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'Limoncella' apple, an Italian apple cultivar: Phenolic and flavonoid contents and antioxidant activity

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Abstract

'Limoncella' is a variety of apple traditionally cultivated in south-central Italy since ancient Roman times. The relationship between phenolic composition and antioxidant activity of apple peel and flesh was investigated. Apple extracts analysed by high-performance liquid chromatography, showed that compared with flesh, peel was richer in all the quantified phenols. The most abundant polyphenols were the flavanols catechin and epicatechin, and the chalcones phloridzin and phloretin xyloglucoside.

The extracts were evaluated for their radical-scavenging activity by the DPPH· and H_2O_2 methods, and the antioxidant activity through reduction of Mo(VI) and inhibition of the formation of TBARS species.

Keywords: cv 'Limoncella' apple; Flavonoids; Phenols; Antioxidant activity; Radical-scavenging activity

1. Introduction

Antioxidants help to neutralise free radicals, which are unstable molecules that are linked to the development of a number of degenerative diseases and conditions including cancer, cardiovascular disease, cognitive impairment, immune dysfunction, cataracts and macular degeneration (Leborgne, Maziere, Maziere, & Andrejak, 2002; Folts, 2002). Fruits and vegetables are sources of natural antioxidants and among them apples have one of the highest levels of antioxidant activity (Chinnici, Bendini, Gaiani, & Riponi, 2004).

Among the phytochemicals, phenols, have received a great deal of attention because of their antioxidant activity (Tsuda et al., 1994). Many studies show that apple is a rich resource of phenolic compounds, especially flavonoids, whose activity and concentration differ with cultivar, maturity stage, environmental conditions and the part of the fruit (Kjersti, Hvattum, & Skrede, 2004; Kondo, Tsuda, Muto, & Ueda, 2002; Van Der Sluice, Dekker, de Jager, & Jongen, 2001). Flavonoids are characterised largely by the hydroxylation pattern around the central C-ring. Apple flavonoids possess a high number of electron-donor hydroxyl groups and a large number of double bonds, properties that have been shown to increase the overall antioxidant activity (Tsuda et al., 1994; Harborne & Williams, 2000).

Recently we reported the phytochemical study of cv. Annurca apple, a variety cultivated in the south of Italy (Cefarelli, D'Abrosca, Fiorentino, Izzo, & Monaco, 2005; Cefarelli et al., 2006; D'Abrosca, Fiorentino, Monaco, & Pacifico, 2005; D'Abrosca, Fiorentino, Oriano, Monaco, & Pacifico, 2006). Forty-three secondary metabolites belonging to different classes were isolated and characterised. Evaluation of the antioxidant capacity of these substances showed that flavonoids, which exhibited activities comparable to that of α-tocopherol, were responsible for the activity and that the antioxidant power was closely correlated with the structure of the tested molecules.

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Continuing the phytochemical study of native fruits of the Campania Region (Italy), our interest has turned to Limoncella apples, known also as '*Limone*' (*lemon*) apple.

Malus domestica cv 'Limoncella' (Neri, 2004) is a variety of apple traditionally cultivated in south-central Italy since Roman times. This variety, together with 'Annurca', 'Zitella' and 'Mela Rosa' cultivars, is well-adapted to the local climate and slows down its growth in the summer. The Limoncella apple, like the Annurca, has to be stored in open air under a rain shelter for at least two months, to develop its characteristic flavour. It develops a green–yellow skin with a citric taste and keeps until the end of the spring. The fruit is medium in size with an ellipsoidal shape. The yellow–green skin is thick, rather wax-like, and possesses numerous rust lentils. The white flesh is quite firm, juicy, aromatic and lightly acidic, a characteristic responsible for the name of this apple.

In this study, we report the determination of total phenol and flavonoid contents of peel and flesh methanolic extracts. The RP-HPLC analysis of the extracts allowed us to identify and quantify the most abundant polyphenols in the apple. An evaluation of the radical-scavenging (by the DPPH$^{\cdot}$ and H_2O_2 methods) and antioxidant activity (by reduction of Mo(VI) and inhibition of the formation of TBARS species) of the alcoholic extract was also performed.

2. Materials and methods

2.1. Chemicals and reagents

The solvents used in the present work were purchased from Riedel-de-Haën (Germany). Soxhlet cartridges are obtained from Waters spa (Italy). Folin-Ciocalteau's reagent, sodium carbonate, gallic acid, quercetin dihydrate, catechin, epicatechin, quercetin-3-β-D-glucoside, rutin trihydrate, caffeic acid, phloridzin dihydrate, chlorogenic acid hemihydrate, DPPH$^{\cdot}$ (2,2-diphenyl-1-picryl-hydrazyl), Tween 40 and peroxidase from horseradish were purchased from Fluka (Germany). Hydrogen peroxide, ammonium molybdate tetrahydrate, sodium nitrite, aluminum chloride, thiobarbituric acid (TBA), tannic acid and thrichloracetic acid (TCA) were acquired from Riedel-de-Haën (Germany). Phlorethin-2′-xyloglucoside was previously isolated and characterised from cv. Annurca apple (Cefarelli et al., 2006).

2.2. Fruit collection

Limoncella apple fruits were collected in San Salvatore Telesino, near Benevento (Italy), in October 2005 when the fruit had just been harvested (green peel) and then stored in a climatic cell at 0 °C and 98% humidity.

2.3. Extraction procedure

Apples were peeled and both peel and flesh were divided into three aliquots of 50 g, sliced, frozen in liquid nitrogen, powered in a mortar and lyophilised using an FTS System Flex-Dry instrument. The obtained powders were exhaustively extracted using a Soxhlet apparatus for 4 h with methanol. The solution was dried using an evaporator to obtain the crude extracts, which were analyzed by HPLC with UV detection.

2.4. Determination of total phenols

The amount of total phenols in peel and flesh extracts were determined according to the Folin-Ciocalteu procedure (Kähkönen et al., 1999). To samples (50 mg in 1 ml) in test tubes were added 1.0 ml of Folin-Ciocalteu's reagent and 8 ml of sodium carbonate (7.5%). The tubes were mixed and allowed to stand for 1 h. An aliquot of each sample (300 μl) were poured into 1700 μl of water. Absorption at 765 nm was measured. The total phenolic content was expressed as gallic acid equivalents in milligrams per gram of fresh material. The total phenols of the samples are expressed in milligrams per serving of gallic acid equivalents (GAE). All samples were analysed in triplicate.

2.5. Determination of total flavonoids

The flavonoid content of the methanolic extracts were measured using a colorimetric assay (Zhishen, Mengcheng, & Jianming, 1999). A known volume (0.5 ml) of the extract or standard solution of quercetin was added to a 10 ml volumetric flask. Distilled water was added to make a volume of 5 ml. At zero time, 0.3 ml of 5% w/v $NaNO_2$ was added to the flask. After 5 min, 0.6 ml of 10% w/v $AlCl_3$ was added and after 6 min, 2 ml of 1 M NaOH was added to the mixture followed by the addition of 2.1 ml distilled water. Absorbance was read at 350 nm against the blank (water) and flavonoid content was expressed as mg quercetin equivalents (QE) in 100 g of fresh material.

2.6. HPLC analysis

One hundred micrograms of the MeOH extract were dissolved in 1 ml of MeOH and analysed by UV–Vis RP-HPLC. The HPLC apparatus consisted of a pump (Beckman 127 System Gold), a UV–Vis detector (Beckman 166) and a Shimadzu Chromatopac C-R6A recorder. HPLC analyses of polyphenols were performed using a reverse-phase Gemini C18 column (Phenomenex, 250 × 4.6 mm i.d., 5 μm) with guard column (4.0 × 2.0 mm i.d.). The mobile phase was a mixture of A (H_2O) and B (MeCN/MeOH, 4:1) at a gradient of A:B from 49:1 to 7:3 in 20 min, then isocratic mode for 10 min, and finally 100% B for 5 min. The flow rate was 0.7 ml min^{-1}, and detection was at 260 nm. Retention times (min) of the compounds were as follows: 27.91 (catechin), 29.71 (epicatechin), 31.44 (quercitin-3-β-D-glucoside), 32.32 (rutin), 33.83 (caffeic acid), 34.93 (phloridzin), 37.12 (phlorethin-2′-xyloglucoside), 42.50 (chlorogenic acid).

2.7. DPPH radical scavenging activity

The scavenging activity of samples was measured in accordance with the method of Brand-Williams (Brand-Williams, Cuvelier, & Berset, 1995). The method was based on the reduction of methanolic DPPH· in the presence of a hydrogen-donating antioxidant. DPPH· solution was an intense violet colour and showed an absorption band at 515 nm. Adsorption and colour lowered when DPPH· was reduced by an antioxidant compound. The remaining DPPH· corresponded inversely to the radical-scavenging activity of the antioxidant. DPPH· (2 mg) was dissolved in 54 ml of MeOH. Aliquots of investigated extract (50, 100, 200, 300, 500 and 1000 μg) were dissolved in 1 ml of MeOH. Then 1.0 ml of each solution was added to 1.0 ml of DPPH· solution at room temperature. The absorbance at 515 nm was measured against a blank (1 ml MeOH in 1.0 ml of DPPH· solution) using a UV-1601 Shimadzu spectrophotometer. The results were expressed as percentage of reduction of the initial DPPH· adsorption by test samples:

% of reduction of the initial DPPH· adsorption $= A_{\mathrm{DPPH(t)}} - A_{\mathrm{sample}\ (t)} / A_{\mathrm{DPPH}\ (t)} \times 100$,

$A_{\mathrm{DPPH(t)}}$ is absorbance of DPPH· at time t and $A_{\mathrm{sample\ (t)}}$ is absorbance of sample at t the same time.

2.8. H_2O_2 scavenging activity

Hydrogen peroxide-scavenging activity was performed by the method of Pick and Keisari, reported by Sroka and Cisowski (2003). One hundred microlitres of 0.003% hydrogen peroxide, 700 μl of 0.1 M phosphate buffer and 100 μl of 0.1 M sodium chloride were added to aliquots of the investigated extract (50, 100, 200, 300, 500 and 1000 μg), previously dissolved in 100 μl of H_2O. The reaction mixture was incubated for 20 min at 37 °C. Then 1 ml of 0.2 mg/ml phenol red dye with 0.1 mg/ml horseradish peroxidase in 0.1 M phosphate buffer was added. After 15 min, 100 μl of 0.5 M NaOH were added and absorbance was measured at 610 nm using a UV-1601 Shimadzu spectrophotometer. The results were expressed as percentage of reduction of H_2O_2 adsorption by test samples:

Percentage of reduction of H_2O_2 adsorption

$$= A_{H_2O_2} - A_{\mathrm{sample}(t)} / A_{H_2O_2} \times 100$$

2.9. Evaluation of total antioxidant activity

Spectrophotometric evaluation of antioxidant activity through the formation of a phosphomolybdenum complex was carried out according to Prieto et al. (Prieto, Pineda, & Aguilar, 1999). Sample solutions containing reducing extract (50, 100, 200, 300, 500 and 1000 μg in 500 μl of dimethylsulfoxide) were combined in an Eppendorf tube with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95 °C for 90 min. After the samples had cooled to room temperature, the absorbance of aqueous solutions of each was measured at 820 nm against a blank.

2.10. Determination of TBARS

End products of polyunsaturated fatty acid oxygenation react with thiobarbituric acid (TBA) to form a red adduct. Determination of thiobarbituric acid reactive substances (TBARS) was performed by the method of Kulisic et al. (Kulisic, Radonic, Katalinic, & Milos, 2004). TBA reagent was prepared as follows: for reagent A, 375 mg of TBA and 30 mg of tannic acid were dissolved in 30 ml of hot water; for reagent B, 15 g of trichloroacetic acid was dissolved in 70 ml of 0.3 M hydrochloric acid. Then, 30 ml of reagent A was mixed with 70 ml of reagent B. Next, 5.2 μl of rapeseed oil were emulsified with 15.6 mg of Tween 40 initially dissolved in 2 ml of 0.2 M Tris–HCl buffer (pH 7.4). The emulsion was irradiated at 254 nm for 60 min at 25 °C. Then, 100 μl of aqueous solutions of test aliquots of sample extracts (50, 100, 200, 300, 500 and 1000 μg in 300 μl of solvent) were added to 1 ml of the reaction mixture. The samples were irradiated for another 30 min. After addition of 2 ml of TBA reagent, all test tubes were placed into a boiling water bath for 15 min and then centrifuged using a Beckman GS-15 R centrifuge for 3 min at 1500 *g*, and the absorbance of the supernatant was measured at 532 nm. Inhibition of lipid peroxidation was measured as a percentage against a blank containing no test sample.

3. Results and discussion

Peels and fleshes of 'Limoncella' apples, previously frozen in liquid nitrogen, powered in a mortar and lyophilised, were extracted by Soxhlet extraction in MeOH. After removal of the solvent, we obtained peel and flesh crude extracts.

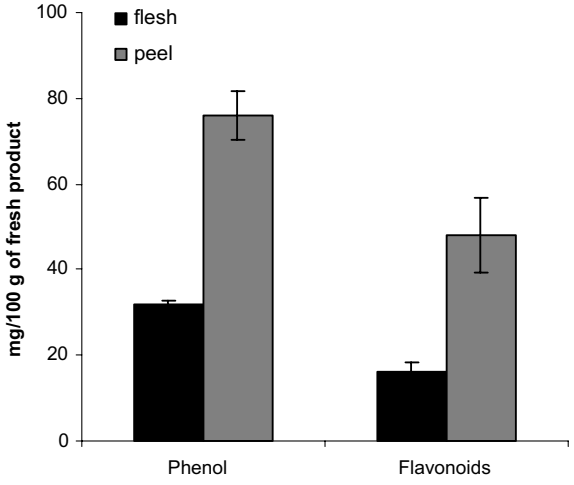

Fig. 1. Total phenolic and flavonoids contents of Limoncella apple.

The phenolic and flavonoid contents of both the peel and flesh extracts of Limoncella apples are reported in Fig. 1.

In the peel extract the amount of phenolic substances was equal to 76.0 mg of gallic acid equivalents (GAE) in 100 g of fresh product. The amount of phenolic compounds decreased in the flesh extract to 31.7 mg GAE per 100 g. A significant difference was also observed in the total flavonoids of the skin and pulp of the cultivar: flavonoid content in the skin was 47.8 mg of quercetin equivalents (QE) in 100 g of fresh material, and in the pulp it was 16.0 mg QE.

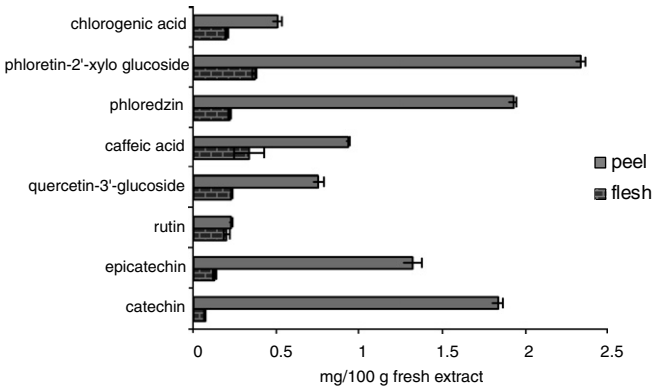

Fig. 2. HPLC profile of polyphenols from 'Limoncella' apples.

In Fig. 2, the principal phenol constituents of the extracts are reported. The phenols were selected on the basis of the literature data of different cultivars of apples. Apples were showed to contain high amounts of flavonoids: phloridzin and phloretin-2′-xyloglucoside were the most abundant compounds. Peel was rich in the flavan-3-ol isomers catechin and epicatechin.

Evaluation of antioxidant activity was conducted by four methods. When DPPH radical scavenging capacity was tested, we observed a linear increase of activity with tested amounts of sample. Reductions equal to 78% and 55% were obtained for 1 mg of peel and flesh extracts, respectively.

In the H_2O_2 radical-scavenging assay a similar but weaker effect was observed: diverse amounts of sample extract possessed a reductive power on pro-oxidant of 15–35% (Fig. 3).

Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex showed an exponential increase of activity with tested amounts of sample. In particular the percentage reduction of Mo(VI) to Mo(V) was equal to 92% for 1 mg of assayed sample extract. Methanolic extracts inhibited exponentially

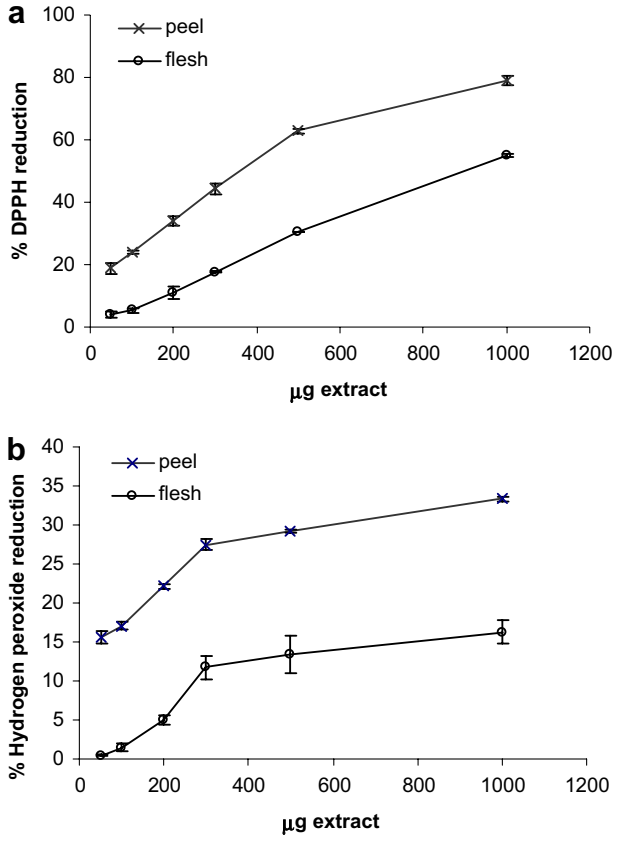

Fig. 3. Radical-scavenging activity of peel and flesh extracts from Limoncella apples. (a) DPPH radical-scavenging activity and (b) H_2O_2 radical-scavenging activity.

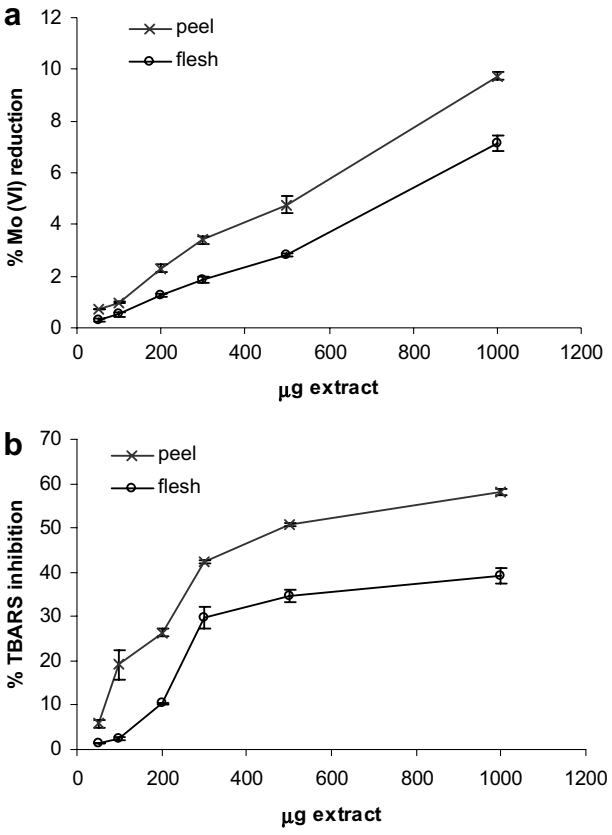

Fig. 4. Antioxidant activity of peel and flesh extract from Limoncella apples. (a) Reduction of Mo(VI) and (b) inhibition of TBARS substances.

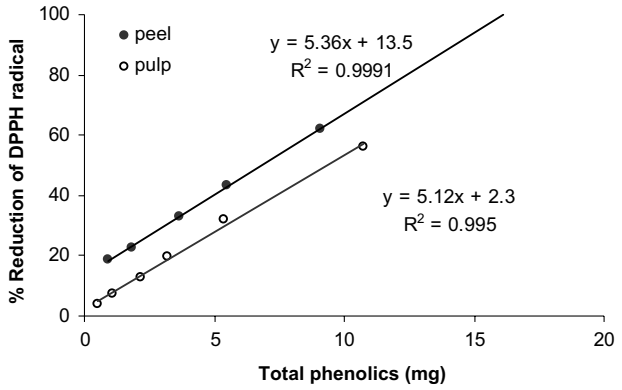

Fig. 5. Correlation between radical-scavenging activity and total phenolics content by linear regression analysis.

the formation of TBARS only at the highest studied amounts. Inhibition was estimated to be equal to 40–60% (Fig. 4).

Phenolic compounds tend to accumulate in the dermal tissues of plant bodies because of their potential roles in protection against ultraviolet radiation, to act as attractants in fruit dispersal, and as defence chemicals against pathogens and predators (Liu, Liu, & Chen, 2005). In this study, significantly higher levels of total phenolics and total flavonoids were detected in the skin of apples. The correlation coefficient between total phenolics and DPPH radical-scavenging activity was found to be 0.933 (Fig. 5).

Wolfe et al. (Wolfe, Wu, & Liu, 2003) reported that apple peel is a rich source of antioxidants and has significantly higher amounts of phenolic compounds, antioxidant activity and antiproliferative activity than the flesh of apples. They suggested that regular consumption of apple peels may result in reduced risks of cardiovascular diseases and cancer. The radical-scavenging and antioxidant properties of methanolic extracts of Limoncella apple reinforce the need for phytochemical study of these fruits.

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