethanol:water:hydrochloric acid 70:29:1 (v/v/v). Blanks, with and without ethanol and hydrochloric acid, was always performed to detect any interference of the solvent with the test. Quantitative determination of the formed 3-NT was performed by HPLC–UV using an external standard calibration curve (six data points, $n = 3$, $r^2 = 0.999$). The amount of 3-NT formed was plotted against the concentration of total phenols in the extracts (mg GAE L^{-1}) and against the concentration of pure compounds (μM). The peroxynitrite scavenging ability of the extracts was expressed as the concentration of total phenols (mg GAE L^{-1}) of extract needed to achieve 50% inhibition of $ONOO^-$ mediated tyrosine nitration (IC_{50}). As regards to the pure compounds, IC_{50} is expressed as μM concentration.

2.8. Antioxidant activity. DPPH•

This method was performed using DPPH• (Brand-Williams et al., 1995), a stable free radical compound with a strong absorption at 515 nm. When an antiradical agent is added to DPPH•, the free electron is paired up and the color (purple) is lost (yellow). This effect is measured as a decrease in absorbance. An aliquot of 0.1 mL of five different dilutions of each extract and different concentrations of each pure compound were added to 3.9 mL of DPPH• methanolic solution (12×10^{-5} M), and vortexed. Absorbance at 515 nm (A_{515}) was measured at different time intervals on a UV–vis Agilent HP 8453 spectrophotometer until the reaction reached a plateau, in order to find the time required to achieve the steady state. The initial concentration of DPPH• was controlled for every experiment by using a calibration curve made by measuring the absorbance at 515 nm of standard samples of DPPH• at different concentrations. The equation of the curve was $Abs_{515} = 0.0085 \times [DPPH^\bullet]$ ($R^2 = 0.99810$), as determined by linear regression. The % remaining concentration of DPPH• was calculated as $DPPH^\bullet_{rem} = [DPPH^\bullet_{ss}]/[DPPH^\bullet_0] \times 100$, where $DPPH^\bullet_{rem}$ is the concentration of remaining DPPH•, $DPPH^\bullet_{ss}$ is the concentration of DPPH• at the steady state and $DPPH^\bullet_0$ is the initial concentration of the radical.

The polyphenols concentration of the extracts (mg GAE L^{-1}) and the concentration of pure standard solutions (μM) were plotted against % $DPPH^\bullet_{rem}$ and the amount of polyphenols or pure standard to decrease the $DPPH^\bullet_0$ concentration of 50% (IC_{50}) was determined from the resulting exponential equation.

2.9. High pressure liquid chromatography

The HPLC system consisted of a Dionex P 680 Pump coupled with a Dionex UVD 170U/340U UV/VIS detector. Data processing was performed by using the software Chromeleon 6.5 (Dionex, Germany) running on a PC coupled with the HPLC system. Separation was performed on a reversed-phase C18 column (Acclaim 120, 5 μm, 4.6 × 250 mm, Dionex) coupled with a C18 guard cartridge (Acclaim 120, 5 μm, 4.3 × 10 mm, Dionex) at room temperature.

Elution conditions for the detection of phenolic compounds in both skin and seeds grape extracts was as follows: eluent A water, eluent B methanol, eluent C acetic acid, flow rate 0.8 mL min^{-1}, 20 μL injection volume. The detection was performed at the maximum UV–vis absorptions of the five compounds: at 280 nm for catechin and epicatechin, at 306 nm for resveratrol, at 360 nm for rutin and quercetin. Identification was based on comparing retention times and UV–vis spectral data of the peaks detected to those of original reference standard compounds. Quantification was accomplished using external calibration with pure standards. The calibration curves (five data points, $n = 2$) were linear with $R^2 = 0.999$.

Elution program for the detection of phenolic compounds in grape seed extracts was as follows: initial conditions B 15% and C 5%, B 25% in 15 min, B 85% in 5 min, which was kept isocratic for 10 min and B 15% in 5 min, which was kept isocratic for 5 min.

Elution program for the detection of phenolic compounds in grape skin extracts was as follows: initial conditions B 15% and C 5%, B 25% in 15 min, B 45% in 5 min, B 60% in 20 min, B 95% in 5 min, which was kept isocratic for 10 min and B 15% in 10 min, which was kept isocratic for 5 min.

Elution conditions for the detection of 3-NT were as follows: 20 mM phosphate buffer (pH 3.2)/methanol 92:8; flow rate 1 mL min^{-1} in isocratic mode, 20 μL injection volume; UV detention at 356 nm.

2.10. Antioxidant interactions of phenolics

In order to evaluate the possible interactions among the five phenols investigated, combinations of two or three compounds and all of them together were studied using both methods. In particular the combinations of catechin and epicatechin, which are present in grape seed samples, and quercetin, rutin and resveratrol, which are present in grape skin samples were analyzed. In the case of peroxynitrite assay, pure compounds

were paired one each other at the same level (25 μM) in order to give a total phenol concentration of 50 μM, in the case of two compounds, 75 μM in the case of three compounds and 125 μM in the case of all the five compounds.

In the case of DPPH$^{\bullet}$ radical, the combinations were obtained by combining, respectively, two, three or all the five compounds at the same concentration (2 μM), in order to have a total phenol concentration of 4 μM in the case of two compounds, 6 μM in the case of three compounds and 10 μM in the case of all the five compounds.

The antioxidant activity of these solutions was measured under the same experimental conditions of the individual pure compounds and expressed as the percent relative inhibition of 3-NT formation and of DPPH$^{\bullet}$ inhibition.

2.11. Statistics

Sampling proceeds on three independent replications ($n = 3$) for each genotype and analytical parameter. Results presented in tables and graphs were reported as means ± standard deviation (SD). Data were subjected to ANOVA and differences among varieties and pure compounds were tested by *post-hoc* comparison test (LSD) at $P = 0.05$. Interaction between pure phenolic compounds was tested statistically by *t*-test procedure.

3. Results and discussion

3.1. General

In this study we investigated the catechin, epicatechin, quercetin, rutin and resveratrol content in the acidic ethanolic extracts of 10 red grape varieties grown in Tuscany. In particular, we evaluated the antiradical capacity of these five compounds towards peroxynitrite and DPPH$^{\bullet}$ radicals and their possible interactions.

Results showed that the antioxidant activity of grape extract is not only dependent on total phenolic content and that phenolic compounds can act in synergy, antagonism or can independently affect the total activity of mixtures.

Data concerning characteristics of the 10 grape genotypes, as berry fresh and dry weight, berry skin dry weight, seeds per berry and seed dry weight, are listed in Table 1.

3.2. Polyphenols content

The phenolic composition is an important quality parameter of red grape. Apart from the genetic background, the maturation and ripening stage and water status are able to impact the level of polyphenols, indicating that cultural practices can be used to modify composition. In our experimental conditions we tried to minimize these factors collecting samples from the same experimental field at the same ripening stage.

The extraction procedures can also largely affect the determination of the phenolic content of the samples, as described by other authors (Downey et al., 2007). In our experimental method we used an acidic ethanolic solvent for the extraction of our samples and our results should be read in relation to this important aspect.

In all grape varieties/clones studied the total phenolic content was quite variable (Fig. 1). Total phenolics in skin extracts were lower than in seed extracts. Only the Colorino del V.no showed a different behavior, having a similar level of phenolic compounds in skin and seed extracts.

As regards the skin, Cabernet Sauvignon, Montepulciano, Colorino del V.no and Canaiolo showed the highest values (5304 ± 482, 5206 ± 382, 5126 ± 236 and 5091 ± 149 mg GAE/100 g of skin DM, respectively), while Merlot and the Sangiovese clones AP SG1, F9, ISV 2 and ISV RC1 showed the lowest ones (4078 ± 184, 3905 ± 317, 3989 ± 90, 3924 ± 247, 3770 ± 275 mg GAE/100 g of skin DM, respectively). The total phenolic content of the seeds ranged between 8708 ± 430 and 5033 ± 40 mg GAE/100 g of seeds DM. Montepulciano showed the highest value, while Colorino del V.no and ISV RC1 showed the lowest one and the average was 6689 ± 306 mg GAE/100 g of seeds DM.

The variability found in total phenolics confirmed the hypothesis that genotypes affect the polyphenol content in grape skin and seeds. Sangiovese clones were similar as regards to the total phenolic content of grape skin, while there was some variability regarding the seed extracts.

Results of the analysis of total anthocyanins of the skin extracts are reported in Fig. 2. Colorino del V.no was the variety with the highest anthocyanins content (3929 ± 184 mg ME/100 g of skin DM), while Canaiolo and Sangiovese clone AP SG 1 were the ones with the lowest level (respectively, 1594 ± 276 and 1902 ± 110 mg ME/100 g of skin DM). Merlot and Cabernet Sauvignon were statistically equivalent, the average being 2852 ± 95 mg ME/100 g of skin DM. The others varieties had intermediate values and they were not significantly different.

Five polyphenols present in grape skin (resveratrol, rutin and quercetin) and seeds (catechin and epicatechin) were chosen because of their biological and pharmacological interest and their contents were determined by reversed-phase HPLC (Table 2). Their amounts were in line with those reported in literature.

Table 1
Weight data and number of seeds of grape varieties studied

Variety/clone	Weight parameters[a]				
	Berry fresh weight (g)	Berry dry weight (g)	Berry skin dry weight (g)	Seeds per berry (no.)	Seed dry weight (g)
AP SG 1	1.77 ± 0.04 b	0.43 ± 0.01 b	0.09 ± 0.00 b	2.53 ± 0.27 ab	0.023 ± 0.00
F9	2.05 ± 0.01 a	0.46 ± 0.01 a	0.10 ± 0.01 b	2.50 ± 0.22 b	0.025 ± 0.00
ISV 2	1.56 ± 0.01 d	0.41 ± 0.01 c	0.10 ± 0.01 b	2.30 ± 0.19 bc	0.024 ± 0.00
ISV RC1	1.31 ± 0.04 ef	0.28 ± 0.01 f	0.09 ± 0.02 b	2.07 ± 0.31 cd	0.024 ± 0.00
Merlot	1.35 ± 0.04 e	0.36 ± 0.01 d	0.06 ± 0.00 c	1.68 ± 0.14 e	0.027 ± 0.00
Cabernet Sauv.	1.27 ± 0.01 f	0.31 ± 0.00 e	0.07 ± 0.00 c	1.79 ± 0.19 de	0.024 ± 0.00
Canaiolo	1.63 ± 0.08 c	0.37 ± 0.02 d	0.10 ± 0.02 b	2.38 ± 0.06 bc	0.028 ± 0.00
Colorino V.no	1.07 ± 0.02 g	0.28 ± 0.01 f	0.07 ± 0.01 c	2.88 ± 0.22 a	0.025 ± 0.00
Foglia Tonda	1.54 ± 0.00 d	0.40 ± 0.00 c	0.09 ± 0.01 b	2.42 ± 0.31 bc	0.022 ± 0.00
Montepulciano	2.09 ± 0.02 a	0.46 ± 0.00 a	0.13 ± 0.01 a	1.53 ± 0.08 e	0.027 ± 0.00
P ANOVA	***	***	***	***	ns

[a] Values are the means ± SD ($n = 3$). Data were analysed by ANOVA (***; $P < 0.001$) and within each column different letters indicate statistically different values according to *post-hoc* comparison (LSD-test) at $P = 0.05$.

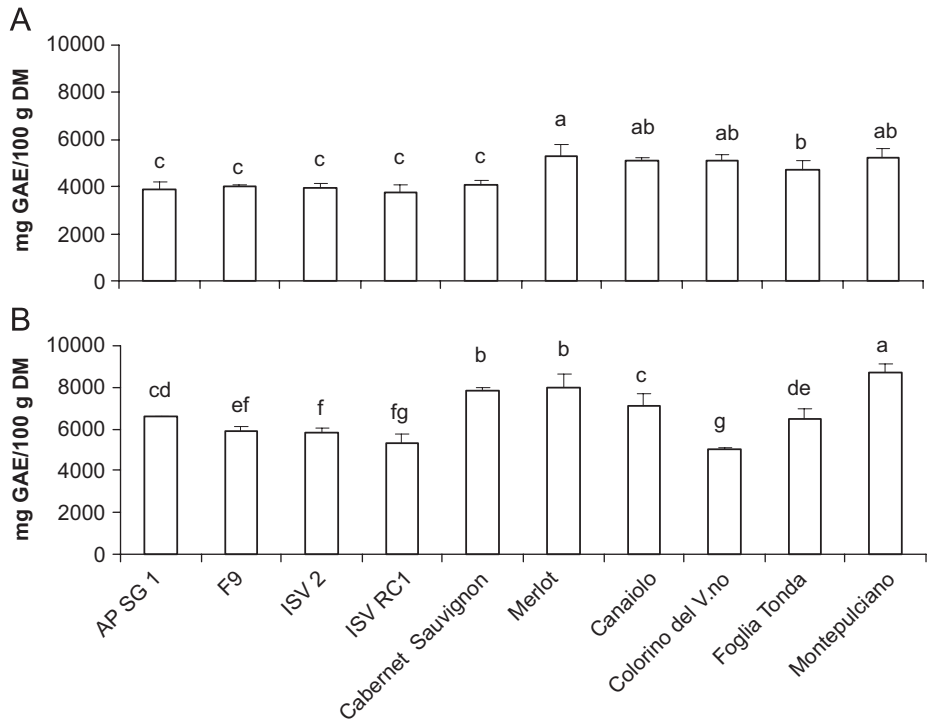

Fig. 1. Total phenolic content of skin (A) and seeds (B) of grape varieties and clones investigated. Values are the means ± SD ($n = 3$). Data were analyzed by ANOVA ($P < 0.001$) and different letters indicate statistically different values according to *post-hoc* comparison (LSD-test) at $P = 0.05$.

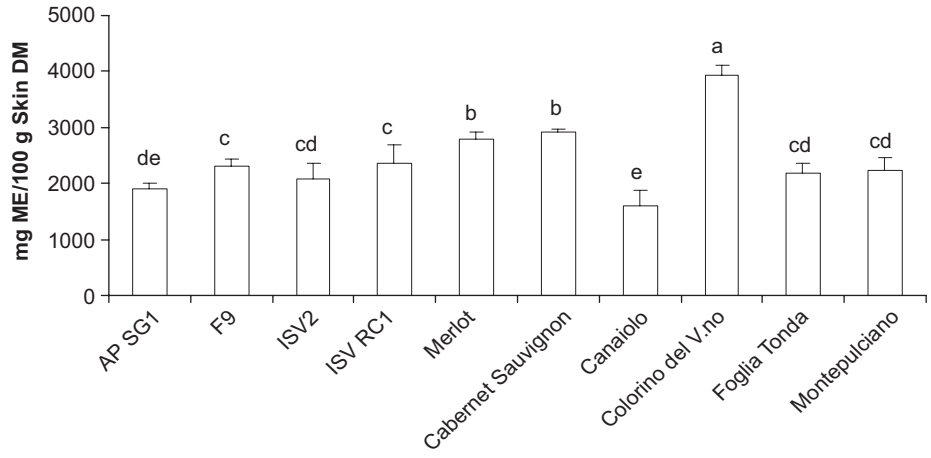

Fig. 2. Total anthocyanin content of skin of grape varieties and clones investigated. Values are the means ± SD ($n = 3$). Data were analyzed by ANOVA ($P < 0.001$) and different letters indicate statistically different values according to *post-hoc* comparison (LSD-test) at $P = 0.05$.

In general, catechin and epicatechin are among the major compounds in grape seeds (Fuleki and Ricardo da Silva, 1997; Revilla and Ryan, 2000). Montepulciano had the highest values of catechin (205.7 ± 19.0 mg/100 g of seeds DM) while Canaiolo was the variety with the highest level of epicatechin (205.7 ± 3.1 mg/100 g of seeds DM). The Sangiovese clones and Foglia Tonda variety showed the lowest values for both compounds (the average values were 74.38 ± 5.4 and 81.7 ± 4.4 mg/100 g of seeds DM for catechin and epicatechin, respectively). Merlot and Cabernet Sauvignon varieties showed high and similar level of catechin (138.8 ± 0.4 and 141.8 ± 7.9 mg/100 g of seeds DM, respectively) and epicatechin (131.8 ± 9.7 and 127.6 ± 4.4 mg/100 g of seeds DM, respectively) in seed extracts.

Cabernet Sauvignon and Merlot were the cultivar in which catechin was the main compound and these results confirmed those reported by other authors (Santos-Buelga et al., 1995; Monagas et al., 2003; Yilmaz and Toledo, 2004; Guendez et al., 2005; Rodriguez Montealegre et al., 2006). Colorino del V.no, Foglia Tonda and Montepulciano also presented this characteristic, whereas all the four Sangiovese clones studied and Canaiolo had epicatechin as the main compound, as also reported by Monagas et al. (2003) in two (Tempranillo and Graciano) Spanish original *V. vinifera* varieties.

As regards to the presence of catechin and epicatechin in skin and seeds, it is commonly known that flavan-3-ols are located in both grape skin and seeds; however, skin contains much lower concentrations of flavan-3-ols than seeds (Revilla and Ryan, 2000; González-Manzano et al., 2004; Rodriguez Montealegre et al., 2006). Revilla and Ryan (2000) reported that the amount of catechin and oligomeric procyanidins in red grape skin samples

Table 2
Catechin and epicatechin content of seeds and resveratrol, rutin and quercetin content of skin of grape genotypes studied

Variety/clone	Phenolic content (mg/100 g DM)[a]				
	Seeds		Skin		
	Catechin	Epicatechin	Resveratrol	Rutin	Quercetin
AP SG 1	96.9 ± 5.9 d	106.8 ± 1.6 d	0.7 ± 0.04 e	149.1 ± 15.1 b	1.05 ± 0.16 a
F9	74.4 ± 0.9 e	98.4 ± 9.9 de	0.8 ± 0.16 de	158.5 ± 3.3 ab	1.00 ± 0.18 a
ISV 2	72.9 ± 7.0 e	88.0 ± 0.7 e	0.8 ± 0.04 de	169.0 ± 7.3 a	1.07 ± 0.20 a
ISV RC1	60.3 ± 8.3 e	67.9 ± 6.2 f	0.6 ± 0.16 e	160.2 ± 16.2 ab	1.04 ± 0.10 a
Merlot	138.8 ± 0.4 bc	131.8 ± 9.7 c	10.5 ± 1.84 c	89.9 ± 10.2 c	0.69 ± 0.07 bc
Cabernet Sauv.	141.8 ± 7.9 b	127.6 ± 4.4 c	25.5 ± 1.16 a	88.6 ± 5.3 c	0.60 ± 0.01 bc
Canaiolo	140.7 ± 2.2 bc	205.7 ± 3.1 a	2.8 ± 0.02 d	41.3 ± 2.3 e	nd
Colorino V.no	124.4 ± 0.1 c	89.0 ± 8.6 e	16.4 ± 1.57 b	40.3 ± 3.9 e	0.50 ± 0.01 de
Foglia Tonda	67.4 ± 5.1 e	47.2 ± 3.5 g	1.3 ± 0.07 de	60.4 ± 3.2 d	0.29 ± 0.06 d
Montepulciano	205.7 ± 19.0 a	164.6 ± 16.8 b	10.9 ± 0.59 c	53.2 ± 4.5 de	0.76 ± 0.05 b
P ANOVA	***	***	***	***	***

[a] Values are the means ± SD ($n = 3$). Data were analysed by ANOVA (***$P < 0.001$) and within each column different letters indicate statistically different values according to *post-hoc* comparison (LSD-test) at $P = 0.05$.

investigated was too low to be quantified by HPLC–UV, and only a very small concentration was detected. Rodriguez Montealegre et al. (2006) quantified flavan-3-ols in grape skin by fluorescence in order to avoid interferences with the other compounds and to lower detection limits. Therefore we decided to quantify the amount of monomeric catechin and epicatechin only in grape seeds by HPLC–UV.

Resveratrol was found at very low level in all the Sangiovese clones and in Foglia Tonda (Foglia Tonda > F9 ≈ ISV 2 > AP SG1 ≈ ISC RC1, the average was 0.8 ± 0.18 mg/100 g of skin DM). The amounts of these varieties were comparable to the results of previous works (Romero-Pérez et al., 2001; Careri et al., 2003; Sun et al., 2006). Sun et al. (2006) showed that the content of *trans*-resveratrol in grape skin of Castelão, Syrah and Tinta Roriz varieties was not significantly different and the average was around 20 mg kg^{-1} of dry skin. Cabernet Sauvignon was the variety with the highest amount of resveratrol (25.5 ± 1.16 mg/100 g of skin DM) and also in Merlot, Colorino del V.no, and Montepulciano *trans*-resveratrol was abundant. The latter confirmed that stilbenes content is largely dependent on grape varieties. Revilla and Ryan (2000) reported that the levels of *trans*-resveratrol in grape skin samples were extremely low. They explained this result as the consequence of the fact that grapes produce stilbenes in response to mold infections and physiological stresses. If these stresses are not present, the levels of stilbenes in grapes remain low. Sun et al. (2006) quantified *trans*-resveratrol in grape seeds, even though at a low concentration (6.8 mg kg^{-1} of dry seed). We did not detect any *trans*-resveratrol in grape seeds samples, as reported by other authors (Yilmaz and Toledo, 2004).

The presence and the real amount of quercetin and its glycoside rutin in grape skin are quite critical and controversial. In some articles quercetin and rutin have been found in grape skin (Careri et al., 2003; Chafer et al., 2005; Gómez-Alonso et al., 2007), while in others (Cheynier and Rigaud, 1986; Makris et al., 2006; Rodriguez Montealegre et al., 2006) quercetin was detected only in its glycosilated forms and rutin was not reported.

In our 10 red grape skin samples the concentrations of quercetin and of its glycoside rutin showed almost the same trend. The amount of quercetin was considerably low, as shown in Table 2. Comparable levels of quercetin were also found by Careri et al. (2003), suggesting that the prevalent form of quercetin is not the aglycon. This is also confirmed by Rodriguez Montealegre et al. (2006) that reported only glycosides quantification within the group of flavonols in the skin extracts of Cencibel, Cabernet Sauvignon, Merlot and Shiraz varieties. They explained the presence of flavonol aglycones in wine or skin extract as a consequence of progressive hydrolysis of glycosides.

The Sangiovese clones had the highest content of rutin among all varieties and they were not significantly different (the average value was 159.2 ± 10.5 mg/100 g of skin DM). Merlot and Cabernet Sauvignon content was not different and the average was 89.2 ± 7.8 mg/100 g of skin DM. The Italian variety Montepulciano and the native Tuscan varieties Canaiolo and Colorino del V.no were the ones with the lowest rutin values: 53.2 ± 4.5, 41.3 ± 2.3 and 40.3 ± 3.9 mg/100 g of skin DM, respectively. Quercetin showed the same trend of rutin. The average of the quercetin content of the Sangiovese clones was 1.04 ± 0.16 mg/100 g of skin DM, while the average of Merlot and Cabernet Sauvignon was 0.64 ± 0.04 mg/100 g of skin DM. Canaiolo had amount of quercetin under instrument detection limits.

3.3. Antioxidant activity of pure compounds and grape extracts

The antioxidant activity of grape skin and seed extracts and of five pure compounds has been studied in different systems, but none of them deal with the protection against tyrosine nitration induced by peroxynitrite. We used two *in vitro* models in order to measure the activities towards different radicals: the stable, synthetic radical ($DPPH^{\bullet}$) and a physiological anion ($ONOO^{-}$) able to form, at physiological conditions, two radicals strictly involved in various cellular damage processes, including tyrosine nitration. The IC_{50}, the amount of polyphenols, expressed as mg GAE L^{-1}, needed to inhibit the 3-NT formation or $DPPH^{\bullet}$ activity by 50%, was considered as measure of the antiradical capacity of crude extracts.

All the examined extracts were significantly active as antioxidant agents. The results of both $ONOO^{-}$ and $DPPH^{\bullet}$ tests for the grape skin and seed extracts are listed in Table 3.

Merlot and Sangiovese skin extracts had high antiradical capacity in both assays, which was confirmed by Kallithraka et al. (2005). As regards $ONOO^{-}$ assay, Merlot together with AP SG1, Cabernet Sauvignon and Foglia Tonda showed the highest (lowest IC_{50}) antiradical activity (11.0 ± 0.7, 11.8 ± 0.8, 12.0 ± 0.5 and 12.4 ± 0.4 mg GAE L^{-1}, respectively), while Canaiolo had the lowest (highest IC_{50}) activity (25.2 ± 0.9 mg GAE L^{-1}).

The analyses of the seed extracts as $ONOO^{-}$ scavengers showed very low variability and IC_{50} values ranged from 11.0 ± 1.2 mg GAE L^{-1} of Cabernet Sauvignon to 15.3 ± 2.5 mg GAE L^{-1} of Sangiovese clone AP SG1. The average was 12.9 ± 1.3 mg GAE L^{-1}.

Table 3
Antioxidant activity of grape skin and seeds measured by $ONOO^-$-mediated tyrosine nitration and by $DPPH^\bullet$ free radical

Variety/clone	IC_{50} (mg GAE L^{-1})[a]			
	Skin		Seeds	
	$ONOO^-$	$DPPH^\bullet$	$ONOO^-$	$DPPH^\bullet$
AP SG1	11.84±0.80 bc	2.16±0.08 cd	15.35±2.49 a	3.02±0.27 abc
F 9	13.36±1.35 b	2.38±0.18 bcd	13.77±2.24 ab	2.82±0.29 bc
ISV 2	12.74±1.34 b	2.47±0.40 bc	13.66±1.13 ab	2.47±0.16 c
ISV RC1	13.39±0.85 b	1.93±0.13 cd	11.87±0.88 bc	1.74±0.14 d
Merlot	10.98±0.65 c	1.74±0.01 d	11.77±0.75 bc	2.90±0.37 bc
Cabernet Sauv.	11.97±0.51 bc	2.44±0.03 bc	10.99±1.20 c	2.55±0.10 bc
Canaiolo	25.17±0.94 a	3.59±0.62 a	11.07±0.34 c	3.49±0.10 a
Colorino V.no	13.13±0.23 b	2.16±0.17 cd	14.57±1.95 a	3.20±0.37 ab
Foglia Tonda	12.37±0.39 bc	2.97±0.18 ab	14.62±1.26 a	2.81±0.36 bc
Montepulciano	12.93±2.04 b	2.27±0.06 bcd	11.76±0.98 bc	2.68±0.19 bc
P ANOVA	***	***	**	***

[a] Values are the means±SD ($n=3$). Data were analysed by ANOVA ($^{**}P<0.01$ and $^{***}P<0.001$) and within each column different letters indicate statistically different values according to *post-hoc* comparison (LSD-test) at $P=0.05$.

Table 4
Comparison of pure phenolic compounds in terms of their radical scavenging activities

Compound	IC_{50} (μM)[a]	
	$ONOO^-$	$DPPH^\bullet$
Catechin	55.7±0.5 d	6.7±0.4 cd
Epicatechin	56.5±0.6 d	6.8±0.2 c
Resveratrol	74.1±1.0 c	57.8±0.1 a
Rutin	92.4±1.0 b	7.4±0.2 c
Quercetin	48.8±0.5 d	5.5±0.1 d
Trolox	137.6±12.7 a	13.7±1.1 b
P ANOVA	***	***

Trolox was used as reference.
[a] Values are the means±SD ($n=3$). Data were analysed by ANOVA ($^{***}P<0.001$) and within each column different letters indicate statistically different values according to *post-hoc* comparison (LSD-test) at $P=0.05$.

Both skin and seed extracts of all the varieties investigated showed high capacity to scavenge the $DPPH^\bullet$. IC_{50} values for the grape skin extracts varied from 1.7±0.0 mg GAE L^{-1} of Merlot to 3.6±0.6 mg GAE L^{-1} of Canaiolo, the average being 2.4±0.2 mg GAE L^{-1}. Cabernet Sauvignon skin extract was less active towards $DPPH^\bullet$ than Merlot, as reported by other authors (Kallithraka et al., 2005).

The seed extracts showed a different trend. The Sangiovese clone ISV RC1 had the highest antiradical activity with 1.7±0.1 mg GAE L^{-1}, whereas the Tuscan variety Canaiolo was the less active (3.5±0.1 mg GAE L^{-1}). The average of all seed extracts IC_{50} was 2.8±0.2 mg GAE L^{-1}.

With the only exception for Canaiolo seed extract towards $ONOO^-$ and for Colorino del V.no skin extract towards $DPPH^\bullet$, the native Tuscan varieties seemed to have quite low antiradical activity.

When the phenolic compounds of this study were assayed against $DPPH^\bullet$ and peroxynitrite, the all five were found to be active. Results of scavenging activity of pure compounds are listed in Table 4. In both cases we used Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water soluble analog of vitamin E, as a known inhibitor for comparative purposes.

None of the pure compounds interfered with the HPLC determination of 3-NT and all of them exhibited a greater ability to reduce tyrosine nitration than Trolox. Under our experimental conditions the relative antioxidant activity towards $ONOO^-$ decreased from quercetin, catechin and epicatechin (compounds average 53.7±0.5 μM), to resveratrol (74.1±1.0 μM), to rutin (92.4±1.0 μM).

As regards to $DPPH^\bullet$ scavenging activity, pure compounds had lower IC_{50} values than Trolox with the only exception of resveratrol, which showed the higher IC_{50}, which results in the lower scavenging effect on $DPPH^\bullet$. IC_{50} values ranged from 5.5±0.1 to 57.8±0.1 μM; quercetin and catechin were the most active compounds.

The mechanism by which phenolic compounds are able to scavenge free radicals is yet to be exactly established. In any case it seems to be clear that the basic structure of compounds and other structural factors are very important in the scavenging mechanism (Choi et al., 2002; Sadeghipour et al., 2005). As reported by Heijnen et al. (2001), the aromatic OH groups are the reactive centers, primarily 3′,4′-dihydroxy catechol group, and their activity can be enhanced by electron donating effects of other substituents. For flavonoid compounds, *O*-dihydroxy groups in the B-ring, the presence of a C 2-3 double bond in conjunction with 4-oxo in the C-ring, the 3- and 5-hydroxy groups and the 4-oxo function in the A and C-rings are associated with antioxidant activity (López et al., 2003). As regards to the antioxidant activity of *trans*-resveratrol, a stilbene compound, the presence of the catechol structure or 4′-hydroxy in ring B and the presence of the *meta*-hydroxy structure in ring A seem to be essential criteria (López et al., 2003).

Therefore, as reported by other authors (Pannala et al., 1997; Ketsawatsakul et al., 2000; Heijnen et al., 2001; Choi et al., 2002; Sadeghipour et al., 2005), quercetin, catechin and epicatechin were the most active peroxynitrite inhibitors. We found that quercetin had the lowest IC_{50} value, which results in the major activity, even towards $DPPH^\bullet$ free radical, as described by Villaño et al. (2007) and it has been reported to have the greatest antioxidant activity evaluated by fluorimetric assay (López et al., 2003) too. Catechin and epicatechin, which were equivalent against both radicals, were weaker than quercetin towards $DPPH^\bullet$. *trans*-resveratrol and rutin showed different trends towards the two radicals. *trans*-resveratrol was the weakest compound in the $DPPH^\bullet$ assay, even weaker that trolox, used as a reference standard compound, in accordance with the order of reactivity obtained by other authors (Villaño et al., 2007). In the case of peroxynitrite it was a stronger antioxidant than trolox. Rutin was stronger than trolox with both methods, but with $DPPH^\bullet$ it was more efficient than *trans*-resveratrol. It has been observed that rutin (quercetin-3-*O*-rutinoside) showed lower antioxidant capacity than its aglycon, quercetin, in both $DPPH^\bullet$ and peroxynitrite assays. In the case of the $DPPH^\bullet$ assay the glycosylation of the hydroxyl group at C-3 did not seem to change the activity notably, and the two compounds were equivalent. In the case of peroxynitrite assay the glycosilation of the 3-hydroxyl group clearly decreased the activity. This could be explained by the steric hindrance created by the saccharides, as reported by López et al. (2003). In fact, Heim et al. (2002) reported that aglycones are generally stronger antioxidant than their corresponding glycosides, but in some assays rutinose is a unique case in which addition of this disaccharide to aglycone does not consistently decrease antioxidant ability.

Sadeghipour et al. (2005) reported inconclusive results for rutin as an inhibitor on 3-NT formation by peroxynitrite oxidation reaction because 3-NT peak overlapped with peak(s) of other compounds. Under our experimental conditions, none of the pure compounds interfered with the determination of 3-NT.

Statistically insignificant or very low correlations (*data not shown*) were observed between total phenolics (mg GAE/100 g of

skin or seeds DM), total anthocyanins (mg ME/100 g of skin DM), catechin, epicatechin, resveratrol, rutin and quercetin content (mg/100 g of skin or seeds DM) and antioxidant activity in grape skin and seed extracts as measured by peroxynitrite and DPPH$^{\bullet}$ (IC_{50} mg L^{-1}). Merlot grape skin extract, for example, was the most active but it was not the one with the highest phenolic content. It also was not the one with the highest level of resveratrol, rutin and quercetin.

This fact is also confirmed by other authors that, using the same and different model systems, showed a low or a statistically insignificant correlation between free radical scavenging capacity and phenolic content, suggesting that the presence of further phenolic components or interactions may be involved in the antioxidant potential (Kallithraka et al., 2005; Ruberto et al., 2007).

3.4. Phenolic interactions in scavenging processes

As possible interactions among phenolic compounds could be considered a reason at the base of the low correlation between antioxidant activity of crude extracts and phenolic content, we assayed combinations of two, three and five compounds towards both radicals to investigate these possible interactions. We established that in the case of the DPPH$^{\bullet}$ assay, under our experimental conditions, all six combinations resulted in generate antagonistic interactions on the DPPH$^{\bullet}$ scavenging activity (Table 5). The inhibition of DPPH$^{\bullet}$ activity was in fact lower than expected by adding the antioxidant activities values of individual compounds at 2 μM.

The combinations showed possible interactions of the compounds in their ability to reduce tyrosine nitration by peroxynitrite. In particular the combinations of rutin with *trans*-resveratrol and of rutin and *trans*-resveratrol with quercetin showed synergistic interaction in the $ONOO^{-}$ assay, in which we found that the inhibition obtained was higher than the sum of the individual effects. On the contrary, the combinations of quercetin with resveratrol and of all five compounds together were less active than expected. The combinations of catechin with epicatechin and quercetin with rutin showed that the activity does not exceed that expected by adding the antioxidant activity values of individual compounds at 25 μM (Table 6).

These results indicate that phenolic compounds present in a mixture can interact and that their interactions can affect the total antioxidant capacity of a mixture. Moreover using different *in vitro* system we obtained different results. This could be due to the different way through which phenolic compounds are able to scavenge different radicals, as in this case DPPH$^{\bullet}$ and radicals generated from the peroxynitrite degeneration. These differences are also at basis of the antioxidant activity of grape extracts and justify the different results obtained from the use of different grape varieties and from the use of two different *in vitro* test.

Phenolic interactions have been evaluated in very few papers using different systems.

Table 5
Antioxidant interactions on inhibition of DPPH$^{\bullet}$ free radical activity of pure compounds combinations[a]

Combination	Expected inhibition[b] (%)	Observed inhibition (%)	Test statistic, t_0	Interaction[c] ($P<0.05$)
Catechin (C)		18.8 ± 0.6		
Epicatechin (E)		18.5 ± 0.3		
Quercetin (Q)		22.3 ± 0.3		
Rutin (R)		17.1 ± 0.3		
Resveratrol (Re)		2.4 ± 0.0		
C+E	37.2 ± 1.0	20.1 ± 0.0	30.46	Antagonism
Q+R	39.4 ± 0.6	20.7 ± 0.6	35.90	Antagonism
Q+Re	24.6 ± 0.3	19.4 ± 0.6	12.64	Antagonism
R+Re	19.5 ± 0.3	18.1 ± 0.7	3.27	Antagonism
Q+R+Re	41.8 ± 0.6	25.9 ± 0.9	25.06	Antagonism
C+E+Q+R+Re	79.0 ± 1.6	52.3 ± 1.0	24.65	Antagonism

[a] Values are the means ± SD ($n = 3$). Final concentrations of individual phenols were 2 μM. resulting in a total addition level of 4 μM in the combinations of two compounds, 6 μM in the combinations of three compounds and 10 μM in the combination of five compounds.
[b] Calculated by summation of individual antioxidant effects obtained at 2 μM addition level.
[c] The calculated *t*-test statistic values were compared with a Students *t*'s critical value ($t_{\text{critical at } 0.025,4} = 2.78$).

Table 6
Antioxidant interactions on inhibition of tyrosine nitration by peroxynitrite of pure compounds combinations[a]

Combination	Expected inhibition[b] (%)	Observed inhibition (%)	Test statistic, t_0	Interaction[c] ($P<0.05$)
Catechin (C)		26.5 ± 0.7		
Epicatechin (E)		24.8 ± 1.5		
Quercetin (Q)		31.6 ± 0.1		
Rutin (R)		9.2 ± 0.2		
Resveratrol (Re)		18.3 ± 0.9		
C+E	51.3 ± 2.2	52.1 ± 0.3	0.63	None
Q+R	40.8 ± 0.3	41.3 ± 0.7	1.38	None
Q+Re	49.9 ± 0.7	47.1 ± 1.5	2.78	Antagonism
R+Re	27.5 ± 0.7	38.8 ± 1.6	11.14	Synergisme
Q+R+Re	59.0 ± 0.6	65.8 ± 2.8	4.07	Synergisme
C+E+Q+R+Re	> 100.0 ± 1.6	91.9 ± 1.2	16.07	Antagonism

[a] Values are the means ± SD ($n = 3$). Final concentrations of individual phenols were 25 μM resulting in a total addition level of 50 μM in the combinations of two compounds, 75 μM in the combinations of three compounds and 125 μM in the combination of five compounds.
[b] Calculated by summation of individual antioxidant effects obtained at 25 μM addition level.
[c] The calculated *t*-test statistic values were compared with a Students *t*'s critical value ($t_{\text{critical at } 0.025,4} = 2.78$).

Meyer et al. (1998) tested the interactions between catechin, quercetin, cyanidin, caffeic acid and ellagic acid. They concluded that, with both two and three compound combinations, the antioxidant effects of the hydroxyphenols tested were additive and that ellagic acid exerted a significant antagonistic effect on the antioxidant activity of all the combinations containing catechin. They attributed this effect to hydrogen-bonding between catechin *o*-dihydroxyls and ellagic acid carbonyls. In a more recent paper, Heo et al. (2007) measured the antioxidant capacity by using ABTS radical test of combinations of two or three selected phenolics at various concentrations. They reported that there was no synergistic effect among the phenolics they studied, but only an additive effect was observed.

Thus, although we demonstrated that phenolics interactions can positively or negatively affect the antioxidant activity of natural mixtures, the scavenging properties of phenolic combinations is questionable and more detailed studies are necessary to substantiate this hypothesis.

4. Conclusion

In summary, it may be possible to consider grape skin and seeds as a good and easily accessible source for nutraceutical compounds. For the first time the peroxynitrite induced tyrosine nitration test has been used to demonstrate the antiradical activity of grape extracts and results showed that this could be considered a reliable method, comparable to other antiradical tests, and more representative of human physiological conditions. The five phenolic compounds investigated, secondary compounds interesting for their biological and pharmacological activities, have been demonstrated to be strong antioxidant agents and the analyses of their combinations let us understand how interactions among compounds are important for the biological activity of crude extracts. Further studies must be performed in order to understand their real potential as nutraceuticals, both as single compounds and as components of natural mixtures. Moreover, that being so, it seems to become more and more important to study the possible interactions of these interesting compounds with anthocyanins, the most abundant phenolic compounds in grape skin.

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