Comprehensive characterization of phytochemicals and biological activities of the Italian ancient apple 'Mela Rosa dei Monti Sibillini'

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Abstract

This study was carried out to characterize extracts from nine samples of the apple 'Mela Rosa dei Monti Sibillini' (MR) and to assess the antioxidant and anti-inflammatory activities. The extracts were analysed by High Performance Liquid Chromatography coupled with photodiode array detector and mass spectrometry (HPLC-DAD-MS) for 20 phytochemicals. The lyophilized material (ELM) was richer in polyphenolic compounds than the dried ones (EDM). The MR extracts contained noteworthy amounts of the investigated analytes compared to one sample of the commercial varieties Annurca, Golden Delicious and Granny Smith used as reference. Principal component analysis (PCA) revealed that the part of the fruit seems to have a significant influence on the chemical composition of the final extract; thus, the peel extracts exhibited higher levels of phenolic compounds, especially epicatechin, procyanidin B2 and phloridzin, and triterpenes than the pulp ones. In general, the lyophilized material showed higher antioxidant activity than the dried material. The strong antioxidant capacity of the MR has also been revealed by the DPPH, ABTS, FRAP and Folin-Ciocalteau assays. The ELM of MR significantly reduced reactive oxygen species compared with lipopolysaccharide (LPS)-activated mouse brain microglia cells (BV-2 cells). Real-time polymerase chain reaction (RT-PCR) analysis demonstrated that the EDM and ELM of MR extracts were effective in reducing pro-inflammatory cytokines and enzymes in BV-2 and peripheral blood mononuclear cells (PBMC). These results contribute to the exploitation of this ancient variety as a source of nutraceuticals.

Keywords: apple; phytochemicals; polyphenols; triterpenes; antioxidant capacity; anti-inflammatory activity.

1. Introduction

The regular consumption of fruits and vegetables, as the main source of polyphenols in the diet, is related to a lower incidence of chronic degenerative diseases such as cancer, cardiovascular and neurodegenerative diseases (Scalbert, Johnson, & Saltmarsh, 2005). Apple (*Malus domestica* Borkh.), a fruit from the Rosaceae family with many different varieties, is the most cultivated fruit across the world, ranking second for the total phenolic content after cranberry but having the highest free polyphenolic portion (Sun, Guo, Fu, Li & Li, 2013 ; Sun, Chu, Wu & Liu, 2002).

The Mela Rosa dei Monti Sibillini (MR) is a small traditional apple variety which is cultivated in the Sibillini Mountains, Marche region, Central Italy, between 400 and 900 m of altitude (Nkuimi Wandjou et al., 2019). Due to its extension, some initiatives such as the augmentation of its cultivation and the valorisation of its uniqueness and typical characteristics have been put in place to revalorize this ancient fruit.

Apple is a source of bioactive compounds due to the high amount and quality of polyphenols it contains (Vrhovsek, Rigo, Tonon & Mattivi, 2004). Five classes of phenolics are usually found in apples: flavan-3-ols/procyanidins, flavonols, phenolic acids, dihydrochalcones and anthocyanins, (Tsao, Yang, Young & Zhu, 2003; Kschonsek, Wolfram, Stöckl & Böhm, 2018) with the flavan-3-ols/procyanidins as the most represented class (Andre et al. 2012). Among fruit polyphenols, the apple ones have shown the strongest antioxidant activity (Yuri, Neira, Quilodran, Motomura & Palomo, 2009).

Pentacyclic triterpenes, which are valuable plant secondary metabolites, are also present in apple peel, leaves and stem bark (Jäger, Trojan, Kopp, Laszczyk & Scheffler, 2009). They have shown some pharmacological effects such as anti-inflammatory, hepatoprotective and anticancer activity without significant toxicity (Jäger et al., 2009). Notably, ursolic and oleanolic acids are the most abundant triterpenes present in the apple peel (Andre et al., 2012).

Several literature reports documented the beneficial health effects of some apple phytochemicals. For instance, procyanidins from the flavan-3-ols class have the ability to suppress the production of reactive oxygen species (ROS) and the expression of pro-inflammatory agents by the inhibition of some pathways such as the NFκB one in *in vitro* study (Bak, Truong, Kang, Jun & Jeong, 2013). Phloretin, a drug penetration enhancer from the class of dihydrochalcones is known to have *in vitro* antioxidant, anti-inflammatory, antitumoral, antiosteoclastogenic and antibacterial activities (Behzad, Sureda, Barreca, Nabavi, Rastrelli & Nabavi, 2017). Its glycosylated form, phloridzin, increases protection against UV radiation and is a competitive inhibitor of the sodium glucose co-transporter type 1 and 2 being promising as antidiabetic agent (Gaudout, Megard, Inisan, Esteve & Lejard, 2006). Chlorogenic acid, besides its strong anti-inflammatory and antioxidant activities, has also shown *in vitro* and *in vivo* anti-bacterial properties (Sun et al., 2013; Boyer & Liu, 2004), Ursolic acid has demonstrated *in vitro* and *in vivo* anti-inflammatory, anticancer and anti-obesity activities (Venugopal & Liu, 2012). *In vitro* and *in vivo* studies have shown that oleanolic acid reduces hyperglicemia and oxidative stress and inhibits the production of proinflammatory factors triggering inflammation (Camer, Yu, Szabo & Huang, 2014). Apple phytochemicals have shown some positive effects on diseases such as Alzheimer's disease, cognitive decline of normal aging, diabetes, weight management, bone health and gastrointestinal protection from drug injury in animal model and epidemiologic studies (Andre et al., 2012).

Nevertheless, the great health benefits of the apple extract are certainly due to the synergy of all the phytochemicals that compose it (Liu, 2003). In the light of the above, apple extracts may thus be used as a source of nutraceuticals useful to prevent or reduce deleterious degenerative diseases.

The study of the characteristics of the different varieties of apple is very important since the consumers are interested to know the composition of the foods in order to choose the ones that are adequate and beneficial to their health (Yuri et al., 2009). Thus, our study assessed the chemical composition along with the antioxidant and anti-inflammatory activities of the peel and pulp extracts from different samples (9) of MR in order to valorise and promote this overlooked apple variety throughout the national territory. For the

purpose two different sample preparation techniques, namely drying and lyophilization, were studied and compared for phytochemicals and biological efficacy. In addition, one sample of other cultivars such as Annurca, which is a traditional apple cultivar from the south of Italy, and two commercial varieties, namely Golden Delicious and Granny Smith, were included in the study to have reference values.

2. Materials and methods

2.1. Sampling

Nine samples of MR and one sample of Annurca were obtained from cultivations sited the Marche region, central Italy, at an altitude between 250 m and 500 m (**Table 1 SM**). The MR and Annurca apples were harvested at mature stage in October-November 2018. The Golden Delicious and Granny Smith apples were purchased in a local market and were from the Trentino Alto Adige, north of Italy. The storage of the MR and Annurca samples was made at ambient temperature according to the traditional methods while the commercial varieties were processed immediately after purchasing.

A portion of the apples was peeled and dried separately (peel and pulp) using a Biosec De Luxe B12 dryer (Albrigi Luigi, Verona, Italy) at 45 ± 5°C for 18 h. Another portion was peeled and ground separately (peel and pulp) with liquid nitrogen, then lyophilised using an Edwards Pirani 1001 freeze dryer. Both lyophilised and dried samples (pulp and peel) were then pulverized using an IKA-WERK MFC DCFH 48 (2 mm-size particles, Staufen, Germany). They were then immediately subjected to the extraction procedures.

2.2. Preparation of extracts

After extractions with different solvents (water, ethanol, methanol, water + methanol, and water + ethanol) and chromatographic preliminary tests carried out with HPLC-DAD-MS, the solvent with the highest extraction efficiency revealed to be methanol (Zheng, Hwang & Chung, 2009; Karaman, Tütem, Başkan & Apak, 2013). The procedure of extraction with methanol was the same for both dried and lyophilized

material. Twenty-five ml of methanol were added to 5 g of the weighted material in an Erlenmeyer and were then submitted to sonication at a frequency of 59 KHz and power 100% (Ultrasonic Cleaner Astrason® Heat System) for 45 min at room temperature. After filtration, 20 ml of methanol was then added to the residue for a second sonication for 20 min. The 2 filtrates were then gathered and concentrated with a rotavapor for at least one hour at a temperature of 30 ± 5˚C. The crude extracts (pulp and peel) obtained from the dried (EDM) and lyophilized materials (ELM) were then weighted and the yield calculated (**Table 2 SM**).

2.3. Chemicals and reagents

The analytical standards of (+)-catechin hydrate, (-)-epicatechin, procyanidin A2, procyanidin B2, cyanidin-3-glucoside, rutin, quercetin-3-D-galactoside, kaempferol, kaempferol-3-glucoside, quercetin, *p*-coumaric acid, 3-O-caffeoylquinic acid, 5-O-caffeoylquinic acid, caffeic acid, gallic acid, *trans*-ferulic acid, phloretin, phloridzin, ursolic acid, and oleanolic acid were purchased from Sigma-Aldrich (Milan, Italy). Dulbecco's modified Eagle medium (DMEM), Roswell Park Memorial Institute medium (RPMI)-1640, fetal bovine serum (FBS), penicillin, streptomycin, glutamine, Concanavalin A (ConcA), Lipopolysaccharide (LPS) from *Escherichia coli* serotype O127:B8, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), H_2O_2, dimethyl sulfoxide (DMSO), were purchased from Sigma-Aldrich (Milan, Italy). Phytohemagglutinin (PHA) and carboxyfluorescein diacetate succinimidyl ester (CFSE) were purchased from Biochrom^{AG} (Berlin, Germany) and BioLegend (San Diego, CA), respectively.

The stock standard solutions were prepared by dissolving 10 mg of the analyte in 10 ml of methanol and stored in a glass-stoppered bottle at 4°C in the dark. Standard working solutions, at various concentrations, were daily prepared by appropriate dilution of aliquots of the stock solutions in water. HPLC-grade ethanol and acetonitrile were purchased from Sigma-Aldrich (Milan, Italy), while HPLC-grade formic acid 99-100% was bought from J.T. Baker B.V. (Deventer, Holland). For sample preparation and chromatographic analysis, deionized water ≥ 18 MΩ/cm resistivity purified with a Milli-Q system (Millipore, Bedford, USA)

was used. All solvents and solutions were filtered through a 0.45-µm PTFE filter from Supelco (Bellefonte, PA, USA) before use.

2.4. HPLC analysis of polar constituents

High Performance Liquid Chromatography with photodiode-array and Mass spectrometry (HPLC-DAD-MS) studies were performed using a Hewlett-Packard HP-1090 Series II (Palo Alto, CA, USA), equipped with a vacuum degasser, a binary pump, an autosampler and a model 1046A HP photodiode array detector (DAD) and a mass spectrometer detector Trap SL (Bruker, Billerica, MA, USA) equipped with an electrospray ionization (ESI) source. The chromatographic separation was accomplished on a Synergi Polar-RP C18 (4.6 mm x 250 mm, 4 µm) analytical column from Phenomenex (Chesire, UK). The column was preceded by a security cartridge. The mobile phase for HPLC-DAD analyses was a mixture of (A) water with 0.1% formic acid (v/v) and (B) methanol with 0.1% formic acid (v/v), flowing at 1 ml/min in gradient conditions: 0-15 min, 20% B; 15-45 min, 100% B; 45-60 min, 20% B. The column temperature was set at 30°C and the injection volume was 10 µl. UV-Visible spectra were recorded in the range 210-520 nm for the 20 compounds, where 272 nm was used for gallic acid, 325 nm for 3-O-caffeoylquinic acid, 5-O-caffeoylquinic acid, caffeic acid, *p*-coumaric acid and *trans*-ferulic acid, 280 nm for (+)-catechin hydrate, (-)-epicatechin, phloretin and phloridzin; 230 nm for procyanidin A2 and procyanidin B2, 520 nm for cyanidin-3-glucoside, 265 nm for rutin, quercetin-3-D-galactoside and kaempferol-3-glucoside, 365 nm for kaempferol and quercetin, 210 nm for ursolic and oleanolic acids.

2.5. Method validation

The method was validated by determining linearity, repeatability, recovery, limits of detection (LODs) and limits of quantification (LOQs). Calibration curves of the analyzed compounds were constructed injecting 1-50 mg/l of standard solutions at five different concentrations, i.e. 1, 5, 10, 25 and 50 mg/l in HPLC-DAD technique. Five replicates for each concentration were performed and the relative standard deviation (RSDs)

ranged from 1.6 to 3.6% for run-to-run precision and from 2.9 to 6.8% for day-to-day precision. All the calibration curves of the analyzed phenolic compounds showed a correlation coefficient greater than 0.9959. The obtained recoveries for all compounds, evaluated spiking the samples at two different levels of concentration (5 and 25 mg/kg) with a standard mixture of the twenty compounds, were in the range 83-96% and 89-106%, respectively, with a relative standard deviation percentage (RSD %) < 9% (n = 3) in all cases (Data not shown). Limits of detection (LODs) and quantification (LOQs) for phenolic compounds were in the range 0.02–0.42 mg/l and 0.1–1.2 mg/l, respectively. Retention time stability was utilized to demonstrate the specificity of the HPLC-DAD method. Reproducibility of the chromatographic retention time for each compound was examined five times per day over a 5-day period (n=25). The retention times using this method were stable with a percent RSD value ≤2.01%. The recovery value was obtained using the following formula: ((Ase-Asblank)/Astd) X 100, where Ase is the area of the sample enriched with a known concentration of all the standard compounds, Ablank is the area of analyte detected in the sample, Astd is the area of a standard mixture of all the compounds dissolved in methanol.

2.6. In vitro antioxidant capacity assays

All the pulp and peel extracts were evaluated for their *in vitro* antioxidant activity by the means of standard methods using a SPECTROstar Omega (BMG LABTECH GmbH, Ortenberg, Germany) microplate reader. The samples stock solutions were prepared as follows: 20 mg of the peel and 40 mg of the pulp crude apple extracts were dissolved in 1 ml of methanol.

2.6.1. Quantification of total phenolic content

The total phenolic content (TPC) of the methanolic apple extracts was determined by the Folin-Ciocalteu spectrophotometric method described by Singleton and Rossi with some modifications (Singleton & Rossi, 1965; Censi, Vargas Peregrina, Lacava, Agas, Lupidi, Sabbieti & Di Martino, 2018). Different volumes, i.e. 12, 25, 50 and 100 µl of the dissolved extracts were introduced in 4 Eppendorfs and water was added

to bring the volume to 100 µl. Then 300 µl of Folin-Ciocalteu reagent (1 ml Folin-Denis' reagent in 4 ml H_2O) and 50 µl of Na_2CO_3 saturated solution were added. After centrifugation at 10000 rpm for 5 min and introduction of the supernatant in 96-well microplate, the absorbance of each well was determined at 765 nm. The results were expressed as the average of four measurements. The standard calibration curve was elaborated using gallic acid. The results were expressed as mg of gallic acid equivalents per g (mg GAE/g) of extract (dry weight).

2.6.2. Free radical scavenging activity

The free radical scavenging activity was evaluated using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) on microplate analytical assay as described by Censi et al (2018). Two hundred µl of DPPH in methanol was added to 50 µl aliquot of samples at different concentrations (20, 10, 5, 2.5, 1.25, 0.625, 0.312 mg/ml and 40, 20, 10, 5, 2.5, 1.25, 0.625 mg/ml for peel and pulp extracts, respectively) and standard at various concentrations prefilled in the well of the microplate. The absorbance of each well was measured at 517 nm against a blank, prepared using methanol in addition to the DPPH reagent to throw off any inherent solvent activity.

2.6.3. Radical cation decolorization assay

The total radical scavenging activity was measured using 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay (Censi et al., 2018). The $ABTS^{\bullet+}$ solution was freshly prepared by the oxidation of ABTS (10 mg) by MnO_2 (0.75 g) in the presence of water (4 ml), followed by 30 min of incubation away from light at room temperature. The working solution was obtained by filtration and dilution with methanol of the previous mixture to have an absorbance around of AU 1 at 734 nm. The extract concentrations used were the same with the DPPH assay and 200 µl of the ABTS solution was added in the wells. After 15 min of incubation, the absorbance of each well was determined at 734 nm.

2.6.4. Ferric reducing antioxidant power (FRAP)

The total antioxidant activity was also determined through the Ferric Reducing Antioxidant Capacity (FRAP) (Censi et al., 2018). Due to their high concentrations, the sample stock solutions were diluted (dilution ratio 1:5 for the peels and 1:2 for the pulp) for this analysis. The FRAP reagent was prepared by mixing the three pre-made solutions below:

(1) 1.25 ml of stock solution of TPTZ (2,4,6-tripyridyl-s-triazine) (9.4 mg) in 3 ml of 40 mM HCl;

(2) 1.25 ml of stock solution of $FeCl_3$ 6 H_2O (9.8 mg) in 3 ml of H_2O;

(3) 7.5 ml acetate buffer pH 3.6 (1.23 g of sodium acetate in 50 mL of water acidifying with acetic acid).

Two hundred µl of the FRAP reagent was added to 50 µl aliquot of sample at different concentrations (4, 2, 1 mg/ml and 20, 10, 5 mg/ml for peel and pulp extracts, respectively) and standard at various concentrations prefilled onto wells of a 96-well plate. After 15 min of reaction, the plate was read at 593 nm.

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used in DPPH, ABTS and FRAP assays as the calibration standard. The antioxidant capacity of the apple extracts (peel and pulp) was expressed in mg Trolox equivalent/g (mg TE/g) of sample, and for the DPPH and ABTS also as IC_{50} (concentration of the tested material required causing a 50% decrease in initial DPPH and ABTS concentration).

2.7. Cellular antioxidant and anti-inflammatory activities

2.7.1. Cell culture

BV-2 murine microglial cells were kindly provided by Prof. Elisabetta Blasi (University of Modena and Reggio Emilia, Modena, Italy). Cells were maintained in DMEM with 10% heat inactivated FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine and mantained at 37°C in a humidified 5% CO_2/95% air atmosphere. Exponentially growing BV-2 cells were treated with various concentrations

(1-100 μg/ml) of the different MR extracts for 24 h, and then were stimulated with LPS (100 ng/ml) for 24 h. *Ex vivo* horse peripheral blood mononuclear cells (PBMCs), isolated from fresh heparinized blood samples (20 ml/horse), were obtained from 5 donors (Italian trotter, females) reared in a hilly area of Umbria (Ministry of Health: Authorization n. 391/2019 – PR. Risp. a prot. 67080.7). The number of live lymphocytes, suspended in complete RPMI with 10% heat inactivated FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine (complete medium) was determined using a counting chamber and a trypan blue dye exclusion procedure (Strober, 2001). The final concentration of live cells was adjusted to 4×10^6/well (2 ml) and cells were treated with different apple extracts for 24 h, and then were stimulated with LPS (100 ng/ml) for 24 h.

2.7.2. MTT viability assay

Cell viability was evaluated by measuring MTT reduction as previously reported (Angeloni, Malaguti, Rizzo, Barbalace, Fabbri & Hrelia, 2015). Briefly, after treatments, cells were incubated with 0.5 mg/ml of MTT solution for 1 h at 37°C. After incubation, MTT solutions were removed and 100 μl of DMSO was added. The absorbance was recorded at $\lambda = 595$ nm using a microplate spectrophotometer (VICTOR3 V Multilabel Counter; PerkinElmer, Wellesley, MA, USA).

2.7.3. Lymphocyte proliferation assay

For the measurement of cell proliferation, the PBMC were prestained with carboxyfluorescein diacetate succinimidyl ester (CFSE) cell tracer and then cultured in complete medium for 5 days at 37°C in 5% CO_2/95% air atmosphere. Proliferation stimuli were 5 μg/ml of Conc A or 1.2 μg/ml of PHA in presence or absence of 10 μg/ml of sample 2 of MR or Annurca extracts (EDM and ELM). Flow cytometry analyses were performed on a standard FACSCalibur™ flow cytometer (Becton Dickinson, Mountain View, CA,

USA) operated by the CELLQuestPro™ software. Within a tight lymphocyte gate, 10,000 cells were acquired and the data were saved in the list mode (Caprioli et al., 2016).

2.7.4. Intracellular ROS levels

The formation of intracellular ROS was evaluated using the fluorescent DCFH-DA probe as previously reported (Marrazzo, Angeloni, Freschi, Lorenzini, Prata, Maraldi & Hrelia, 2018). Briefly, at the end of each experiment, BV-2 cells were incubated with 100 µl of a 10 µM DCFH-DA solution prepared in Dulbecco's Modified Eagle's medium (DMEM) containing1% of fetal bovine serum (FBS) without phenol red for 30 min. Then, DCFH-DA was removed and replaced by PBS. DCF fluorescence was measured using 485 nm excitation and 535 nm emission with a microplate spectrofluorometer (VICTOR3 V Multilabel Counter, PerkinElmer, Wellesley, MA, USA).

2.7.5. RNA extraction

Total RNA was extracted from BV-2 cells pre-treated for 24 h with the different extracts and then stimulated with LPS (100 ng/ml) for 24 h using RNAeasy Mini Kit (QIAGEN GmbH, Hilden, Germany), following the manufacturer's protocol. The yield and purity of the RNA were measured using NanoVue Spectrophotometer (GE Healthcare, Milano, Italy).

2.7.6. Real-Time polymerase chain reaction (PCR) assay

One µg of total RNA was reverse-transcribed to cDNA using iScript cDNA Synthesis Kit (BIO-RAD, Hercules, CA, USA), following the manufacturer's instructions. The subsequent PCR was performed in a total volume of 10 µl containing 2.5 µl (12.5 ng) of cDNA, 5 µl SsoAdvanced Universal SYBR Green

Supermix (BIO-RAD) and 0.5 µl (500 nM) of each primer. The primers used (SIGMA-ALDRICH, Milan, Italy) are reported in **Tables 3SM** and **4SM**; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as reference gene for BV-2 murine microglial cells, whereas RN18S as reference gene for *ex vivo* horse PBMCs.

2.8 Statistical analysis

In order to understand the relationships among the apple samples analysed based on chemical compositions and to determine the main constituents influencing the chemical variability, a covariance data matrix (960 data) composed of 48 apple samples (i.e., 12 dried peel samples, 12 peel lyophilized samples, 12 dried pulp samples and 12 pulp lyophilized samples) and 20 variables for phytochemicals was subjected to principal component analysis (PCA) using STATISTICA 7.1 (Stat Soft Italia S.r.l., Vigonza, Italy). Eigenvalues were calculated and score and loading plots including all apple samples and the 20 analytes determined by HPLC were generated. All the biological experiments were performed at least in triplicate and values were expressed as mean ± standard error. In experiments with BV-2 cell cultures and PBMCs, one-way ANOVA was used to compare differences among groups followed by Dunnett's or Bonferroni's test (Prism 5; GraphPad Software, San Diego, CA). Differences at the level $p < 0.05$ were considered statistically significant.

3. Results and discussion

3.1. Phenolic and triterpene profiles

In order to observe the influence of enzymatic degradation on the stability of polyphenols, two dehydration methods were applied for the sample preparation: i) freezing with liquid nitrogen to neutralize the action of the enzymes followed by lyophilization, or ii) drying at 45±5°C for 18 h. Twenty compounds belonging to 6 classes were quali-quantitatively analysed and the results are reported in the **Tables 1** and **2**. As an

example, the HPLC-DAD chromatogram of the standard mixture of the 20 analytes has been reported in Fig 1SM.

The most abundant groups in the peel extracts were triterpenes, followed by flavan-3-ols and dihydrochalcones. On average, the most represented compounds in the peel of the 9 samples of MR were ursolic acid (583.4-15088.8 mg/kg), oleanolic acid (320.5-10835.5 mg/kg), epicatechin (938.1-3999 mg/kg), procyanidin B2 (819.3-2732.7 mg/kg) and phloridzin (387.7-2012.1 mg/kg) both in EDM and ELM. On the other hand, in the pulp samples we found epicatechin (494.5-1725.2 mg/kg), procyanidin B2 (292.1-1262.2 mg/kg) and chlorogenic acid (136-2212.5 mg/kg) as the main compounds. Ursolic acid was highly represented in the peel of the EDM sample 1 (15088.8 mg/kg of the extract) followed by the ELM sample 6 (12541.8 mg/kg). The ELM sample 8 (10831 mg/kg) and the EDM sample 1 (10719.5 mg/kg) also showed a rather significant amount of this compound. Within the ELM samples, epicatechin and procyanidin B2 were found in a very high amount in the samples 5 (3999 and 2732.7 mg/kg, respectively) and 6 (3976.4 and 2421.4 mg/kg, respectively); on the other hand, in the EDM samples epicatechin was most represented in the samples 6 (2083.5 mg/kg) and 9 (2049.9 mg/kg), whereas procyanidin B2 was abundant in the samples 1 (2041.4 mg/kg) and 5 (1324.2 mg/kg). Our results showed a great quantitative variation between the samples of MR. It also brought out that the ELM samples were averagely richer in polyphenols than the ones of the EDM samples since freezing followed by lyophilisation preserves the bioactive compounds from enzymatic degradation (Boyer & Liu, 2004).

In the Annurca extracts ursolic acid (9094.3 mg/kg ELM; 6236.6 mg/kg EDM) and oleanolic acid (5101.9 mg/kg ELM; 2504 mg/kg EDM) were the most abundant compounds. On the other hand, the most abundant phenolic compounds were epicatechin (1854.9 mg/kg ELM, 1147.7 mg/kg EDM), cyanidin-3-glucoside (1583.5 mg/kg ELM, 958.1 mg/kg EDM), chlorogenic acid (1243 mg/kg ELM, 1042.3 mg/kg EDM) and phloridzin (1012.8 mg/kg ELM, 649.3 mg/kg EDM). In Golden Delicious extracts, epicatechin (1878.8 mg/kg ELM, 1945.7 mg/kg EDM), procyanidin B2 (1469.9 mg/kg ELM, 1529.3 mg/kg EDM), quercetin-3-D-galactoside (827 mg/kg ELM, 860.5 mg/kg EDM) and kaempferol-3-glucoside (806.4 mg/kg ELM,

838.9 mg/kg EDM) were the most represented analytes. The Granny Smith extracts were rich in epicatechin (1997.7 mg/kg ELM, 1306.2 mg/kg EDM), kaempferol-3-glucoside (1327.8 mg/kg ELM, 694.2 mg/kg EDM) and procyanidin B2 (1176.1 mg/kg ELM, 843.3 mg/kg EDM). The study of Kschonsek et al. (2018) on the dehydrated peels of the Golden Delicious and Granny Smith varieties revealed that the most abundant compounds in both cases were quercetin-3-D-galactoside, quercetin, procyanidin B2 and chlorogenic acid. The differences highlighted in our samples with respect to those reported in literature may be justified by different factors such as cultivar, geographic origin, solar radiation, temperature, agronomic techniques etc. (Tsao et al., 2003; Yuri et al., 2009), as well as by the extraction method applied (Alberti, Zielinski, Zardo, Demiate, Nogueira & Mafra, 2014).

Thus, the hallmark of extracts from some samples of MR (i.e. peel EDM samples 1 and 8; peel ELM samples 2, 6 and 8), when considering the composition of the reference samples (Annurca and Golden Delicious), seems to be a high content of triterpene acids. This result may contribute to the implementation of MR orchards due to the health effects of these phytochemicals.

In the pulp extracts from all the samples studied, the most abundant compounds were chlorogenic acid, epicatechin and procyanidin B2. The peel samples with the highest content of phenolic compounds (3797.7–12687.5 mg/kg) were the samples 5 and 6 of MR for the ELM, and Golden Delicious and sample 1 of MR for EDM. The triterpenes were abundant in the Granny Smith and sample 6 of MR of the ELM and in the sample 1 of MR and Granny Smith of the EDM. Overall, the total polyphenolic concentrations of the pulp extracts, ranging from 1573.6 to 4209.3 mg/kg, were in all cases lower than the ones of the peel ones (3797.7-12679.8 mg/kg) (Tsao et al., 2003).

In average, the MR had the highest polyphenol content in the ELM peel (10343.0 mg/kg) followed by the Annurca peel sample (8962.6 mg/kg), Golden Delicious (7182.9 mg/kg) and Granny Smith (6083.5 mg/kg). On the other hand, in the EDM peel the Golden Delicious extract showed the best content in polyphenols (7473.2 mg/kg) followed by Annurca (6324.5 mg/kg), MR (5509.1 mg/kg) and Granny Smith (3797.7

mg/kg). As regards the triterpene content, in both ELM and EDM peel samples, the Granny Smith extract showed the highest level (27816.2 and 17668.4 mg/kg respectively). In the pulp samples, the MR and the Anuurca were the ones with the highest level of polyphenols in both ELM and EDM peel (2696.4 and 2896.4 mg/kg for ELM; 2649.9 and 2127.7 mg/kg for EDM respectively).

Our results on the phenolic profile of the apple peel are different from those reported by Kschonsek et al. (2018) who studied some old (10) apple cultivars in which the most abundant compound was quercetin-3-O-galactoside (80-1909 mg/kg lyophilized material, LM), with concentrations slightly higher than the MR samples (11.5-1313 mg/kg ELM). On the other hand, the concentrations of epicatechin (22-178 mg/kg LM), procyanidin B2 (44-96 mg/kg LM), phloridzin (15-638 mg/kg LM) and chlorogenic acid (39-181 mg/kg LM) are far lower than the ones detected in our study in the MR samples. The analyses of the latter polyphenols on 21 apple peels by Huber et al. (Huber & Rupasinghe, 2009) have given concentrations higher in average than the ones of Kschonsek et al. (2018), but nevertheless lower than the ones of our study.

On the above, MR may be considered of high quality for its phytonutrient profile and therefore may be used as a potential source of nutraceuticals.

In order to understand the variability of chemical data in all samples analysed considering all factors at the same time, namely variety, fruit part and processing of material, a PCA was carried out and score and loading plots were generated (**Fig. 1**). Overall, the PCA graphs explained 95.21% of data variability, with the highest contribution (90.64%) given by the first principal component. In this respect, the components showing the highest variance were the triterpenes ursolic and oleanolic acids (**Fig. 1, B**). On this basis, two main groups of samples have been identified, with the ones taking place in the left-hand side of the score plot coming from the pulp and having an overlapping composition, whereas those in the right-hand side of the same plot coming mostly from the peel and showing a higher level of variance (**Fig. 1, A**). It is worth mentioning that the plant processing and the apple variety did not allow to cluster samples in different

groups. From the representation of the two main clusters, we can conclude that all kinds of peel extracts contain higher levels of phenolic compounds, especially epicatechin, procyanidin B2 and phloridzin, and triterpenes. Regarding the extracts obtained from peel, one sample from lyophilized Granny Smith and three samples from MR (2 dried and 1 lyophilized) exhibited the highest concentration of triterpene acids. In conclusion, from PCA analysis only the part of the fruit seems to have a significant influence on the chemical composition of the final extract.

3.2. In vitro antioxidant capacity

In this study, we assessed the antioxidant capacity of the dried and lyophilized methanolic apple extracts (pulp and peel) of 4 different varieties: MR (9 samples), Annurca, Golden Delicious and Granny Smith. The antioxidant activity of the peel and pulp extracts are reported in **Tables 3** and **4**, respectively.

In the EDM peel, the total polyphenols content (TPC) was in the range 26324.6 to 40874.3 mg GAE/kg with the samples 5 and 9 of MR having the highest TPC and the sample 7 the lowest one. While in the ELM, the range was 36502.9 to 55752.8 mg GAE/kg, with samples 3 and 5 of MR having the highest TPC and the Annurca sample being the poorest one. Thus, almost all the lyophilized apple peel samples were more potent than the dried peel samples, but this was not noticed in the pulp extracts. In fact, in these extracts, the EDM samples showed higher TPC values than the ELM apart from the Golden Delicious and Granny Smith samples. Birtic et al. (Birtic, Régis, Le Bourvellec and Renard, 2019) reported that new molecules may be formed during drying due to inter- and intramolecular bonding between polyphenols and other macromolecules. The highest content of water in the pulp respect to the peel may facilitate these reactions. The range of the TPC was from 8892.3 to 20989.3 mg GAE/kg for the EDM, with sample 9 of MR being the richest and the Golden Delicious the poorest. In the ELM, the range was between 7887.2 (sample 3) to 13476.6 mg GAE/kg (sample 9).

The FRAP assay showed that all the lyophilised peel samples displayed higher antioxidant capacity compared with the dried peel samples. This was in accordance with results of the Folin-Ciocalteu assay.

The antioxidant capacity of the peels resulted 1 to 10 times greater compared with that of pulps and this may be certainly due to its higher amount of polyphenols (Yuri et al., 2009). The TPC of the ELM peels of Golden Delicous and Granny Smith are very high (8 times) compared to the results of Kschonsek et al. (2018) (5219.0 mg and 5810.0 mg GAE/kg, respectively). Mari et al. (Mari, Tedesco, Nappo, Russo, Malorni & Carbone, 2010) in the characterisation of phenolic compounds reported that the TPC of the ELM peels of the Annurca apple was 2 times higher than the one of the Golden Delicious, but this was not detected in our study, where the value of the TPC of Golden Delicious was slightly higher than the one of Annurca. The TPC resulting from the Folin-Ciocalteu assay and the total polyphenols from HPLC analysis are quite different, since apple contains a huge number of compounds and thus, their complete identification is almost impossible by HPLC techniques due to the unavailability of the corresponding standards (Karaman et al., 2013).

The greatest antioxidant activity was demonstrated by the EDM peel of the samples 5 and 9 of MR; in the case of ELM peel, the samples 3 and 5 of MR and the Granny Smith sample according to the different assays. The sample 9 showed the highest antioxidant activity among the EDM pulp samples, while the samples 3 and 9 of MR were the most active among the ELM samples.

These analyses demonstrated the noteworthy antioxidant capacity of the MR samples. In addition, when comparing the polyphenol content using both Folin-Ciocalteu assay and HPLC analyses, the lyophilized material, in particular that from peel, showed averagely higher values than the dried one.

3.3. Cellular anti-inflammatory activity

To investigate the anti-inflammatory activity of the peel extracts, samples 1 and 2 of MR, and Annurca were selected as reference samples in murine BV-2 cell analysis, whereas, sample 2 of MR and Annurca were utilized in PBMC assay. Samples 1 and 2 have been chosen because they possess intermediate characteristics between all the apple extracts, as determined by chemical and antioxidant characterization. We decided to study both the EDM and ELM extracts obtained by the peels, as they possess a higher

concentration of bioactive compounds and a higher antioxidant capacity in respect to the extracts obtained from the pulps. Potential cytotoxicity of the extracts was determined by treating BV-2 cells with increasing concentrations (1-100 μg/ml) of the extracts for 24 h, after which cell viability was assessed by MTT assay (**Fig. 2 SM**). Both the extracts (ELM and EDM) obtained by sample 1 and ELM of sample 2 did not show any toxic effect, meanwhile EDM of sample 2, and the extracts obtained from Annurca (both EDM and ELM) showed a significant, but slight toxicity at 50 and 100 μg/ml. On this basis, we decided to carry out the subsequent experiments using concentrations up to 10 μg/ml. LPS, a component of the outer membrane of gram-negative bacteria, is an endotoxin widely used in inflammatory cellular and animal models (Batista, Gomes, Candelario-Jalil, Fiebich & de Oliveira, 2019). LPS, besides increasing pro-inflammatory mediators, induce oxidative stress (He, Yan, Wen, Zhang, Liu, Liu & Xiao, 2019). To study the antioxidant effects of the different extracts against LPS-induced oxidative stress, BV-2 murine cells were pre-treated with the extracts (1-10 μg/ml) for 24 h, then exposed to 100 nM LPS and after 24 h intracellular ROS levels were evaluated by DCFH-DA assay (**Fig. 2**). Both EDM and ELM of Annurca extracts did not show any antioxidant effects. In fact, Annurca treated cells had ROS levels comparable to LPS exposed cells. On the contrary, ELM of sample 1 and both the extracts obtained by sample 2 significantly reduced ROS levels with respect to LPS treated cells. Of note, at 10 μg/ml, ELM of sample 1, and EDM and ELM of sample 2 completely counteracted oxidative stress, maintaining ROS level to values comparable to control cells.

These data are not completely in agreement with the results of the FRAP assay. In particular, in the FRAP assay, ELM of Annurca extract showed a higher antioxidant capacity in respect to ELM of sample 1. In BV-2 treated cells, ELM of sample 1 significantly counteracted LPS-induced ROS; meanwhile ELM of Annurca had no effect (**Fig. 2**). Moreover, in the FRAP assay EDM of sample 2 and EDM of Annurca showed comparable antioxidant activity but in BV-2 cells EDM of sample 2 was more effective than EDM of Annurca.

This discrepancy could be due to the fact that in the cell system, bioactive compounds do not work only as direct antioxidants but also modulate pathways that lead to an indirect antioxidant effect (Bernatoniene & Kopustinskiene, 2018).

This also could explain why some extracts with higher antioxidant *in vitro* activity has no antioxidant activity in the cell system. As the concentration of 10 μg/mL resulted the most effective in counteracting oxidative stress, the next experiments were carried out using this concentration.

Given the strong antioxidant effects of samples 1 and 2 on LPS-stimulated microglia cells, we decided to further investigate their anti-inflammatory activity by measuring, in LPS-stimulated BV-2 microglial cells, the expression of inflammatory mediators.

Neuroinflammation, caused by activated glial cells, is very important in the development of neurodegeneration (Hong, Kim & Im, 2016). Upon activation, microglia cells release pro-inflammatory cytokines including IL-1β and TNF-α, which would lead to the damage of neurons and further activate the inflammatory cascade (Lull & Block, 2010). It has been shown that IL-1β and TNF-α are significantly up-regulated in patients with neurodegenerative diseases (Kaur, Sharma & Deshmukh, 2019).

Moreover, the activated microglial cells also overexpress enzymes, such as inducible nitric oxide synthase (iNOS), cyclooxygenase (COX-2), that produce harmful compounds, which may lead to neuronal death and induce the development of neurodegeneration (Dulla, Kurauchi, Hisatsune, Seki, Shudo & Katsuki, 2016).

On this basis, we investigated the expression of pro-inflammatory genes, including IL-1β, TNF-α, as well as the levels of pro-inflammatory enzymes, such as iNOS and COX-2.

A RT-PCR analysis was carried out to determine if the extracts inhibit the expression of pro-inflammatory genes and inducible enzymes. As expected, mRNA expression levels of IL-1β, TNF-α, iNOS, and COX-2 were significantly increased in LPS-stimulated BV-2 microglial cells (**Fig. 3A**). Interestingly, only ELM of sample 1 was able to significantly reduce the expression of IL-1 β, meanwhile all the extracts significantly reduced TNF-α expression with respect to LPS treated cells. The expression of iNOS was

significantly reduced with respect to LPS only by the ELM of samples 1 and 2, and COX-2 expression was significantly reduced by all the extracts except EDM of Annurca.

To further investigate whether in different cell models the extracts may differently modulate the anti-inflammatory responses, extracts from sample 2 (MR) and Annurca (EDM and ELM) were tested in horse *ex vivo* PBMCs. Sample 2 was chosen because both its extracts (EDM and ELM) were able to significantly reduce the ROS production to values comparable to control cells.

This type of leukocytes was chosen because, circulating in the blood stream throughout the body, represent the first systemic cell line acting in the innate immune/anti-inflammatory responses. Besides the pro-inflammatory cytokines above mentioned, also interleukin (IL-6 and IL-10) expressions were tested. IL-6 acts as a pro-inflammatory cytokine in monocytes and macrophages in PBMC culture (Brandt & Pedersen, 2010); IL-10, instead, represents a classical anti-inflammatory cytokine (Iyer & Cheng, 2012).

Finally, horse PBMCs were utilized for a lymphocyte proliferation assay to evaluate a possible immunogenic activity of the studied extracts or their ability to trigger a more complex and adaptive immune response.

PBMCs cells were pre-treated for 24 h with the different extracts (10 μg/ml) and then stimulated with LPS (100 ng/ml) for 24 h. Likewise, mRNA expression levels of IL-1α, IL-6, IL-10 and TNF-α were significantly increased in LPS-stimulated PBMCs (**Fig. 3B**). In these immune-competent cells all the extracts were able to significantly reduce the IL-1, IL-10 and TNF-α cytokine expression with respect to LPS treated cells. Interestingly, only the extracts from the sample 2 of MR (EDM 2 and ELM 2) were able to significantly reduce the expression of the pro-inflammatory IL-6. The inflammatory environment triggered by LPS, as testified by the increased expression of all the pro-inflammatory cytokines tested, promoted also an increased expression of IL-10 to downregulate the inflammatory process. In particular, in the EDM 2 treated cells it seems that to the higher expression of IL-10 correspond the higher ability to modulate the expression of pro-inflammatory IL-6 (**Fig. 3B**).

In the lymphocyte proliferation assay (**Fig. 3 SM**) no effects were found for CTRL or PHA stimulated cells treated with the different extracts, whereas cells triggered with Conc A (B^+ cells) and ELM 2 extract showed an opposite trend with respect to the ones treated with both EDM and ELM of Annurca. Although these data deserve to be further investigated, MR extracts seem to be able not only to downregulate the inflammatory process but also to modulate the adaptive immune response and this makes these extracts possible allies in the prevention of autoimmune disease.

In general, these results evidenced a high anti-inflammatory effect of EDM and ELM extracts of MR. Furthermore, the extraction method appeared to be able to influence the biological activity of the samples.

4. Conclusions

In the present work we developed and validated an HPLC-DAD method to simultaneously quantify 20 analytes in different extracts obtained from MR, Annurca and commercial varieties of apple. Principal component analysis (PCA) confirmed that the part of the fruit is the major factor affecting the chemical composition of the final extract. In this regard, the peel samples exhibited higher levels of triterpenes and phenolic compounds, especially epicatechin, procyanidin B2 and phloridzin, than the pulp ones. Notably, the peels were 2-3 times richer in polyphenols than pulps and 1 to 10 times more potent for antioxidant activity. The freeze-lyophilized material, avoiding enzymatic degradation, allowed to obtain peel extracts richer in polyphenols as found by HPLC analysis and endowed with higher antioxidant capacity in both chemical and cellular assays. Nevertheless, the pulp dried extract, contrary to what expected, have demonstrated a higher total polyphenol content in the Folin-Ciocalteu assay. Some samples of the MR, apart from their high concentration in bioactive compounds, also showed a noteworthy antioxidant and anti-inflammatory activity. In conclusion, our findings support the recovery of this ancient apple variety of central Italy through an implementation of its cultivation area and industrial exploitation. Notably, this fruit revealed to be a promising source of nutraceuticals aimed at preventing oxidative and inflammatory stress conditions.

Conflicts of interest

Authors declare that there are no conflicts of interest.

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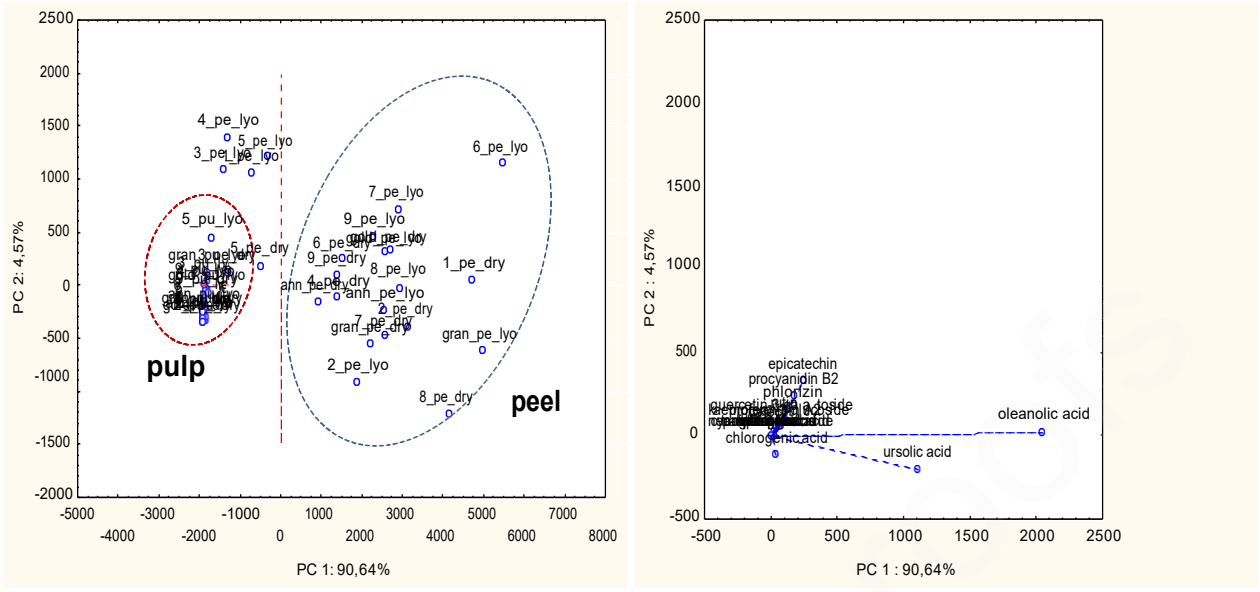


Fig. 1. A) Principal Component Analysis (PCA) score plot depicting the clustering of apple samples based on the chemical composition as determined by HPLC-DAD analysis. B) The PCA loading plot for the chemical constituents representing 90.64% of the variation in the first principal component and 4.57% in the second principal component. Abbreviations used for samples: pe, peel; pu, pulp; dry, from dried material; lyo, from lyophilized material; ann, Annurca; gold, Golend delicious; gran, Granny Smith. Numbers 1-9 correspond to those reported in Tables 1-2.

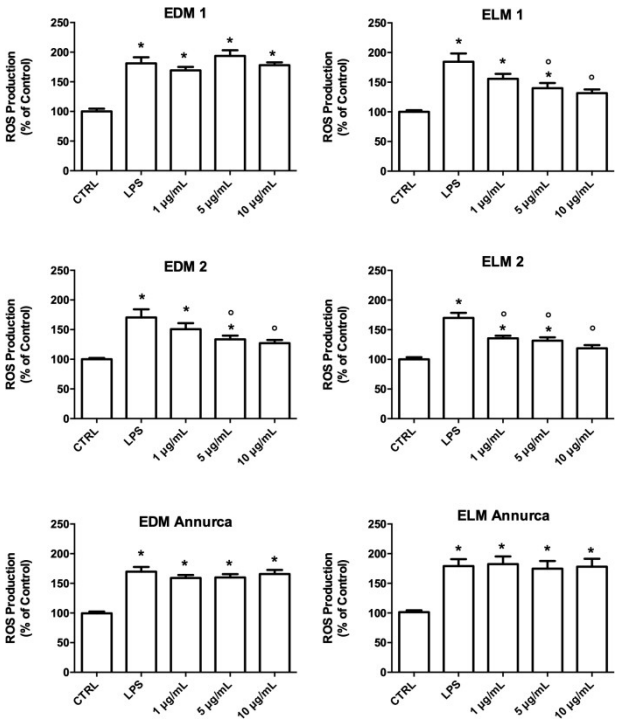


Fig. 2 Antioxidant effect of the extracts against LPS-induced ROS production in BV-2 cells. Intracellular ROS levels were determined with the peroxide-sensitive probe DCFH-DA. Data are expressed as a percentage compared to control and are presented as mean ± SD of at least 3 independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. *$p < 0.05$ with respect to control; °$p < 0.05$ vs LPS

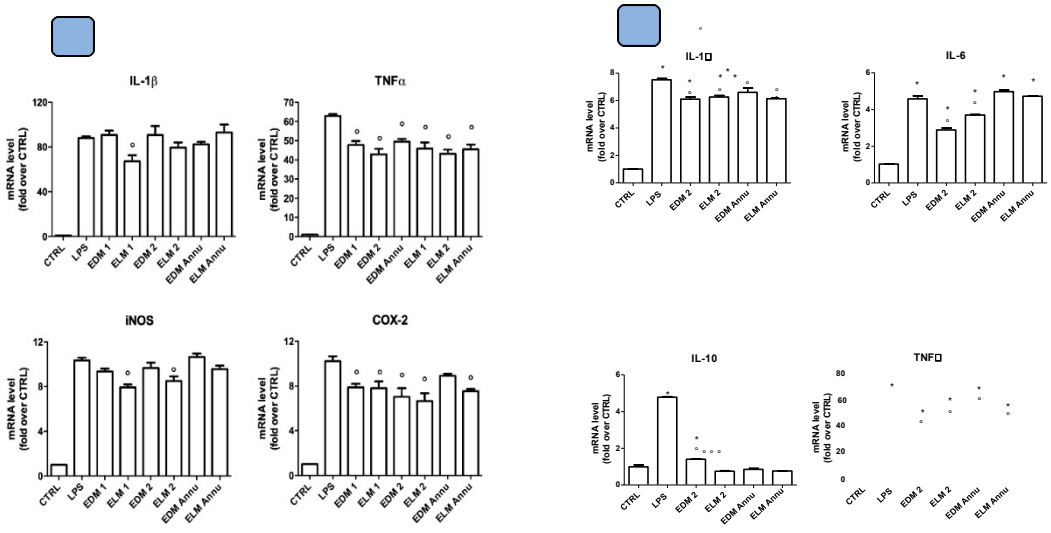


Fig 3 -A. Anti-inflammatory effect of the extracts (concentration 10 µg/mL) against LPS-induced inflammation in BV-2 cells. Real time-PCR was performed to detect IL-1β, TNF-α, iNOS, and COX-2 mRNA levels. Data are expressed as relative abundance compared to untreated cells. Each bar represents mean ± SEM of three independent experiments. Data were analyzed with one-way ANOVA followed by the Bonferroni's test. ◦ $p < 0.05$ vs. LPS.

B. Anti-inflammatory effect of the extracts (concentration 10 µg/mL) against LPS-induced inflammation in horse PBMCs. Real time-PCR was performed to detect IL-1β, IL-6, IL-10 and TNF-α mRNA levels. Data are expressed as relative abundance compared to untreated cells. Each bar represents mean ± SEM of three independent experiments. Data were analyzed with one-way ANOVA followed by the Bonferroni's test. * $p < 0.05$ vs. control, ◦ $p < 0.05$ vs. LPS

Table 1 Concentrations expressed in mg/kg of the extracts obtained from the dried and lyophilised peel of Mela Rosa, Annurca, Golden Delicious and Granny Smith samples.

pounds	Dried														Lyophilized											
	Mela Rosa											Annu	Gold	Gran	Mela Rosa										Annu	Gold	Gran
	1	2	3	4	5	6	7	8	9	Mean	Dev Std %				1	2	3	4	5	6	7	8	9	Mean	Dev Std %			
roxybenzoic acids																												
ic acid	8.8	6.8	25.5	7.9	6.7	8.4	3.9	6.8	10.2	9.4	66.5	0.0	0.0	5.0	6.5	3.9	4.1	4.1	8.2	0.0	0.0	4.7	0.0	3.5	84.6	0.0	0.0	0.0
an-3-ols																												
chin	333.5	136.1	190.4	223.1	309.7	301.2	221.2	60.0	364.2	237.7	41.8	180.2	152.5	201.7	470.0	235.9	260.8	260.4	552.0	426.2	320.4	126.7	403.0	339.5	39.3	529.7	146.6	283.0
atechin	2042.9	938.1	1280.1	1257.0	1772.0	2083.5	1630.1	1388.1	2049.9	1604.6	25.7	1147.7	1954.7	1306.2	2735.5	1740.2	3059.2	3053.9	3999.0	3976.4	3007.5	2454.9	2869.5	2988.5	23.4	1854.9	1878.8	1997.7
ianidin B2	2041.4	855.0	858.5	874.0	1324.2	1177.9	1204.6	819.3	1201.6	1150.7	33.5	495.2	1529.3	843.2	2089.8	1655.9	2266.4	2262.5	2732.7	2421.4	2386.6	1867.0	1995.1	2186.4	14.8	708.1	1469.9	1176.1
ianidin A2	252.4	230.1	209.0	280.9	224.6	174.0	174.9	112.5	222.1	208.9	23.7	218.5	292.5	119.1	301.5	272.7	715.7	714.4	565.0	503.8	562.8	379.3	267.5	475.9	37.5	294.9	281.1	252.3
hocyanins																												
nidin 3-glucoside	27.7	7.1	16.9	19.7	19.3	9.8	7.7	70.1	9.8	20.9	94.1	958.1	7.4	0.0	50.8	51.9	103.5	103.3	79.4	123.7	43.9	415.7	39.2	112.4	104.8	1583.5	7.2	0.0
onols																												
n	306.2	572.2	509.9	656.8	342.0	687.2	553.2	377.7	545.1	505.6	26.8	653.5	768.0	145.6	0.0	882.0	1034.5	1032.7	972.9	1384.9	1422.4	667.9	917.6	923.9	45.4	717.4	738.1	285.7
rcetin-3-O-ctoside	101.1	235.6	359.3	496.8	360.4	444.0	220.2	246.2	363.3	314.1	39.3	411.4	860.5	347.6	11.5	429.8	751.3	749.9	1313.8	1158.8	922.3	632.5	650.6	735.6	52.3	372.0	827.0	521.7
pferol-3-glucoside	258.2	293.0	408.9	253.6	340.8	197.2	126.3	134.3	272.1	253.8	36.1	484.4	838.9	694.2	358.6	486.9	811.8	810.3	1015.7	472.4	336.6	394.5	296.7	553.7	46.6	569.8	806.4	1327.8
rcetin	6.2	5.2	16.6	6.5	6.7	3.8	8.0	5.1	14.6	8.1	55.2	53.1	0.0	0.0	21.3	9.1	14.4	14.3	8.5	4.3	14.4	6.3	20.9	12.6	47.9	23.2	0.0	12.3
pferol	2.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.6	0.6	198.7	0.0	0.0	0.0	6.9	0.0	0.0	0.0	0.0	7.9	0.0	0.0	6.0	2.3	151.5	0.0	0.0	0.0
rocinnamic acids																												
chlorogenic acid	6.0	0.0	0.0	7.2	4.4	4.5	4.2	4.2	4.3	3.9	62.5	0.0	0.0	0.0	12.0	0.0	0.0	0.0	9.8	0.0	4.2	4.7	5.8	4.1	112.1	0.0	0.0	0.0
rogenic acid	120.9	1551.7	84.0	90.2	143.6	163.7	137.1	2125.9	196.0	512.6	149.5	1042.3	636.1	47.5	240.9	1726.3	97.8	97.7	154.9	167.2	109.8	2287.6	156.6	559.9	148.9	1243.0	611.3	50.0
eic acid	21.0	0.0	13.6	18.7	11.7	13.0	18.8	100.2	18.6	24.0	122.2	22.2	31.6	0.0	26.2	0.0	0.0	0.0	14.9	5.5	17.4	89.7	19.9	19.3	146.0	35.5	30.4	0.0
umaric acid	4.8	7.6	0.0	4.7	3.4	2.2	0.0	0.0	0.0	2.5	109.8	0.0	0.0	0.0	2.3	0.0	0.0	0.0	6.2	3.7	2.1	0.0	0.0	1.6	139.0	0.0	0.0	0.0
s-Ferulic acid	3.7	2.2	9.6	6.0	7.3	2.9	6.1	6.1	8.0	5.8	42.3	8.5	7.5	0.0	13.8	5.1	39.7	39.7	15.3	13.0	10.7	4.4	5.9	16.4	84.1	7.8	7.2	0.0
ydrochalcones																												
ridzin	1315.0	507.3	527.1	387.7	600.5	872.8	398.5	585.6	562.9	639.7	45.3	649.3	394.2	87.4	1809.5	892.0	1628.3	1625.5	1228.1	2012.1	730.2	1897.9	773.8	1399.7	35.9	1012.8	378.9	176.9
retin	16.1	0.0	10.9	3.2	3.2	10.4	11.7	0.0	0.0	6.2	99.6	0.0	0.0	0.0	3.7	0.0	27.4	27.4	3.3	6.1	3.1	0.0	0.0	7.9	142.7	10.2	0.0	0.0
l polyphenols	**6868.8**	**5347.9**	**4520.3**		**4593.9**	**5480.3**	**6156.5**	**4726.6**	**6042.2**	**5845.2**	**5509.1**	**14.5**	**6324.5**	**7473.2**	**3797.7**	**8160.7**	**8391.5**	**10814.9**	**10796.1**	**12679.8**	**12687.5**	**9894.5**	**11233.9**	**8427.8**	**10343.0**	**17.0**	**8962.6**	**7182.9**	**6083.5**

erpenes																												
nolic acid	6301.5	3996.2	598.5	3361.9	2340.1	3132.9	6527.3	8654.7	3543.2	4272.9	57.5	2504.0	3296.4	6374.6	811.6	10835.5	321.1	320.5	1398.0	6194.2	4658.1	6276.7	4278.2	3899.3	91.2	5101.9	3168.4	9732.7
lic acid	15088.8	10719.5	1361.7	5383.3	2121.7	7188.4	9006.2	9617.5	7307.5	7532.7	56.7	6236.6	9058.5	11293.8	1911.8	7667.8	584.4	583.4	3366.3	12541.8	8722.0	10831.0	9069.4	6142.0	74.6	9094.3	8706.7	18083.5
l triterpenes	**21390.4**	**14715.8**	**1960.2**	**8745.2**	**4461.8**	**10321.4**	**15533.5**	**18272.2**	**10850.6**	**11805.7**	**53.5**	**8740.6**	**12354.9**	**17668.4**	**2723.5**	**18503.3**	**905.5**	**903.9**	**4764.3**	**18736.0**	**13380.1**	**17107.8**	**13347.6**	**10041.3**	**76.1**	**14196.2**	**11875.0**	**27816.2**

Table 2 Concentrations expressed in mg/kg of the extracts obtained from the dried and lyophilised pulp samples of Mela Rosa, Annurca, Golden Delicious and Granny Smith.

	Dried														**Lyophilised**												
	Mela Rosa														**Mela Rosa**												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**Mean**	**Dev Std %**	**Annu**	**Gold**	**Gran**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**Mean**	**Dev Std %**	**Annu**	**G**
Hydroxybenzoic acids																											
Gallic acid	4.8	5.7	3.9	3.8	5.6	6.4	4.1	8.0	11.8	6.0	42.5	4.5	6.1	11.3	2.4	2.2	0.0	2.7	2.0	2.0	5.7	3.1	4.9	2.8	60.8	1.7	2
Flavan-3-ols																											
Catechin	183.5	131.7	261.8	144.9	285.3	336.6	271.3	123.1	366.1	233.8	38.9	108.0	20.3	15.1	214.5	164.8	176.2	184.9	340.1	236.7	195.1	131.0	416.9	228.9	40.1	119.4	53
Epicatechin	586.9	616.5	791.7	676.1	982.9	1208.7	820.5	938.5	1410.9	892.5	31.0	624.7	331.2	772.5	833.7	494.5	881.1	814.3	1292.2	738.7	769.3	706.2	1725.2	917.2	40.2	701.5	57
Procianidin B2	338.9	325.6	455.4	429.2	827.5	714.0	472.0	588.2	1015.6	574.0	40.9	367.7	342.5	686.2	431.0	387.0	538.6	471.5	784.3	503.7	535.1	292.1	1262.2	578.4	50.1	608.1	50
Procianidin A2	30.0	27.2	38.0	41.0	68.9	65.2	39.3	41.6	88.0	48.8	41.9	44.0	31.1	66.6	66.5	32.6	49.3	53.0	97.6	33.8	55.9	29.4	106.7	58.3	47.6	56.4	41
Anthocyanins																											
Cyanidin 3-glucoside	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0
Flavonols																											
Rutin	88.9	63.5	82.9	65.3	99.5	125.8	86.0	98.8	123.9	92.7	23.8	75.9	21.3	20.1	108.7	84.5	109.7	71.4	117.4	82.7	61.7	100.3	124.7	95.7	22.6	91.8	25
Quercetin-3-O-galactoside	7.1	3.8	8.7	8.1	14.8	8.0	9.0	10.1	13.1	9.2	35.1	7.0	1.8	7.1	9.9	4.1	8.8	9.1	14.8	6.8	10.7	5.0	20.4	9.9	50.9	7.3	2
Kampferol-3-glucoside	24.6	17.5	36.9	24.7	48.5	30.3	38.3	12.4	36.4	30.0	37.7	25.9	43.7	19.3	37.0	19.3	49.2	27.4	73.1	32.2	22.7	17.1	55.4	37.0	50.7	44.9	43
Quercetin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0
Kampferol	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0
Hydrocinnamic acids																											
Neochlorogenic acid	2.7	2.4	3.0	1.9	4.3	3.3	3.1	13.8	5.8	4.5	82.7	2.0	5.4	0.0	3.1	3.5	2.3	4.0	4.3	4.3	4.7	6.9	7.1	4.5	35.8	1.9	2
Chlorogenic acid	311.9	1438.7	306.5	136.0	260.8	492.0	317.7	2212.5	529.6	667.3	104.1	841.0	708.2	349.5	384.2	1946.8	244.6	228.2	273.6	387.5	167.9	1779.9	338.8	639.1	109.4	1230.5	64
Caffeic acid	14.0	0.0	9.8	16.1	15.2	21.5	10.1	42.2	0.0	14.3	88.4	0.0	0.0	0.0	14.6	0.0	14.6	14.3	26.5	15.9	21.0	29.1	16.5	16.9	49.4	0.0	0

p-Coumaric acid	1.0	59	0.0	1.0	1.1	1.1	0.0	2.3	1.0	1.5	121.2	0.9	0.0	1.5	0.0	6.1	0.0	1.1	1.1	1.0	1.0	4.8	0.8	1.8	122.4	0.8	1
trans-Ferulic acid	1.1	1.2	1.1	1.5	1.1	1.6	1.1	2.5	1.8	1.4	33.5	1.5	1.0	0.9	2.0	2.5	4.5	1.6	1.8	1.6	1.2	2.1	1.8	2.1	44.5	1.3	1
Dihydrochalcones																											
Phloridzin	72.1	73.1	61.3	49.6	64.5	101.0	63.5	115.4	64.5	73.9	28.3	24.6	60.9	19.5	136.9	135.5	98.5	68.8	122.4	97.3	58.7	112.6	103.7	103.8	26.1	30.8	39
Phloretin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0
Total polyphenols	**1667.6**	**2712.9**	**2060.9**	**1599.0**	**2679.7**	**3115.4**	**2135.9**	**4209.3**	**3668.5**	**2649.9**	**33.7**	**2127.7**	**1573.6**	**1969.6**	**2244.3**	**3283.4**	**2177.4**	**1952.3**	**3151.2**	**2144.0**	**1910.7**	**3219.6**	**4185.0**	**2696.4**	**29.3**	**2896.4**	**194**
Triterpenes																											
Oleanolic acid	47.7	123.6	144.6	71.9	85.3	95.0	149.9	68.6	32.7	91.0	45.3	101.8	34.5	49.5	39.7	107.7	107.2	142.5	75.8	56.6	178.9	124.0	19.7	94.7	54.1	82.2	74
Ursolic acid	40.6	29.8	77.4	29.4	137.7	36.2	80.2	28.3	48.3	56.4	64.4	111.2	50.2	66.8	35.5	17.3	47.2	82.9	90.7	58.0	23.3	49.8	37.5	49.1	50.6	66.5	11
Total triterpenes	**88.3**	**153.4**	**222.0**	**101.2**	**223.0**	**131.1**	**230.0**	**96.9**	**81.0**	**147.4**	**42.2**	**212.9**	**84.7**	**116.3**	**75.2**	**125.0**	**154.4**	**225.3**	**166.5**	**114.6**	**202.2**	**173.8**	**57.2**	**143.8**	**38.9**	**148.7**	**18**

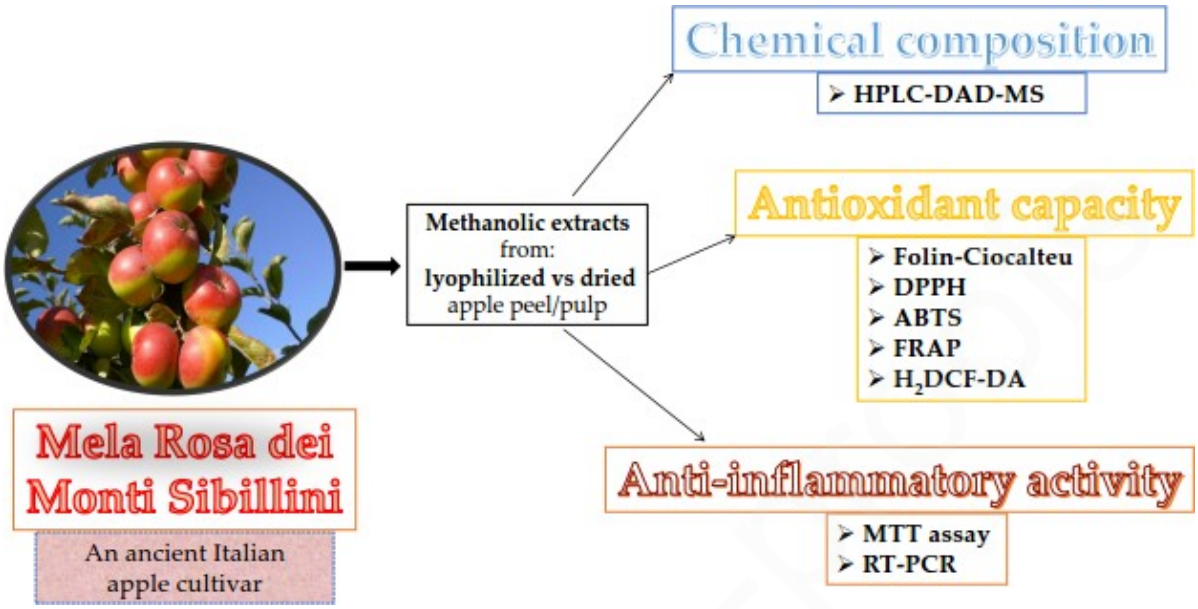
Table 3 Total phenolic content and antioxidant capacity of methanolic extracts of apple peels.

	Dried samples						*Lyophilized samples*					
	FOLIN	**DPPH**		**ABTS**		**FRAP**	**FOLIN**	**DPPH**		**ABTS**		**FRAP**
		TEAC	**IC_{50}**	**TEAC**	**IC_{50}**			**TEAC**	**IC_{50}**	**TEAC**	**IC_{50}**	
Sample	mgGAE/kg	mgTE/g	mg/ml	mgTE/g	mg/ml	mgTE/g	mgGAE/kg	mgTE/g	mg/ml	mgTE/g	mg/ml	mgTE/g
1	36173.0 ± 3189.9	14.2 ± 1.2	5.7 ± 0.5	23.0 ± 4.0	2.0 ± 0.3	10.0 ± 2.0	42839.2 ± 7062.6	14.1 ± 1.6	2.2 ± 0.3	10.0 ± 0.1	1.5 ± 0.0	10.7 ± 1.7
2	35983.2 ± 8878.2	18.4 ± 2.0	3.9 ± 0.4	24.8 ± 0.9	2.8 ± 0.1	11.1 ± 2.1	45307.8 ± 5461.6	18.9 ± 0.8	1.5 ± 0.1	10.8 ± 0.8	1.4 ± 0.1	15.7 ± 2.9
3	34061.9 ± 6075.8	19.1 ± 0.2	3.7 ± 0.0	23.4 ± 0.2	3.0 ± 0.0	9.5 ± 1.7	55752.8 ± 9584.7	22.1 ± 0.2	1.4 ± 0.0	13.5 ± 0.1	1.1 ± 0.0	16.9 ± 3.5
4	28016.5 ± 12594.2	11.1 ± 1.3	6.4 ± 0.8	17.4 ± 0.4	4.0 ± 0.1	7.5 ± 1.8	44059.9 ± 10682.0	14.4 ± 0.5	3.5 ± 0.1	12.4 ± 0.6	1.2 ± 0.1	14.0 ± 2.4
5	40874.3 ± 2923.0	15.4 ± 0.5	4.6 ± 0.2	21.9 ± 1.4	3.2 ± 0.2	12.7 ± 3.6	53056.4 ± 5939.5	24.6 ± 1.6	2.0 ± 0.1	20.6 ± 1.4	0.7 ± 0.1	22.1 ± 4.7
6	32802.3 ± 4463.2	8.7 ± 1.1	8.2 ± 1.0	20.4 ± 0.41	3.4 ± 0.1	9.4 ± 1.8	49459.5 ± 8390.5	17.4 ± 0.7	2.9 ± 0.1	19.8 ± 0.5	0.8 ± 0.0	17.4 ± 3.2
7	26324.6 ± 1576.4	11.0 ± 0.5	6.4 ± 0.3	16.1 ± 4.3	3.0 ± 0.8	7.9 ± 1.0	46610.1 ± 4417.3	15.2 ± 1.0	3.3 ± 0.2	18.1 ± 0.7	0.8 ± 0.0	15.8 ± 3.3
8	32389.2 ± 4107.7	17.1 ± 4.5	4.4 ± 1.2	17.6 ± 0.5	4.0 ± 0.1	10.1 ± 1.8	44242.0 ± 2672.6	12.0 ± 1.4	2.5 ± 0.3	17.4 ± 1.4	0.9 ± 0.1	16.6 ± 3.8
9	39619.8 ± 3850.6	22.9 ± 1.4	3.1 ± 0.2	28.8 ± 4.1	2.5 ± 0.4	11.6 ± 2.3	39570.4 ± 6226.2	7.9 ± 0.1	3.8 ± 0.1	17.0 ± 1.5	0.9 ± 0.1	13.2 ± 2.4
Ann	33403.2 ± 2178.0	13.1 ± 2.2	5.5 ± 1.0	21.3 ± 0.3	3.3 ± 0.1	10.8 ±2.0	36502.9 ± 3753.0	9.1 ± 1.2	3.4 ± 0.5	15.2 ± 0.5	1.0 ± 0.0	13.4 ± 2.4
Gold	30672.9 ± 1080.1	10.0 ± 1.3	4.1 ± 0.5	7.1 ± 0.2	2.1 ± 0.1	9.9 ± 1.7	40542.4 ± 13200.8	6.8 ± 0.3	5.9 ± 0.3	25.3 ± 0.1	2.0 ± 0.0	10.2 ±3.0
Gran	35114.7 ± 3132.2	12.9 ± 1.0	3.1 ± 0.3	9.2 ± 1.1	1.7 ± 0.2	14.5 ± 3.1	40706.4 ± 10787.7	7.0 ± 1.0	5.8 ± 0.8	30.5 ± 0.2	1.6 ± 0.0	11.9 ± 2.8

Table 4 Total phenolic content and antioxidant capacity of methanolic extracts of apple pulps

	Dried samples						*Lyophilized samples*					
	FOLIN	**DPPH**		**ABTS**		**FRAP**	**FOLIN**	**DPPH**		**ABTS**		**FRAP**
		TEAC	**IC_{50}**	**TEAC**	**IC_{50}**			**TEAC**	**IC_{50}**	**TEAC**	**IC_{50}**	
Sample	mgGAE/kg	mgTE/g	mg/ml	mgTE/g	mg/ml	mgTE/g	mgGAE/kg	mgTE/g	mg/ml	mgTE/g	mg/ml	mgTE/g
1	11987.1 ± 3611.5	1.9 ± 0.3	22.4 ± 4.2	4.2 ± 0.0	7.2 ± 0.0	6.0 ± 0.9	9572.8 ± 1344.8	4.0 ± 0.4	19.0 ± 3.1	8.1 ± 0.0	5.0 ± 0.0	8.5 ± 2.1
2	13751.4 ± 2555.2	2.4 ± 0.1	16.7 ± 0.9	5.5 ± 0.0	5.4 ± 0.0	9.9 ± 1.5	9912.3 ± 2948.6	4.4 ± 0.0	16.0 ± 0.3	8.5 ± 0.1	4.7 ± 0.1	9.6 ± 1.5
3	13601.7 ± 772.9	2.1 ± 0.1	19.4 ± 0.7	5.9 ± 0.1	5.1 ± 0.1	7.0 ± 1	11618.0 ± 1172.4	8.7 ± 0.9	9.0 ± 1.7	11.2 ± 0.2	3.6 ± 0.1	9.8 ± 2
4	10425.1 ± 1071.8	2.4 ± 0.1	16.5 ± 0.4	4.7 ± 0.2	6.4 ± 0.2	6.6 ± 1.9	7887.2 ± 1087.7	4.4 ± 0.2	16.6 ± 1.6	8.1 ± 1.1	5.0 ± 0.7	7.9 ± 1.5
5	15083.7 ± 2711.4	3.5 ± 0.0	11.4 ± 0.1	8.1 ± 0.5	3.7 ± 0.25	10.9 ± 2.7	12605.3 ± 1185.7	7.1 ± 0.1	10.1 ± 0.3	14.8 ± 1.0	2.7 ± 0.2	9.5 ± 2.1
6	13636.9 ± 1122.7	3.1 ± 0.1	13.0 ± 0.3	7.6 ± 0.3	4.0 ± 0.13	9.9 ± 2.5	9225.4 ± 1318.3	5.2 ± 1.2	17.4 ± 6.4	7.4 ± 0.0	5.4 ± 0.0	5.5 ± 1
7	13684.7 ± 471.2	2.7 ± 0.5	15.3 ± 2.6	6.8 ± 0.2	4,4 ± 0.1	8.6 ± 2	8798.0 ± 1581.6	2.4 ± 0.0	30.2 ± 0.9	8.7 ± 0.2	4.6 ± 0.1	3.7 ± 1
8	18048.3 ± 2478.8	3.7 ± 0.1	10.9 ± 0.2	7.4 ± 0.0	4.1 ± 0.0	11 ± 2.8	10172.9 ± 1853.2	3.2 ± 0.0	21.9 ± 0.1	7.3 ± 0.1	5.5 ± 0.1	3.2 ± 0,7
9	20989.3 ± 2376.3	5.3 ± 0.3	7.6 ± 0.5	12.3 ± 0.1	2.5 ± 0.0	11.3 ± 2.6	13476.6 ± 1788.8	4.9 ± 0.1	14.6 ± 0.8	15.2 ± 0.2	2.6 ± 0.0	5.4 ± 1.8
Ann	12326.6 ± 2028.7	1.3 ± 0.0	31.6 ± 0.3	4.9 ±0.0	6.2 ± 0.0	7.6 ± 1.7	8527.7 ± 595.3	4.0 ± 0.1	18.0 ± 0.9	8.8 ± 0.3	4.5 ± 0.1	3.8 ±1.1
Gold	8892.3 ± 585.0	2.1 ± 0.3	19.4 ± 2.7	6.3 ± 0.1	7.9 ± 0.1	6.8 ± 1	11400.7 ± 1885.9	1.9 ± 0.0	20.9 ± 0.3	5.7 ± 0.2	7.0 ± 0.2	7.7 ± 1.5
Gran	10809.3 ± 1671.9	2.5 ± 0.5	16.9 ± 3.6	10.4 ± 0.1	4.8 ± 0.1	8.8 ± 1.6	11862.9 ±1436.5	3.0 ± 0.1	13.5 ± 0.6	9.4 ± 0.1	4.2 ± 0.0	9.5 ± 2.2

Graphical abstract



Highlights

- The ‘Mela Rosa dei Monti Sibillini’ is an ancient apple cultivar of central Italy.
- Two sample preparation procedures were used for extracts.
- 20 phytochemicals were quantitatively determined by HPLC-DAD-MS.
- The fruit part influences the chemical composition of the final extract.
- The antioxidant and anti-inflammatory activities were measured.

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