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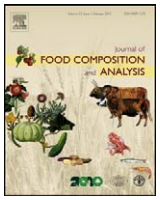

Original Article

Antiradical potential of ancient Italian apple varieties of *Malus* × *domestica* Borkh. in a peroxynitrite-induced oxidative process

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ABSTRACT

Ancient apple cultivars may be an important source of genes for apple breeding programs and for the production of value-added apple cultivars. We evaluated the biochemical properties of six apple varieties grown in the Casentino area (Tuscany, Italy). Two commercial varieties (Golden Delicious and Stark Delicious) and four local varieties (Mora, Nesta, Panaia-red and Ruggine) were selected and their methanolic extracts were analysed for total phenolics and flavonoids. Moreover the content of catechin, epicatechin, rutin, chlorogenic and caffeic acid, five major phenolic constituents, was determined through HPLC-UV analysis. The radical scavenging capacity of the methanolic extracts was assessed using two *in vitro* tests: the blanching of the stable DPPH radical and the inhibition of tyrosine nitration induced by peroxynitrite. Three of the four local varieties had the highest content of total phenols and flavonoids. Ruggine and Panaia-red apple had the highest content of the five phenolic compounds investigated and the highest antioxidant activity towards both radicals. The commercial varieties were characterized by lower phenolic content and antioxidant activity compared to old varieties. Finally, a significant correlation between phenolic content and antioxidant activity was found.

1. Introduction

Epidemiological studies have widely shown that diet plays a crucial role in the prevention of chronic diseases, and one of the most important messages of modern nutrition research is that a diet rich in fruit and vegetables is strongly associated with the risk reduction of cancer, diabetes, cardiovascular and Alzheimer's disease and age-related functional decline (Donaldson, 2004).

It has been proposed that oxidative stress could be implicated in the pathogenesis of these diseases, whose incidence increases as age increases (Mendoza-Núñez et al., 2007). Free radicals are present in biological systems and may oxidize all the biological molecules present in our body, such as nucleic acids, proteins, lipids, and initiating degenerative diseases (Cook and Samman, 1996; Harborne and Williams, 2000; Heim et al., 2002).

Antioxidants are substances that neutralize free radicals and their negative effects. They act at different stages (prevention, interception and repair) and by different mechanisms: reducing agents by donating hydrogen, quenching singlet oxygen, acting as chelators and trapping free radicals (Devasagayam et al., 2004).

Research studies report that fruits, vegetables, grains and other plant food are an important source of polyphenols, bioactive non-nutrient plant compounds that have been found to provide a very strong antioxidant and free radical scavenging activity. The antioxidant activity of dietary polyphenols is considered to be much greater than that of the essential vitamins; therefore it contributes significantly to the health benefits of fruits (Tsao et al., 2005). Other studies also suggest that there are many different substances that are protective in fruits and vegetables, so that the entire effect cannot be simply attributed to a particular component or phytochemical, but to the complex mixture of compounds and to the complex interactions among them.

Apple (*Malus* × *domestica* Borkh.) is one of the most commonly consumed fruits worldwide (Shoji et al., 2004), and it contains a large amount of natural phenolic phytochemicals. Apple polyphenols have been reported to have various *in vitro* and *in vivo*

Abbreviations: CE, catechin equivalents; DPPH, 2,2-diphenyl-1-picryl-hydrazyl; FW, fresh weight; GAE, gallic acid equivalents; $ONOO^-$, peroxynitrite; TE, Trolox equivalents; TPC, total phenolic content.

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Table 1
Apple samples: physicochemical parameters at harvest time.

Cultivar	Average weight (g)		Flesh firmness (kg/cm^2)		Soluble solids (°Brix)		Acidity (g/L)		pH	
Golden Delicious	116.6 ± 20.8	a	5.8 ± 0.4	b	13.6 ± 0.7	b	4.1 ± 0.6	bc	3.6 ± 0.1	ab
Stark Delicious	129.5 ± 24.0	a	7.7 ± 0.4	a	14.1 ± 1.0	b	3.0 ± 0.6	c	3.9 ± 0.1	a
Mora	81.6 ± 19.2	b	7.5 ± 0.6	a	15.9 ± 0.6	b	8.3 ± 0.9	a	2.9 ± 0.1	c
Nesta	81.7 ± 29.8	b	7.2 ± 0.6	a	16.9 ± 1.8	b	5.9 ± 1.1	abc	3.5 ± 0.1	ab
Panaia-red	126.3 ± 35.9	a	5.8 ± 0.4	b	17.1 ± 1.2	b	6.2 ± 0.7	abc	3.2 ± 0.1	c
Ruggine	42.8 ± 15.5	c	7.7 ± 0.8	a	23.4 ± 4.4	a	7.4 ± 4.5	ab	3.7 ± 0.7	a
P ANOVA	***		***		***		**		***	

Sampling was done at optimal commercial maturity, corresponding to: 25th–30th September for Golden Delicious and Stark Delicious; 20th–30th October for Mora, Nesta, Panaia-red and Ruggine. Values are the means ± SD ($n = 6$). Data were analysed by ANOVA and within each column different letters indicate statistically different values according to post hoc comparison (Tukey's HSD).
** $P < 0.01$.
*** $P < 0.001$.

physiological properties: antiallergenic and anti-caries activity, and inhibitory activity against some enzymes and receptors involved in pathological processes (Shoji et al., 2004).

Peroxynitrite ($ONOO^-$) is nowadays considered as one of the most relevant radical generators involved in pathophysiological and toxicological processes. This anion is a product of the reaction between nitric oxide and superoxide and it is a strong 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 pH, 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).

This work provides information on antioxidant compounds present in commercial and old apple varieties. Analysis of total phenolic and total flavonoid content and antioxidant activity is very important to understand the nutraceutical potential of apple and their possible use as source of genes for apple breeding program and cultivar selection. Moreover, to our knowledge, this is the first time that apple 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).

2. Materials and methods

2.1. Chemicals and reagents

Gallic acid, caffeic acid, chlorogenic acid, catechin, epicatechin, rutin, Trolox, Folin–Ciocalteu reagent, tyrosine, 3-nitro-tyrosine, 2,2-diphenyl-1-picridazil ($DPPH^\bullet$), HPLC grade methanol and acetic acid were purchased from Sigma–Aldrich (Milan, Italy). All chemicals were of analytical or higher grade and the aqueous solution were prepared by using ultra-pure water purified by Milli-Q System (Millipore, Milan, Italy).

2.2. Fruit collection

The *Malus* × *domestica* genotypes used in this study were chosen among six apple varieties: two commercial (Golden Delicious and Stark Delicious) and four old local (Mora, Nesta, Panaia-red and Ruggine) varieties. The investigation was run in 2006 in the Casentino area (Tuscany, Italy) and apples were collected at commercial maturity. The optimal harvest period was from 25 to 30 September for Golden Delicious and Stark Delicious, and from 20 to 30 October for Mora, Nesta, Panaia-red and Ruggine varieties. Six replicates of each variety were collected and each replicate was made up of four apples.

In Table 1 we summarize the physicochemical parameters at harvest time of the six varieties: fresh weight (g), soluble solids (°Brix), firmness (kg cm^{-2}), titrable acidity (g malic acid L^{-1}) and pH.

2.3. Extraction of phenolics

Phenolics were extracted from 60 g of fresh material (peel and pulp) using 80% aqueous methanol. The samples were homogenized in a blender and centrifuged for 10 min at 3300 rpm (2500 × *g*), using a Sigma 302K Centrifuge (Bicasa, Milan, Italy), with a swing-out rotor. The precipitate was extracted again with the same solvent to a final volume of 200 ml. Extracts were stored at −20 °C and analysed within a month since extraction.

Extracts were injected into an HPLC system (described in Section 2.9) after filtering through a 0.45 μm cellulose filter (Millipore, Milan, Italy).

2.4. Determination of total phenols

Total phenolic content (TPC) of the extracts was measured by the method described by Singleton and Rossi (1965) with some modifications to reduce volumes. Briefly, 150 μl of Folin–Ciocalteu's phenol reagent was added to 30 μl of extract or standard solution of gallic acid and the mixture was shaken. After 8 min, 600 μl of 10% (w/v) Na_2CO_3 solution was added. After mixing the solution was immediately diluted to 3 ml with ultra-pure water and mixed thoroughly. After 120 min of incubation at 20 °C, the absorbance was measured at 765 nm versus water blank. Total phenolic content was expressed as mg gallic acid equivalents (GAE) per 100 g of fresh material. Each analysis was run in triplicate.

2.5. Determination of total flavonoids

Flavonoid content of the methanolic extracts was determined using a colorimetric assay (Zhishen et al., 1999). Briefly, 1 ml of the extract or standard solution of catechin was added to a 10 ml volumetric flask containing 4 ml ultra-pure water. At zero time, 0.3 ml of 5% (w/v) $NaNO_2$ was added to the flask. After 5 min 0.3 ml of 10% (w/v) $AlCl_3$ was added and after 6 min 1 ml of 1 M NaOH was added.

The total was made up to 10 ml with ultra-pure water and thoroughly mixed. Absorbance of the pink mixture was measured at 510 nm versus water blank and flavonoid content was expressed as mg catechin equivalents (CE) per 100 g of fresh material. Each analysis was run in triplicate.

2.6. Synthesis of peroxynitrite

Peroxynitrite was synthesized according to the method of Beckman et al. (1994). Briefly, an acidic solution (0.6 M HCl) of H_2O_2 (0.7 M) was mixed with KNO_2 (0.6 M) on ice and the reaction quenched with ice-cold NaOH (1.2 M). Residual H_2O_2 was removed by mixing with granular MnO_2 prewashed with NaOH (1.2 M). 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 ($\varepsilon_{ONOO^-} = 1670\,M^{-1}\,cm^{-1}$).

2.7. Inhibition of tyrosine nitration by peroxynitrite

This method is based on the determination, by reversed-phase HPLC-UV analysis, of the 3-NT quantity formed by the reaction between free tyrosine and peroxynitrite at the physiological pH.

The reaction was carried out by adding, under vigorous vortexing, peroxynitrite (5–40 μl, 1 mM final concentration) to a solution containing different dilutions of each apple extract or pure compounds at the desired concentration, tyrosine (2 mM) and HCO3$^-$ (50 mM), all 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). Blank, 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 ($Abs_{356} = 54.717 \times [3\text{-NT}] - 0.224$, $R^2 = 0.999$). The peroxynitrite scavenging ability of the extracts was expressed as Trolox equivalents (TE) in 100 g of fresh material. The peroxynitrite scavenging ability of pure compounds was expressed as the concentration (μM) needed to achieve 50% inhibition of $ONOO^-$ mediated tyrosine nitration (IC_{50}).

2.8. DPPH radical scavenging activity

This method was performed using DPPH$^\bullet$, a stable free radical compound with a characteristic absorption at 515 nm (Brand-Williams et al., 1995). When an antiradical agent is added to DPPH$^\bullet$, the free electron is paired up and the colour (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$^\bullet$ methanolic solution (12×10^{-5} M), and vortexed.

In order to find the time required achieving the steady state (T_{ss}), absorbance at 515 nm (Abs_{515}) was measured at different time intervals on a UV–vis spectrophotometer (Lambda 25, PerkinElmer, Italy) until the reaction reached a plateau. Then, the decrease in absorbance was recorded when the reaction reached the steady state. The initial concentration of DPPH$^\bullet$ was controlled for every experiment by using a calibration curve estimated by measuring the absorbance at 515 nm of standard samples of DPPH$^\bullet$ at different concentrations. The equation of the linear regression was $Abs_{515} = 0.0085 \times [DPPH^\bullet]$ ($R^2 = 0.998$).

The DPPH$^\bullet$ radical scavenging effect was expressed as percentage reduction (%R) of the initial DPPH$^\bullet$ absorption by test samples:

$$\%R \text{ of the initial DPPH}^\bullet \text{ absorption} = \left[\frac{A_{DPPH}(t) - A_{sample}(t)}{A_{DPPH}(t)}\right] \times 100$$

where $A_{DPPH}(t)$ is the absorbance of DPPH$^\bullet$ at time t and $A_{sample}(t)$ is the absorbance of the sample at the same time t.

The DPPH$^\bullet$ scavenging ability of pure compounds was expressed as the concentration (μM) needed to decrease the $DPPH^\bullet_0$ concentration of 50% (IC_{50}).

2.9. HPLC analysis

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) running on a PC coupled with the HPLC system. Separation was performed on a reversed-phase C18 column (Acclaim 120, 5 μm, 4.6 mm × 250 mm, Dionex) coupled with a C18 guard cartridge (Acclaim 120, 5 μm, 4.3 mm × 10 mm, Dionex) at room temperature.

Elution conditions for the detection of phenolic compounds in apple 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 four compounds investigated: at 280 nm for catechin and epicatechin, at 320 nm for chlorogenic acid and caffeic acid and at 360 nm for rutin. 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 were linear with $R^2 = 0.999$.

Elution program for the detection of phenolic compounds in apple extracts was as follows: B 5% and C 3% as initial conditions, B 55% in 40 min, B 100% in 10 min, which was kept isocratic for 10 min, and B 5% in 5 min, which was kept isocratic for 5 min.

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

2.10. Statistical analysis

Three analytical determinations were carried out on each extract for every parameter and the analytical measurements were averaged on a sample base. Six independent replications ($n = 6$) were obtained from each genotype and the results presented in tables and graphs were reported as means ± standard deviation (SD). Data were subjected to ANOVA and differences within mean values were tested by *post hoc* comparison test (Tukey's HSD) at $P = 0.05$.

3. Results and discussion

3.1. Polyphenolic composition of apple

The total phenolic and total flavonoid content of the whole fruit extracts of six apple varieties (four old and two commercial varieties) was evaluated and results are reported in Table 2.

Panaia-red and Ruggine, two of the four old varieties analysed, were the ones with the higher total phenolic content (221 ± 28 and 211 ± 38 mg GAE/100 g of fresh weight (FW)), while the Golden

Table 2
Total phenolic and flavonoid content of apple cultivar studied.

Cultivar	Total phenolics		Total flavonoids	
	GAE[a]/100 g FW		CE[a]/100 g FW	
Golden Delicious	104.3 ± 11.2	c	65.0 ± 7.5	c
Stark Delicious	98.6 ± 8.5	c	71.6 ± 6.1	c
Mora	157.6 ± 22.9	b	101.5 ± 16.5	b
Nesta	128.2 ± 16.1	bc	91.8 ± 15.4	bc
Panaia-red	221.2 ± 27.5	a	137.5 ± 24.5	a
Ruggine	211.1 ± 38.0	a	140.3 ± 17.4	a
P ANOVA	***		***	

Values are the means ± SD ($n = 6$). Data were analysed by ANOVA and within each column different letters indicate statistically different values according to post hoc comparison (Tukey's HSD).

[a] GAE, gallic acid equivalents, CE, catechin equivalents.

*** $P < 0.001$.

Table 3
Catechin, epicatechin, rutin, chlorogenic acid and caffeic acid content of apple cultivar studied.

Cultivar	Phenolic content (mg/100 g FW)[a]									
	Catechin		Epicatechin		Rutin		Chlorogenic acid		Caffeic acid	
Golden Delicious	0.74 ± 0.16	c	4.73 ± 0.88	d	0.33 ± 0.08	bc	15.49 ± 2.21	d	1.47 ± 0.19	d
Stark Delicious	1.75 ± 0.50	b	11.07 ± 1.74	bc	0.30 ± 0.08	bc	12.17 ± 1.03	d	0.96 ± 0.23	e
Mora	2.50 ± 0.26	b	9.81 ± 1.23	c	0.62 ± 0.17	a	29.51 ± 1.99	c	2.54 ± 0.21	bc
Nesta	1.76 ± 0.46	b	13.69 ± 1.78	b	0.20 ± 0.06	c	39.93 ± 7.85	b	3.47 ± 0.66	a
Panaia-red	4.45 ± 1.08	a	13.15 ± 1.77	b	0.46 ± 0.10	ab	35.33 ± 7.11	bc	2.87 ± 0.39	b
Ruggine	3.94 ± 0.81	a	19.65 ± 5.37	a	0.41 ± 0.13	a	63.05 ± 17.59	a	2.17 ± 0.42	c
P ANOVA	***		***		***		***		***	

[a] Values are the means ± SD ($n = 6$). Data were analysed by ANOVA and within each column different letters indicate statistically different values according to *post hoc* comparison (Tukey's HSD) at $P = 0.05$.
*** $P < 0.001$.

Delicious, Stark Delicious and Nesta varieties had the lowest values (respectively 104 ± 11, 98 ± 9 and 128 ± 16 mg GAE/100 g FW). The old variety Mora had intermediate values (158 ± 23 mg GAE/100 g FW). The same trend was found in the total flavonoid content. Flavonoids content values ranged from 140 ± 17 of Ruggine to 65 ± 8 of Golden Delicious, the average being 101 ± 15 mg CE/100 g FW.

Apple phenolic content is characterized by some major classes of phenolic compounds present in the peel and in the flesh of this fruit: flavan-3-ols monomers (catechin and epicatechin), flavan-3-ols polymers (procyanidins), dihydrochalcones (phloretin glycosides), flavonols (quercetin glycosides), hydroxycinnamic acids (chlorogenic and caffeic acid) and, in red skin cultivar, anthocyanins (Alonso-Salces et al., 2001).

In Table 3 the principal phenol constituents of the apple extracts are reported. The phenolic compounds were selected on the basis of the literature data (Escarpa and González, 1998) concerning different apple varieties. Among all the phenolics, the flavan-3-ols catechin and epicatechin, the flavonols rutin and the hydroxycinnamic acids chlorogenic and caffeic acid were determined and quantified.

The HPLC pattern of the phenolic compounds was similar in all the examined cultivar (Fig. 1), in accordance to literature data (Escarpa and González, 1998; Napolitano et al., 2004), and the main compounds were found to be chlorogenic acid and epicatechin. This latter was found to be the most abundant phenolic compounds even in all the genotypes studied by others (Chinnici et al., 2004; Khanizadeh et al., 2008).

We found fairly large variability among apple of the same cultivar, as other authors have already observed (Escarpa and González, 1998; Lata et al., 2009).

When the phenolic composition of the six varieties is compared, we could state that commercial Golden Delicious was found to be the variety with the lowest content of almost all the compounds under our evaluation, whereas Ruggine contained the highest level. Stark Delicious had a lower content of caffeic acid than Golden Delicious (respectively 0.96 ± 0.23 and 1.47 ± 0.19 mg/100 g FW) and Nesta and Panaia-red apple presented a larger amount of caffeic acid in their extracts than Ruggine.

The content of chlorogenic acid was very variable within the six varieties and values range from 12.2 ± 1 of Stark Delicious to 63.1 ± 17.6 mg/100 g FW of Ruggine apple. Similar results were found by Lata et al. (2009) that reported chlorogenic acid amount between 6.32 and 79.0 mg per whole fruit, within the group of the 19 apple varieties studied. Quite low levels of rutin were detected, the average value being 0.39 ± 0.1 mg/100 g FW, as also reported by Chinnici et al. (2004) for Golden Delicious.

As underlined also by others (Lata et al., 2009), it is difficult to directly compare the content of apple phenolic among different studies, as many variations can be principally caused by different growth period, geographic location, genetic diversity and many other factors. Apples remain, anyway, a rich source of phenolic compounds.

3.2. Antioxidant activity of apple extracts and pure compounds

Several methods have been proposed to evaluate the antioxidant activity of plant extracts and pure compounds and it is widely accepted that their effectiveness depends on the environmental conditions and procedures used. Each method relates to the

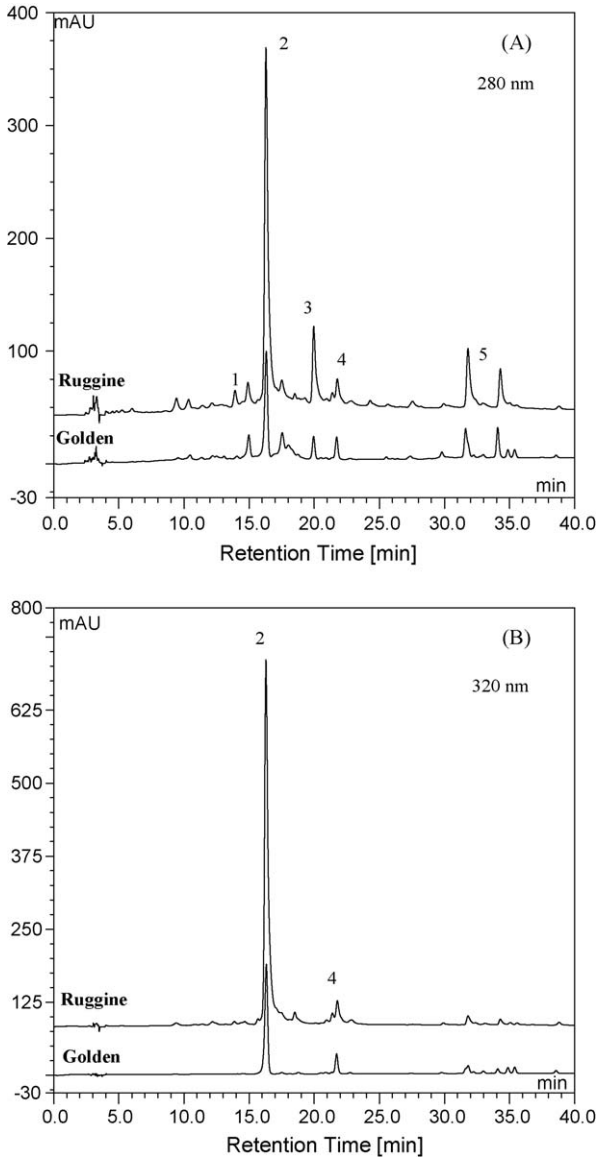

Fig. 1. HPLC chromatogram of Golden Delicious and Ruggine apple extracts at 280 nm (A) and 320 nm (B). Peaks: (1) catechin, (2) chlorogenic acid, (3) epicatechin, (4) caffeic acid, and (5) rutin.

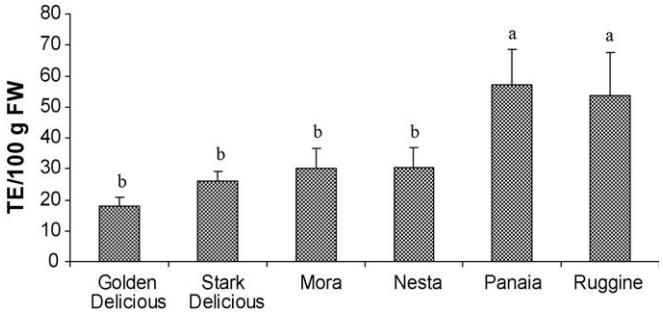

Fig. 2. Antioxidant activity. Inhibition of tyrosine nitration by peroxynitrite. Data were analysed by ANOVA and different letters indicate statistically different values according to *post hoc* comparison (Tukey's HSD) at $P = 0.05$.

generation or use of a different radical that is directly involved in the oxidative process, acting through a variety of mechanisms. Antioxidants act by scavenging these radicals and by reducing their oxidative power. Among the commonly used *in vitro* assays, we selected the DPPH• assay, based on the inactivation of stable synthetic radicals, the DPPH•, first envisaged by Blois (1958) and the inhibition of tyrosine nitration by $ONOO^-$ to determine the antioxidant activity of apple extracts.

We are not aware of any published data on antioxidant capacity of these ancient local (Mora, Nesta, Panaia-red and Ruggine) vs. commercial (Golden Delicious and Stark Delicious) apple varieties.

Figs. 2 and 3 show, respectively, the $ONOO^-$ and DPPH radical scavenging capacity of the six apple variety extracts. All the apple extracts showed inhibiting activity against the nitration of tyrosine by $ONOO^-$. The strongest activity was observed for the local varieties Panaia-red and Ruggine, respectively 57 ± 12 and 54 ± 14 TE/100 g FW. Commercial varieties were statistically equivalent to local varieties Nesta and Mora towards $ONOO^-$ and the average value was 26 ± 5 TE/100 g FW. Therefore we observed that the antioxidant activity of these four varieties was about a half that the one of Panaia-red and Ruggine varieties, according to reported data.

When DPPH• radical scavenging capacity was tested, the strongest activity was observed for the local variety Ruggine. It was able to reduce the radical by 94% after 35 min of incubation, and by 80% after 5 min of incubation. Panaia-red apple showed also a strong activity. In fact, it was able to reduce radical absorbance by 90% after 35 min and 63% after 5 min of incubation. Stark Delicious apple showed a different trend in comparison with $ONOO^-$ assay, exhibiting an high radical absorbance inhibition, statistically equivalent to Panaia-red apple. The local variety Nesta was the one with the lowest activity. DPPH• absorbance was reduced by Nesta apple by 70% after 35 min and by 35% after 5 min of incubation.

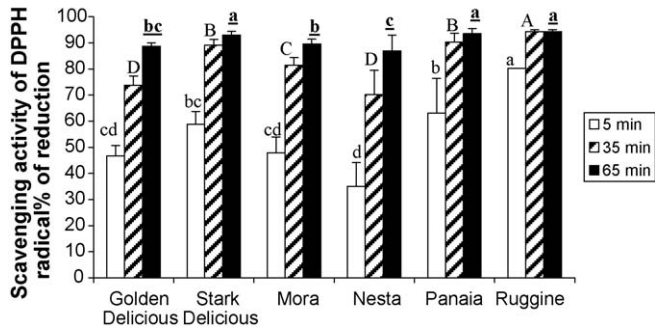

Fig. 3. Antioxidant activity. Inhibition of DPPH radical activity at different times of reaction. Data were analysed by ANOVA and within each time different letters indicate statistically different values according to *post hoc* comparison (Tukey's HSD) at $P = 0.05$.

Table 4
Comparison of pure phenolic compounds in terms of their radical scavenging activities. Trolox was used as reference.

Compound	IC_{50} (μM)[a]			
	$ONOO^-$		DPPH	
Catechin	55.7 ± 0.5	d	6.7 ± 0.4	b
Epicatechin	56.5 ± 0.6	d	6.8 ± 0.2	b
Rutin	92.4 ± 1.0	b	7.4 ± 0.2	b
Chlorogenic acid	74.9 ± 2.8	c	13.6 ± 0.2	a
Caffeic acid	83.1 ± 4.6	bc	12.5 ± 0.3	a
Trolox	137.6 ± 12.7	a	13.7 ± 1.1	a
P ANOVA	***		***	

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

In both assays Ruggine and Panaia-red apple were the varieties with the higher antioxidant capacity. The results of the two tests had a similar trend. Stark Delicious apple showed stronger activity towards DPPH• radical than $ONOO^-$.

When the pure compounds were assayed against DPPH• and peroxynitrite, all the five were found to be active (Table 4). In both cases we used Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble analogue of vitamin E, as a known inhibitor for comparative purposes. None of the pure compounds interfered with the HPLC determination of 3-NT and none of them exhibited a lower ability to reduce tyrosine nitration scavenge radicals than Trolox.

Under our experimental conditions the relative antioxidant activity towards $ONOO^-$ decreased from the statistically equivalent catechin and epicatechin (IC_{50} compounds average 56.1 ± 0.7 μM), to chlorogenic acid, caffeic acid and rutin (IC_{50} compounds average 83.5 ± 2.8 μM). As regards to DPPH• scavenging activity IC_{50} values ranged from 6.7 ± 0.4 to 13.7 ± 1.1 μM. Among the pure compounds analysed the phenolic acids, caffeic and chlorogenic acids, were statistically equivalent to Trolox. Catechin, epicatechin and rutin were the most active compounds and were statistically equivalent.

The basic structure of the phenols and other structural factors play a fundamental role in the mechanism by which these compounds are able to scavenge free radicals (Sadeghipour et al., 2005). The aromatic OH groups are the reactive centres, primarily 3′,4′-dihydroxy catechol group, and their activity can be enhanced by electron donating effects of other substituents (Heijnen et al., 2001). As regards 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).

Catechin and epicatechin had the same behaviour and were the compounds with the highest scavenging activity within the five under analysis. As also reported by other authors (Pannala et al., 1997; Sadeghipour et al., 2005), catechin and epicatechin were the most active peroxynitrite inhibitors. On the contrary, chlorogenic and caffeic acid were the ones with the lowest activity, even being in both cases statistically equivalent (Pannala et al., 1998). As seen in our previous work (Iacopini et al., 2008), rutin had different results when assayed against peroxynitrite and DPPH•. It was statistically equivalent to the phenolic acids and it had the lowest activity towards peroxynitrite, while it had the highest activity towards DPPH•, together with catechin and epicatechin.

3.3. Correlation

In order to prove if the differences concerning phenolic composition and antioxidant activity among the six apple varieties

Table 5
Correlation coefficient (r) and P values of the linear regression between physicochemical and biochemical parameters of apple extracts.

	Weight		Flesh firmness		Soluble solids		Acidity	
	r	P	r	P	r	P	r	P
Total phenols	−0.386	*	–	ns	0.489	**	–	ns
Total flavonoids	−0.454	**	–	ns	0.572	***	–	ns
Catechin	–	ns	–	ns	0.609	***	0.455	**
Epicatechin	−0.540	***	0.437	**	0.852	***	0.492	**
Rutin	–	ns	–	ns	–	ns	–	ns
Chlorogenic acid	−0.727	***	–	ns	0.687	***	–	ns
Caffeic acid	−0.374	*	–	ns	–	ns	0.360	*
$DPPH^{\bullet}_{(65\ min)}$	–	ns	–	ns	0.419	*	–	ns
$DPPH^{\bullet}_{(35\ min)}$	–	ns	–	ns	0.479	**	–	ns
$DPPH^{\bullet}_{(5\ min)}$	–	ns	–	ns	0.466	**	–	ns
$ONOO^-$	–	ns	–	ns	0.569	***	–	ns

Data ($n = 36$) were analysed by linear regression analysis. ns = not significant.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

investigated were related to ripening level, the relationship between the physicochemical and biochemical parameters were also determined through correlation analysis.

Results, reported in Table 5, show that the phenolic content and the antioxidant activity of the extracts had a very low correlation with the physicochemical parameters measured. Epicatechin, chlorogenic acid, caffeic acid, total phenols and flavonoids had a low and negative correlation with weight, and chlorogenic acid showed the highest value ($r = -0.73$). Catechin, rutin and antioxidant activity were not correlated with weight. Flesh firmness was found to have a significant correlation only with epicatechin content, but at a very low level ($r = 0.44$). The same was for the parameter "acidity" that was significantly correlated at low level with catechin, epicatechin and caffeic acid. Soluble solids showed a different trend. This parameter had a significant and positive correlation with almost all the biochemical parameter measured, but in general values were quite low. Epicatechin and chlorogenic acid were the phenolic compounds with the highest correlations (respectively $r = 0.85$ and 0.69). Rutin and caffeic acid were not correlated at all.

As the correlation coefficients between the physicochemical and biochemical parameters of the six apple varieties investigated were generally low or not significant, these results let us say that the physicochemical differences between the cultivar were not influencing factors on the phenolic content and on the antioxidant activity of the apple extracts.

When the correlations between the phenolic content and the antioxidant activity were determined (Table 6), we found that catechin was largely the phenolic compound with the highest correlations with both total polyphenolic content and antioxidant activity, as found in Khanizadeh et al. (2008). In particular it had strong correlations with total phenolics ($r = 0.80$), total flavonoids ($r = 0.81$), $DPPH^{\bullet}$ after 35 min ($r = 0.69$) and $ONOO^-$ ($r = 0.75$). Chlorogenic acid also showed a significant correlation with total phenolic content and with $ONOO^-$ scavenging capacity. Epicatechin showed significant correlations with all the parameters, but at very lower level with respect to catechin. Its highest correlation coefficient was $r = 0.66$ with $ONOO^-$ scavenging activity. Rutin and caffeic acid had weak or not significant correlations. The positive and significant correlation between catechin, epicatechin and chlorogenic acid with $ONOO^-$ assay suggests that these three compounds largely contribute to the radical scavenging capacity of whole apple extract.

There was a positive linear correlation between total phenols and total flavonoids ($r = 0.93$). Total phenols and flavonoids had a significant and high correlation with $ONOO^-$ scavenging capacity of the apple extracts (respectively $r = 0.83$ and 0.79), that is to say that the capacity to inhibit the tyrosine nitration increases with the increase in polyphenols content and that phenolic compounds are directly responsible for the $ONOO^-$ scavenging capacity of the extracts. The correlation with $DPPH^{\bullet}$ antiradical activity was also significant but at lower levels.

The correlation between polyphenolic content and $DPPH^{\bullet}$ antiradical activity after 65 min was very weak, while it seemed to increase when time of reaction was reduced. This could be because after 65 min of reaction all the six apple variety reached the plateau and the % of reduction of the radical (Fig. 3) was very similar (values ranged from 89 to 94%). After 5 and 35 min reactions were not complete and the phenolic composition of the extracts played still a discriminating role in the scavenging capacity of the varieties.

The same performance was observed when the correlation between $DPPH^{\bullet}$ radical scavenging capacity and inhibition of

Table 6
Correlation coefficient (r) and P values of the linear regression between phenolic content and antioxidant activity of apple extracts.

	Total phenols		Total flavonoids		DPPH (65 min)		DPPH (35 min)		DPPH (5 min)		$ONOO^-$	
	r	P	r	P	r	P	r	P	r	P	r	P
Catechin	0.803	***	0.807	***	0.557	***	0.686	***	0.589	***	0.746	***
Epicatechin	0.508	**	0.589	***	0.352	*	0.478	**	0.449	**	0.656	***
Rutin	0.368	*	0.365	*	–	ns	0.371	*	–	ns	–	ns
Chlorogenic acid	0.673	***	0.724	***	–	ns	–	ns	0.471	**	0.634	***
Caffeic acid	0.421	*	0.423	*	–	ns	–	ns	–	ns	0.360	*
Total flavonoids	0.928	***	–	–	0.409	*	0.531	***	0.512	**	0.788	***
Total phenols	–	–	0.928	***	0.396	*	0.537	***	0.584	***	0.830	***

Data ($n = 36$) were analysed by linear regression analysis. ns = not significant.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

tyrosine nitration by $ONOO^-$ was evaluated. There was a significant correlation between the two assays when it was valued with $DPPH^•$ after 5 min ($r = 0.63$, P ANOVA = 0.0000) and after 35 min of reaction ($r = 0.54$, P ANOVA = 0.0007). The correlation decreased as the reaction time increased. In fact the correlation coefficient between $ONOO^-$ and $DPPH^•$ after 65 min of reaction was reduced at $r = 0.36$ and the significance was P ANOVA = 0.0299. Therefore, the two *in vitro* tests can be considered comparable as their results significantly correlate. The loss of correlation after 65 min of reaction ($DPPH^•$) can be due to the fact that at that time the reaction is complete and the phenolics have not more the discriminating role in the scavenging capacity of the varieties that they have after 5 and 35 min of reaction. Even this parameter can be used to strength the idea that the phenolic compounds plays a direct crucial role and are directly responsible for the antioxidant activity of apple extracts.

4. Conclusion

In conclusion, the phenolic content of local and commercial apple varieties was studied and their antioxidant activities were compared using $ONOO^-$ and $DPPH^•$ assays.

To our knowledge, this was the first time that the peroxynitrite-induced tyrosine nitration test has been used to demonstrate the antiradical activity of apple extracts. The reported results show that this method could be considered reliable and comparable to other antiradical tests, but more representatives of human physiological conditions, as it works at physiological pH 7.4, it involves radicals physiologically produced by the human organism and directly correlated with the emergence of pathophysiological and toxicological processes. Moreover the 3-nitro-tyrosine, product of the reaction between tyrosine and peroxynitrite, as it represents a common modification introduced by the biological formation of peroxynitrite, is commonly used as a marker of nitrosative stress (Pacher et al., 2007).

Significant quantitatively differences were observed among apple varieties studied. Generally, old local varieties, in particular Ruggine and Panaia-red, showed higher level of phenolic compounds and of antioxidant activity. The commercial variety Golden Delicious was the one with the lowest values. The antioxidant activity was positively correlated with the total polyphenolic concentration and with the concentration of the principal phenolic compounds present in apple extract: catechin, epicatechin and chlorogenic acid. These data suggest the relevant role played by the genotype in the determination of the polyphenolic content and of the scavenging properties, as recently reported by other authors (Scalzo et al., 2005). The physicochemical variability among the six varieties seemed not to explain the differences in phenolic content and antioxidant capacity.

We therefore conclude that the phenolic content, the radical scavenging and antioxidant properties of old local apple varieties demonstrate that these neglected cultivars could be a good source of phytochemicals, bioactive compounds with important protective properties. These local apple cultivars could be also considered as an important source of genes for apple breeding program and for the production of value-added apple cultivar. So that being, further studies on local and ancient varieties have to be encouraged so that those varieties with the most technological interest can be selected.

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