Protective effect of bioaccessible fractions of citrus fruit pulps against

H₂O₂-induced oxidative stress in Caco-2 cells

Running title: Cytoprotection of citrus fruit pulps in Caco-2 cells

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Abbreviations: N: Navel orange; CC: Cara Cara orange; M: mandarin freshly harvested; M12: mandarin refrigerated stored; BF: bioaccessible fractions; ROS: reactive oxygen species; t-BuOOH: tert-Butyl hydroperoxide; ABTS^{•+}: 2,2'-azino-bis [3 ethylbenzothiazoline-6-sulfonic acid] anion radical; DPPH: 2,2-diphenyl-1-picrylhydrazyl; FRAP: ferric reducing antioxidant power; TEER: transepithelial electrical resistance. MTT: 3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide; MDA: malondialdehyde; DHR: dihydrorhodamine; PI: propidium iodide; Δψ_m: mitochondrial membrane potential changes; DMEM: Dulbecco's Modified Eagle Medium

Abstract

Fruit pulps from Navel (N) and Cara Cara (CC) oranges, and Clementine mandarin freshly harvested (M) and refrigerated stored (M12) were used to evaluate the cytoprotective effect of their bioaccessible fractions (BF) against H₂O₂-induced oxidative stress in Caco-2 cells. BF of samples preserved viability vs. H₂O₂ treated cells, reaching values similar to controls. Lipid peroxidation was reduced to levels of control cells, but M did not reach control values. ROS and mitochondrial membrane potential changes ($\Delta\psi_m$) values were reduced compared with H₂O₂ treated cells, but without achieving control levels. A significant reduction in cell proportions in G1 phase and a significant increase in sub-G1 phase (apoptosis) of cell cycle was shown in H₂O₂ treated cells, and BF allowed a recovery close to control levels. Thus, BF of samples protect the cells from oxidative stress by preserving cell viability, mitochondrial membrane potential and correct cell cycle progression, and diminishing lipid peroxidation and ROS.

Keywords: Citrus fruit pulps, bioactive compounds, antioxidants, *in vitro* digestion, Caco-2 cells, oxidative stress.

1. Introduction

The gastrointestinal tract is the first site where interaction between endogenously generated reactive oxygen species (ROS) and dietary antioxidants takes place *in vivo* (Halliwell, Zhao & Whiteman, 2000). At elevated levels, ROS can adversely affect cell function leading to cell damage and cell death; thus, body's antioxidant system, which is incomplete without exogenous antioxidants such as vitamin C, carotenoids and polyphenols, has to be efficient avoiding the onset of oxidative stress (Bouayed & Bohn, 2010).

Citrus fruits and juices of orange and mandarin are widely consumed worldwide and have attracted attention since a high intake may reduce the risk of degenerative diseases including cardiovascular, neoplastic, and nervous system related diseases (Silalahi, 2002), and also colon carcinogenesis (Miyagi, Om, Chee & Bennink, 2000; Tanaka et al. 2000). These products are rich sources of a complex mixture of antioxidants such as vitamin C, carotenoids (namely β -cryptoxanthin, lutein, zeaxanthin and β -carotene) and polyphenols (mainly flavanones such as hesperidin and naringenin) (Guarnieri, Riso & Porrini, 2007) which may act in concert (additively or synergistically) to exert their antioxidant and anticancer activities (Liu, 2004).

A spontaneous mutant variety from sweet orange, termed Cara Cara, has been characterized and shows distinctive red pulp coloration due to the accumulation of lycopene in the juice vesicles (Lee, 2001; Alquezar, Rodrigo & Zacarias, 2008; Brasili et al. 2017). In addition, it has been reported that certain postharvest conditions promote the accumulation of carotenoids in the skin and pulp of citrus fruits, specifically β -cryptoxanthin in mandarin fruits (Carmona, Zacarias & Rodrigo, 2012; Rodrigo et al., 2015). Based on these data, the profile and bioaccessibility of bioactive compounds

(vitamin C, flavonoids and carotenoids) in fruit juices and fruit pulps from Navel and Cara Cara oranges, and Clementine mandarin freshly harvested and postharvest stored 5 weeks at 12 °C has been determined. In general, vitamin C contents and bioaccessibility were similar in the 4 varieties studied, but higher bioaccessibility on average for pulps versus juices was observed. Likewise, much more content of flavonoids and carotenoids were noticed in pulps than in juices, and higher bioaccessibility was achieved in oranges or mandarins when flavonoids or carotenoids were considered, respectively (De Ancos, Cilla, Barberá, Sánchez-Moreno & Cano, 2017; Rodrigo, Cilla, Barberá & Zacarías, 2015). Therefore, next step would be the evaluation of the bioactivity of citrus fruit pulps (due to their major bioaccessibility of vitamin C, flavonoids and carotenoids *versus* fruit juices) in a biological relevant model (Caco-2 cells), to further characterize their potential functional health benefits.

To this end, fully differentiated Caco-2 cells may serve as a good and valuable model for assessing the physiological response of enterocytes to various oxidant stresses (Bedoya-Ramírez, Cilla, Contreras-Calderón & Alegria-Torán, 2017). The protective effects of citrus fruits or related antioxidants against oxidative stress using Caco-2 cells have been documented in previous studies. In these sense, pre-incubation (24 h) of Caco-2 cells with β -carotene (0.1-50 μ M) failed to inhibit 10 mM H₂O₂-derived oxidative stress (Bestwick & Milne, 1999). On the contrary, Tarozzi et al. 2006 reported that pre-incubation for 24 h with blood orange extracts (6.25-50 mg/mL) protected Caco-2 cells from 3 mM tert-Butyl hydroperoxide (t-BuOOH) oxidative stress decreasing cytotoxicity and ROS levels. Similarly, pre-incubation (1 h) with β -cryptoxanthin (1-25 μ M) protected Caco-2 cells from DNA damage generated by 15 μ M H₂O₂ (Lorenzo, Azqueta, Luna, Bonilla, Domínguez & Collins, 2009). Other cytoprotective studies against oxidative stress with citrus fruits or related bioactive

components such as sweet orange peel extracts (Chen, Chu, Chyau, Chu & Duh, 2012) or sweet orange and citrus fruit unshiu mikan juice powders and their major flavonoids (Jannat, Ali, Kim, Jung & Choi, 2016) in HepG2, flavonoid fraction of bergamot and orange juices in A549 cells (Ferlazzo et al. 2015), citrus flavanones in PC12 cells (Hwang & Yen, 2008) and lutein and zeaxanthin in HLEC cells (Gao et al. 2011) have been reported. However, these studies have not considered the fact that food after consumption undergoes a gastrointestinal digestion process that may affect the native antioxidant potential of the complex mixture of bioactive compounds present in the food matrix before reaching the proximal intestine, thus, probably overestimating their biological activity (Cilla, Perales, Lagarda, Barberá, Clemente & Farré, 2011a). In this respect, to our knowledge, only two studies involving digested fruit beverages made of grape-orange-apricot have evaluated the cytoprotection against H₂O₂-induced oxidative stress in Caco-2 cells, based on cell viability maintenance, GSH-related enzymes activation, more preserved mitochondrial membrane potential and cell cycle distribution, and inhibition of caspase-3 activation (Cilla, Laparra, Alegría, Barberá & Farré, 2008; Cilla, Laparra, Alegría & Barberá, 2011b).

Thus, the aim of the present study was to evaluate the cytoprotective effect of bioaccessible fractions obtained after simulated gastrointestinal digestion of citrus fruit pulps from Navel and Cara Cara oranges, and Clementine mandarins freshly harvested and postharvest stored, against H₂O₂-induced oxidative stress in Caco-2 cells.

2. Materials and methods

2.1 Reagents

Simulated gastrointestinal digestion: Pepsin (porcine, 975 units per mg protein), pancreatin (porcine, activity equivalent to 4 x USP specifications) and bile extract (porcine) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Carotenoids: HPLC-grade methanol, chloroform and acetone were supplied by Scharlau (Barcelona, Spain) and methyl tert-butyl ether (MTBE) by Merck (Darmstadt, Germany). Petroleum ether and diethyl ether were of analytical grade and were supplied by Scharlau (Barcelona, Spain). Commercial standards of β -carotene ($\geq 97\%$), lutein ($\geq 95\%$) and lycopene ($\geq 90\%$) were purchased from Sigma-Aldrich, and β -cryptoxanthin ($\geq 97\%$) and zeaxanthin ($\geq 98\%$) from Extrasynthese (Lyon, France). Standards of phytoene and phytofluene were obtained from peel extracts of Pinalate orange fruits, and of all-*E*-violaxanthin and 9-*Z*-violaxanthin from peel extracts of Navel orange fruits and HPLC purified.

Ascorbic acid analysis: Glacial acetic acid, metaphosphoric acid and formic acid and L(+) ascorbic acid ($\geq 99\%$ purity) were purchased from Panreac Química (Barcelona, Spain).

Flavonoid analysis: Methanol and acetonitrile (HPLC-grade) were provided by Lab-Scan (Dublin, Ireland). Formic acid and hydrochloric acid were purchased from Panreac Química (Barcelona, Spain). Narirutin (naringenin-7-*O*-rutinoside) was obtained from Extrasynthese (France); hesperidin (hesperitin-7-*O*-rutinoside) and apigenin were purchased from Sigma (St. Louis, MO, USA).

Hydrophilic antioxidant capacity analysis: 2,2'-azino-bis [3-ethylbenzothiazoline-6-sulfonic acid] (ABTS^{•+}) anion radical, potassium persulfate (K₂S₂O₈), iron (III) chloride hexahydrate, phosphate buffered saline, hexadecyltrimethyl-ammonium bromide, and 2,2-diphenyl-1-picrylhydrazyl (DPPH[•]), were purchased from Sigma-Aldrich (St. Louis, MO, USA). N-(1-

naphtyl)ethylenediaminedihydrochloride and 2,4,6-tris (2-pyridyl)-s-triazine (TPTZ) were obtained from Fluka Chemie AG (Buchs, Switzerland). Ascorbic acid ($\geq 99\%$ purity) and ethanol were obtained from Panreac Química (Barcelona, Spain).

Caco-2 cell culture: DMEM+GlutaMAXTM-I, phosphate buffered saline (PBS), and trypsin-EDTA solution (2.5 g/L trypsin and 0.2 g/L EDTA) were purchased from Gibco Invitrogen (Carlsbad, USA). Absolute methanol was obtained from Merck (Darmstadt, Germany). The reagents used for preparing buffers were purchased from Panreac Química (Barcelona, Spain), and the rest of reagents used throughout the assays were from Sigma Chemical Co. (St. Louis, MO, USA), including the hydrogen peroxide solution 30% (w/w) used as inducer of oxidative stress.

Water of cellular grade was used for the preparation of reagents used in Caco-2 cells and throughout the *in vitro* digestion assay (Aqua B Braun, Braun Medical, Barcelona, Spain). The rest of the reagents were prepared with Milli-Q water (18.2 M Ω cm resistivity).

2.2 Samples

Fruit pulps from freshly harvested Clementine mandarin (*Citrus clementina* L) (M) and postharvest stored at 12°C (90-95% relative humidity) for 5 weeks (M12), rich in β -cryptoxanthin (Carmona et al. 2012) and from Navel orange (*Citrus sinensis* L) (N) and its spontaneous mutant Cara Cara (CC) with high lycopene and other linear carotenes (phytoene and phytofluene) content (Alquezar et al. 2008), were used to evaluate the cytoprotective effect of their bioaccessible fractions (BF) (the maximum soluble fraction of food-derived compounds in the simulated gastrointestinal media that would be available for absorption) obtained by *in vitro* digestion against H₂O₂-induced

oxidative stress in Caco-2 cells. Bioaccessible bioactive compounds contents have been analyzed in previous reports (De Ancos et al. 2017; Rodrigo et al. 2015).

2.3 *In vitro* digestion

An *in vitro* gastrointestinal digestion procedure mimicking the physiological situation in the upper digestive tract (stomach and small intestine) was used to obtain bioaccessible fraction from 80 g of citrus fruit pulps in order to evaluate BF of carotenoids, polyphenols, ascorbic acid and hydrophilic antioxidant capacity according to the procedure described by Rodrigo et al. (2015).

Briefly, 80 g of citrus fruit pulps or juices were adjusted to pH 2.0 with 6 M HCl (GLP 21 pH-meter, Crison, Barcelona, Spain). The pH was checked after 15 min, and if necessary readjusted to 2.0. Then an amount of freshly prepared demineralized pepsin solution sufficient to yield 0.02 g pepsin/g sample was added. The samples were made up to 100 g with cell culture-grade water (Aqua B Braun, Braun Medical, Barcelona, Spain), and incubated in a shaking water bath at 37°C/120 strokes per minute for 2 h (SS40-2, Gran Instruments, Cambridge, UK). The gastric digests were maintained in ice for 10 min to stop pepsin digestion. For the intestinal digestion stage, the pH of the gastric digests was raised to pH 6.5 by the dropwise addition of 1 M NaHCO₃. Then an amount of freshly prepared and previously demineralized pancreatin-bile salt solution sufficient to provide 0.005 g pancreatin and 0.03 g bile salt/g sample was added, and incubation was continued for an additional 2 h. To stop intestinal digestion, the sample was kept for 10 min in an ice bath. The pH was then adjusted to 7.2 by the dropwise addition of 0.5 M NaOH. Aliquots of 25 g of sample were transferred to polypropylene centrifuge tubes (50 ml, Costar, New York, USA) and centrifuged at 3500 x g for 1 h at 4°C (GT422 centrifuge, Jouan, Saint Nazaire, France). Supernatants (aqueous-micellar

fraction considered the bioaccessible fraction, BF) obtained after *in vitro* digestion were immediately frozen at -80°C and used for cytoprotective assays with cells

2.4 Antioxidants determination

2.4.1 Carotenoids

Carotenoids from BF (25 mL) were extracted, identified and quantified by HPLC-DAD as previously described by [Rodrigo et al. \(2015\)](#).

2.4.2 Flavonoid analysis

Flavonoids from acidified BF (20 mL) were extracted, identified (HPLC-MS-ESI-QTOF) and quantified (HPLC-DAD) according to the procedure described by [De Ancos et al. \(2017\)](#).

2.4.3 Ascorbic acid

Ascorbic acid was extracted and quantified by HPLC according to the procedure described by [De Ancos et al. \(2017\)](#) from 10 mL of acidified BF.

2.4.4 Total antioxidant capacity (ABTS^{•+}, FRAP and DPPH[•])

Total antioxidant capacity was determined according to the method of [Re, Pellegrini, Proteggente, Pannala, Yang & Rice-Evans \(1999\)](#) (for ABTS^{•+}), the method of [Benzie & Strain \(1996\)](#) (for FRAP), and the method of [Sánchez-Moreno, Larrauri and Saura-Calixto \(1998\)](#) (for DPPH[•]), including an adaptation of the methods to 96-well microplate format as previously described by [De Ancos et al. \(2017\)](#).

2.5 Caco-2 cell culture

Caco-2 cells were obtained from the American Type Culture Collection (HTB-38, Rockville, MD, USA), and were used between passages 52 and 67. The cells were

maintained and grown as previously described ([García-Nebot, Cilla, Alegría & Barberá, 2011](#)).

Caco-2 cells were seeded onto 24-well plates for all experiments (Costar Corp., USA) at a density of 2.5×10^4 cells/cm² with 1 mL of DMEM (Dulbecco's Modified Eagle Medium), and culture medium was changed every two days. However, the integrity of the monolayer of differentiated cells (seven days post-seeding) was monitored in Transwell plates by measuring the transepithelial electrical resistance (TEER) (Millicell[®] ERS-2, Millipore) value and microscopically observing the formation of domes (a feature of differentiated Caco-2 cells). Differentiated cells with values higher than $400 \Omega \times \text{cm}^2$ were used in the cytoprotection assays (MTT, lipid peroxidation, ROS, mitochondrial membrane potential and cell cycle). Then, the culture medium was aspirated from the wells, and cell monolayers were washed with PBS warmed to 37 °C. Cell cultures were preincubated (37 °C/5% CO₂/95% relative humidity) for 24 h with bioaccessible fractions of citrus fruit pulps added in 1:6 proportion (v/v) with culture media to preserve cell viability and ensure they were not cytotoxic based on pH (7.4) and osmolarity (339 mOsm/L) which were within the limits tolerated by human cells (pH 7-7.5 and osmolarity (280-330 mOsm/L) ([Cilla, González-Sarriás, Tomás-Barberán, Espín & Barberá, 2009](#)). Control cells only suffered change of the DMEM culture medium in the same treatment period. Afterwards, culture medium was removed and the cells were washed with PBS at 37 °C; the induction of oxidative stress was carried out by exposure to a 5 mM H₂O₂ solution in culture medium for 2 h (37 °C/5% CO₂/95% relative humidity) based on dose- and time-dependent experiments as previously described by [Cilla et al. \(2008 and 2011b\)](#) and [García-Nebot et al. \(2011\)](#).

In order to evaluate the cytoprotective effects of citrus fruit pulps in a model of oxidative stress in cultured cells (Goya, Martín, Ramos, Mateos & Bravo, 2009) we measured: (i) cell viability (MTT test), (ii) an index of cellular oxidative stress (intracellular ROS levels) and (iii) biomarkers of oxidative damage to molecules and/or organelles such as lipids (malondialdehyde), mitochondria (changes in mitochondria membrane potential) and DNA (cell cycle distribution).

2.6 Cell viability (mitochondrial enzyme activities – MTT test)

The mitochondrial functionality of the Caco-2 cells was evaluated by using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide) assay, as previously reported (García-Nebot et al., 2011). This colorimetric method is based on reduction of the tetrazolium ring of MTT by mitochondrial succinate dehydrogenases, yielding a blue formazan product which can be measured spectrophotometrically; the amount of formazan produced is proportional to the number of viable cells. The conversion to insoluble formazan was measured at 570 nm with background subtraction at 690 nm. Control cells were used in each assay.

2.7 Lipid peroxidation

Lipid peroxidation was measured according to Buege & Aust (1978), based on the cellular content of malondialdehyde (MDA). Lysed and homogenized cells were boiled (100 °C water bath for 20 min) under acidic conditions in the presence of 0.5% thiobarbituric acid, 1.5 mM deferoxamine and 3.75% butylated hydroxytoluene. Afterwards, samples were cooled, centrifuged (3500 g, 15 min), and the absorbance was measured at 532 nm in a thermostated UV-VIS spectrophotometer (Lambda 2, Perkin Elmer, Barcelona, Spain). Results were expressed as nmol of MDA/mL cell homogenate. Control cells were used in each assay.

2.8 Intracellular reactive oxygen species (ROS)

The intracellular accumulation of ROS in Caco-2 cells was evaluated using a 2 mM dihydrorhodamine (DHR) solution prepared in dimethylsulfoxide, which is oxidized to fluorescent rhodamine (Cilla et al. 2008). After pre-incubation with bioaccessible fractions of citrus fruit pulps, 1 mL of PBS containing DHR (final concentration 5 μ M) was added, followed by incubation for 30 min in darkness (37 °C/5% CO₂/95% relative humidity). After eliminating the supernatants, cells were washed with PBS and resuspended in a trypsin-EDTA solution (2.5 g/L trypsin, 0.2 g/L EDTA). Fluorescent intensities of DHR ($\lambda_{\text{ex}} = 488$ nm and $\lambda_{\text{em}} = 525$ nm) was determined by flow cytometry (Coulter, EPICS XL-MCL, Miami, USA). Control cells were used throughout each assay. At least 10,000 events per sample were analyzed.

2.9 Mitochondrial membrane potential changes ($\Delta\psi_m$)

Mitochondrial membrane potential ($\Delta\psi_m$) changes were evaluated using DHR and propidium iodide (PI) double labeling, as previously determined by Cilla et al. (2008). Briefly, cultures were washed with PBS and resuspended in a trypsin-EDTA solution. Then, they were incubated with 1 mL of PBS containing DHR, prepared as described above, for 30 min in darkness (37 °C/5% CO₂/95% relative humidity). Afterwards, the media were aspirated and 0.5 mL of a PI staining solution (1 mg/mL of trisodium citrate, 0.05 mg/mL PI, and 1 mg/mL of RNase A) was added with an additional incubation time of 15 min in darkness under the aforementioned conditions. Fluorescent intensities of DHR ($\lambda_{\text{ex}} = 488$ nm and $\lambda_{\text{em}} = 525$ nm) and PI ($\lambda_{\text{exc}} = 488$ nm and $\lambda_{\text{em}} = 620$ nm) were determined by flow cytometry (Coulter, EPICS XL-MCL, Miami, USA). $\Delta\psi_m$ changes were estimated by the relative amount of PI versus DHR

double negative cell populations. Control cells were used throughout each assay. At least 10,000 events per sample were analyzed.

2.10 Cell cycle analysis

DNA distribution in each phase of the cell cycle was determined according to [García-Nebot et al. \(2011\)](#). Cells (2×10^5) were collected after the corresponding treatment period, fixed in ice-cold ethanol: PBS (70:30, v/v) for 30 min at 4 °C, further resuspended in PBS with 40 µg/mL RNase and 100 µg/mL PI, and incubated for 30 min in darkness (37 °C/5% CO₂/95% relative humidity). DNA content (10,000 cells) was analyzed by flow cytometry (Coulter, EPICS XL-MCL, Miami, FL, USA) at $\lambda_{\text{exc}} = 488$ nm and $\lambda_{\text{em}} = 620$ nm. Control events were used in each assay.

2.11 Statistical analysis

The results shown represent mean values $\pm$ standard deviation. These results were calculated from the means of 4 replicates obtained in at least two separate experiments. This means that it was performed one digestion experiment and two independent Caco-2 cell experiments, receiving 4 replicates of the digest for each assay (MTT, lipid peroxidation, ROS, mitochondrial membrane potential and cell cycle). One-way (type of sample) ANOVA was conducted followed by the Tukey *post hoc* test. The level of significance was set at $p < 0.05$ (Statgraphics Plus v. 5.1, Maryland, USA).

3. Results and discussion

3.1 Content of antioxidant bioactive compounds in bioaccessible fractions

Concentrations of bioactive compounds such as vitamin C, vitamin E and carotenoids range between high µM and low mM levels in human plasma and organs, whereas polyphenol concentrations are situated between high nM to low µM levels

([Bouayed & Bohn, 2010](#)). Nevertheless, gastrointestinal concentrations of these bioactive compounds might be much higher than serum/plasma concentrations due to the low bioavailability of polyphenols what implies that larger quantities are delivered to the colon where they and their catabolic products of bacterial fermentation can exert beneficial effects, perhaps preventing oxidative stress linked gastrointestinal diseases ([Deiana et al. 2012](#)).

The concentration of major bioactive compounds used for cell assays from BF diluted (1:6 v/v) in culture medium are shown in **table 1**.

The BF of M12 mandarin and CC orange showed less ascorbic acid and higher carotenoid and polyphenol contents (and consequently higher total bioactive compounds concentration) than their respective counterparts, M mandarin and N orange. The total bioactive compounds (carotenoids + polyphenols + ascorbic acid) in M12 mandarins and CC orange compared with their corresponding counterparts is 1.7 and 3.1 fold higher, respectively. In addition, total antioxidant capacity is, in general, slightly higher in these samples. However, strikingly, these differences in total bioactive compounds were not translated into significant differences in total antioxidant capacity as determined by the ABTS^{•+} and FRAP methods and further in the cytoprotective activity in Caco-2 cells (see below). The results in the present study are in line with those reported by [Tarozzi et al. \(2006\)](#), who indicated that despite the differences in total phenolics, ascorbic acid, total anthocyanins and intracellular antioxidant activity of blood orange extracts from organic versus non-organic agriculture, no statistical differences in cytoprotective activity in Caco-2 cells were found. Similarly, differences in antioxidant activity evaluated by chemical tests (ORAC and Folin-Ciocalteu) in bergamot and orange juices did not imply differences in viability preservation, ROS amelioration, lipid peroxidation and mitochondrial membrane potential preservation in

A549 cells (Ferlazzo et al. 2015). Likewise, Deiana et al. (2012) reported that wine extracts from distinct Sardinian grape varieties with different total phenolic content showed comparable cytoprotective activity in Caco-2 cells (in terms of cell viability and prevention of lipid peroxidation). These authors indicate that the resulting antioxidant capacity of a food extract may be influenced by the interactions (synergy and/or antagonism) among the different classes of bioactive compounds and the presence of radical molecules. Also, they point out that a high phenolic amount of an extract is not crucial for its antiradical activity, and should be ascribed not only to the different classes of phenolic compounds, but probably to the molecular structures of the individual phenolic compounds present in the food extract determined (Deiana et al., 2012).

3.2 Cell viability

MTT test was applied to measure the mitochondrial metabolic rate as an indirect value of cell viability to check the possible cytoprotection of BF of citrus fruit pulps against oxidative stress-induced cytotoxicity. MTT conversion percentages in cell cultures preincubated with BF of citrus fruit pulps with/without oxidative stress are shown in **figure 1**.

Pretreatment of Caco-2 cells for 24 h with the BF of samples (diluted 1:6 v/v in DMEM) resulted in no changes in cell viability compared to control cells, indicating that the concentrations used did not damage cell integrity during the period of incubation. However, after H₂O₂ exposure, cell viability was significantly decreased ($p<0.05$) versus control (33%), due to a decline in mitochondrial enzyme activities. On the contrary, pretreated cells with BF of samples and further exposure to oxidative stress were able to prevent a decline in cell viability reaching basal conditions of the

control cells. As previously indicated, all four BF of citrus fruit pulps exhibited comparable results despite differences in total bioactive compounds (see **table 1**).

In line with the present results, pretreatment 24 h with BF of fruit beverages made of grape-orange-apricot (diluted 1:1 in DMEM, ~50 μ M total phenolics) were able to protect differentiated Caco-2 cells against H₂O₂-induced oxidative stress (5 mM/2 h) (Cilla et al. 2008 and 2011b). Inhibition of cytotoxicity (10-70%) in Caco-2 cells induced by t-BuOOH (3 mM/3 h) after 24 h incubation with blood orange extracts (12.5-50 mg/mL) has also been indicated (Tarozzi et al. 2006). In agreement, pretreatment of HepG2 cells 24 h with sweet orange peel extracts (50-500 μ g/mL) containing the flavonoids hesperidin, hesperetin, nobiletin and tangeretin, restored the cytotoxicity derived by treatment with t-BuOOH (0.2 mM/6 h) (Chen et al. 2012). Similarly, pretreatment with flavonoid extracts of bergamot and orange juices (25 and 50 μ g/mL) ameliorated the decrease in cell viability due to H₂O₂-induced oxidative stress (200 μ M/2 h) in A549 cells (Ferlazzo et al. 2015). In tune with this, 24 h pretreatment with sweet orange and unshiu mikan citrus fruit juice powders (50-200 μ g/mL) as well as major flavonoids (hesperidin, rutin and narirutin) at 2.5-100 μ M dose-dependently protected against the decrease in cell viability evoked by t-BuOOH (200 μ M/2 h) in HepG2 cells (Jannat et al. 2016). Likewise, pretreatment 6 h with citrus flavanones hesperidin, hesperetin and neohesperidin (0.8-50 μ M) protected PC12 cells from H₂O₂-induced oxidative stress (400 μ M/16h) (Hwang et al. 2008). On the contrary, pretreatment of HLEC cells for 48 h with lutein and zeaxanthin (5 μ M) did not prevent H₂O₂ (100 μ M/1h) induced loss of cell viability (Gao et al. 2011). This fact may imply that whole foods containing complex combinations of bioactive compounds, or perhaps specific flavonoids or combinations of them, as indicated by Hwang et al. (2008) and

Jannat et al. (2016), are able to protect from oxidative stress better than carotenoids; suggesting the higher chemo-protective capacity of flavonoids.

3.3 Lipid peroxidation

Lipid peroxidation is responsible for the cleavage of polyunsaturated fatty acids at their double bounds in lipid bilayers, causing disruption of membrane structure and function (García-Nebot et al., 2011). MDA, a three-carbon compound formed by scission of peroxidized polyunsaturated fatty acids is one of the main products of lipid peroxidation (Goya et al. 2009). The MDA levels in cell cultures preincubated with BF of citrus fruit pulps with/without oxidative stress are shown in **figure 2**.

In the absence of an oxidative insult, pretreatment with BF of samples for 24 h did not alter the basal levels of MDA. On the contrary, H₂O₂ significantly increased ($p<0.05$) the MDA levels (13.07 ± 2.25) versus control cells (4.32 ± 0.68). In contrast, pretreatment with BF of citrus fruit pulps before H₂O₂ exposure equally prevented the damage to lipids in all samples, which showed similar levels of MDA than the same samples without stress, and in the case of N and CC oranges completely recovered basal levels.

Accordingly, pretreatment of HLEC cells for 48 h with the carotenoids lutein and zeaxanthin at 5 μ M completely prevented the H₂O₂ (100 μ M/1 h) induced lipid peroxidation (Gao et al. 2011). Similarly, pretreatment of HepG2 cells for 24 h with sweet orange peel extracts (50-500 μ g/mL) decreased the percentage of lipid peroxidation produced by t-BuOOH (0.2 mM/6 h) though not reaching control basal levels; however, hesperetin and nobiletin but not hesperidin (at 10 μ g/mL) completely prevented lipid peroxidation as in control cells (Chen et al. 2012). In agreement, the increase in lipid peroxidation in A549 cells caused by H₂O₂ (200 μ M/2 h) was

counteracted by 18 h pretreatment with the flavonoid fraction of bergamot and orange juices (25 and 50 µg/mL) without reaching control basal levels (Ferlazzo et al. 2015). The antioxidant action of bioactive compounds such as polyphenols and carotenoids depends on both the radical-scavenging ability of aqueous peroxy radicals at the surface of the membrane, and of lipid peroxy radicals within the membrane. Therefore, the hydrophilicity/hydrophobicity of the bioactive compounds could result an essential factor for the resulting cytoprotective activity (Deiana et al. 2012). In this sense, although comparable results were found in the four samples analyzed, the two varieties of oranges (N and CC) displayed more efficiently the protection against lipid peroxidation, reaching basal levels, probably due to their higher content in total polyphenols than mandarins (see **table 1**). Since main polyphenols determined in the BF of citrus fruit pulps comprise glucosides and rutosides, they mainly would act at the surface of the membrane due to the hydrophilicity conferred by the sugar moiety. Other compounds that could contribute to increase the protective action against lipid peroxidation by oranges BFs are the high concentration of ascorbic acid and total carotenoids found in N and CC, respectively.

3.4 Intracellular ROS accumulation

As an index of the overall oxidative stress in living cells, the evaluation of intracellular ROS is useful to gain insights of the redox cell state (Cilla et al., 2008). Intracellular ROS accumulation in cell cultures preincubated with BF of citrus fruit pulps with/without oxidative stress are shown in **figure 3**.

Treatment of Caco-2 cells with BF of samples without oxidative stress was not overtly toxic since no difference was found comparing with control cells. ROS generation, in turn, significantly ($p<0.05$) increased approximately ten-fold when the

cells were challenged with H_2O_2 (22.55 ± 3.13) *versus* control cells (2.53 ± 0.84). When the cells were pretreated with BF of citrus fruit pulps and subsequently exposed to oxidative stress, ROS levels were significantly decreased ($p < 0.05$) in M and M12 mandarins and CC oranges *versus* control stress cells, though without reaching their counterparts without stress and control basal levels. The absence of effect observed in N orange could be attributed to its lowest carotenoid (five-, ten- and fifteen-fold than M, M12 and CC, respectively) and total bioactive content than the other samples. This means that the mechanism of action of bioactive compounds present in the BF of citrus fruit pulps did not act mainly through ROS quenching.

In tune with the present results, pretreatment of HepG2 cells for 24 h with sweet orange peel extracts (50-500 $\mu\text{g/mL}$) and flavonoids hesperidin, hesperetin and nobiletin (10- $\mu\text{g/mL}$) decreased ROS levels evoked by t-BuOOH (0.2 mM/6 h) though not reaching control basal levels ([Chen et al. 2012](#)). In the same line, pretreatment 6 h with citrus flavanones hesperetin, hesperidin and neohesperidin (0.8-50 μM) protected PC12 cells from H_2O_2 (400 μM /16 h) increase in ROS levels, however in some cases not reaching control basal levels ([Hwang et al. 2008](#)). Similarly, the pretreatment 18 h with flavonoid fraction of bergamot and orange juices (25 and 50 $\mu\text{g/mL}$) lessened the ROS production caused by H_2O_2 (200 μM /2 h) in A549 cells ([Ferlazzo et al. 2015](#)). In agreement, juice powders of sweet orange and unshiu mikan citrus fruit (50-100 $\mu\text{g/mL}$) and their key flavonoids hesperidin, rutin and narirutin (2.5-100 μM) pretreatment for 24 h dose-dependently inhibited the ROS generation induced by H_2O_2 (200 μM /2h) in HepG2 cells ([Jannat et al. 2016](#)). A dose-dependent inhibition of intracellular ROS (20-90%) evoked by t-BuOOH (3 mM/3 h) has also been indicated in Caco-2 cells incubated 24 h with blood orange extracts (6.25-50ng/mL) ([Tarozzi et al. 2006](#)). On the

contrary, other studies have shown a failure in the inhibition of intracellular ROS accumulation in H₂O₂-injured Caco-2 cells as in the studies where the cells were subjected to a 24 h pretreatment with β -carotene (0.1-50 μ M) (Bestwick et al. 1999) or with a fruit beverages made of grape-orange-apricot (~50 μ M total phenolics) (Cilla et al. 2008). Likewise, pretreatment 20 h of HepG2 cells with phloroglucinol (4-400 μ M), coffee melanoidin (0.5 μ g/mL) or a selenium derivative (1 μ M) did not prevent ROS production caused by t-BuOOH (Goya et al. 2009; Quéguineur, Goya, Ramos, Martín, Mateos & Bravo, 2012). Nevertheless, in these studies in which antioxidants failed to protect against ROS generation, they were able to show a general cytoprotective effect by other mechanisms different to direct ROS scavenging, including viability maintenance and prevention of lipid peroxidation.

3.5 Mitochondrial membrane potential changes

Mitochondria dysfunction, generally due to an increase in ROS levels and a decrease in GSH content, is manifested by a decrease in mitochondrial membrane potential and reduced ATP generation (Prasad, Ram, Sawhney, Ilavazhagan & Banerjee, 2007). Thus, measurement of $\Delta\psi_m$ allows us to ascertain whether mitochondria have suffered oxidative disturbances. $\Delta\psi_m$ in cell cultures preincubated with BF of citrus fruit pulps with/without oxidative stress are shown in **figure 4**.

Pretreatment of Caco-2 cells 24 h with BF of samples without oxidative stress showed no statistical ($p>0.05$) modifications in $\Delta\psi_m$ compared with control cells, with the exception of CC orange which presented a slight decrease ($p<0.05$) in mitochondrial membrane potential (concluded from the increased proportion of PI versus DHR double negative cells (PI/DHR, %) with respect to control cells). Exposure of Caco-2 cells to H₂O₂ (5 mM/2 h) resulted in a 15-fold drop ($p<0.05$) of mitochondrial membrane

potential (5.63 ± 0.94) *versus* control cells (0.37 ± 0.14). However, pretreatment with BF of citrus fruit pulps and further oxidative stress resulted in a prevention of mitochondrial membrane potential dissipation *versus* control stress cells ($p < 0.05$), though not reaching control basal levels. M12 mandarin was the least effective sample in preserving $\Delta\Psi_m$, while CC orange was able to reduce changes in $\Delta\Psi_m$ to levels found in the same sample without oxidative stress.

The results in the present study are coincident with those of [Chen et al. \(2012\)](#) who reported that pretreatment 24 h with sweet orange peel extracts (50-500 $\mu\text{g/mL}$) and flavonoids hesperidin, hesperetin and nobiletin (10 $\mu\text{g/mL}$) partially prevented $\Delta\Psi_m$ evoked by t-BuOOH (0.2 mM/6 h) though not reaching control basal levels. Similar results were quoted with pretreatment 6 h with citrus flavanones hesperetin, hesperidin and neohesperidin (0.8-50 μM) and further H_2O_2 (400 μM /16 h) induced stress in PC12 cells ([Hwang et al. 2008](#)). In consonance, pretreatment 18 h with the flavonoid fraction of bergamot and orange juices (25 and 50 $\mu\text{g/mL}$) lessened the H_2O_2 (200 μM /2h) induced mitochondrial impairment in A549 cells ([Ferlazzo et al. 2015](#)). A complete prevention of mitochondrial membrane dissipation, in turn, was found with pretreatment 24 h with BF of fruit beverages made of grape-orange-apricot (~ 50 μM total phenolics) in Caco-2 cells against H_2O_2 -induced oxidative stress (5 mM/2 h) ([Cilla et al. 2008](#)). Excessive ROS generation may involve, in the end, apoptotic and necrotic cell death. Thus, changes of $\Delta\Psi_m$ may provide an early indication of the initiation of cellular apoptosis, since programmed cell death is accompanied by the signs of mitochondrial dysfunction including, among others, a loss in inner mitochondrial transmembrane potential, which can be prevented by antioxidants ([Chen et al. 2012](#)). Our findings let us to hypothesize that BF of citrus fruit pulps may behave as cytoprotectants through

additional mechanisms such as modulation of intracellular signaling cascades involved in cell apoptosis.

3.6 Cell cycle progression

Cell cycle implies the sequential transition from one phase of the cycle to another, with checkpoints that control the fidelity in this progress (Burhans & Heintz, 2009). The extent of oxidative stress determines the cell fate leading to cell cycle arrest and DNA repair or the activation of apoptotic pathways (Barzilai & Yamamoto, 2004). In addition, peroxides have been found to initiate checkpoint arrest in several cell types with H₂O₂ inducing a G1 checkpoint response that could be attenuated by antioxidants (Clopton & Saltman, 1995). Oxidative stress-induced alterations in cell cycle distribution in cell cultures preincubated with BF of citrus fruit pulps with/without oxidative stress are shown in **figure 5**.

Pretreatment of Caco-2 cells with BF of samples in the absence of oxidant did not affect the different phases of cell cycle compared to control cells. On the contrary, H₂O₂ evoked a marked decrease (12.8%) in the G1 phase population accompanied by a sharp increase in sub-G1 phase ($8.50 \pm 0.90\%$) compared to control cells ($1.62 \pm 0.55\%$). This increase in sub-G1 from H₂O₂-induced oxidative stress is commonly, though not exclusively, associated with DNA fragmentation occurring during apoptosis (García-Nebot et al. 2011). The treatment of cells with BF of citrus fruit pulps and subsequent oxidative insult showed that all samples were able to restore the proportion of cells in G1 phase and to avoid the rise in sub-G1 (probably related to apoptosis) compared to control stress cells, almost mirroring the same distribution of DNA in all phases of cell cycle as in control cells.

Similar results were reported for BF of fruit beverages made of grape-orange-apricot which partially prevented the decrease in G1 phase and the increase in sub-G1 cell population in Caco-2 cells challenged with H₂O₂ (5 mM/2 h) (Cilla et al. 2011b).

The present results point out that the cascade of events involving lipid peroxidation of cell membranes, increase in ROS levels and mitochondrial dysfunction that may lead to cell cycle perturbation through a reduction in the proportion of cells in G1 phase and the rise in sub-G1 phase due to oxidative stress, were almost completely prevented by bioactive compounds present in BF of citrus fruit pulps allowing the maintenance of viability of Caco-2 cells.

On the other hand, it is of mention to highlight that the results of cytoprotection of the present study with differentiated Caco-2 cells can be more physiologically relevant than others performed with non differentiated cells of cancer origin. In fact, human colon carcinoma Caco-2 cells grown in culture undergo enterocytic differentiation to form a polarized monolayer closely mimicking both morphologically and functionally the human small intestinal epithelium, serving as a suitable model for evaluating the physiological response of enterocytes to oxidative injury (Manna, Galletti, Cucciolla, Molledo, Leone & Zappia, 1997). In this sense, it is known that the redox status of cancer cells is different than those of differentiated ones, making undifferentiated cancer cells more sensitive towards toxicity. For instance, it has been reported that during differentiation, HL-60 cells acquire more resistance to oxidative stress, increasing the content and/or the activity of antioxidants and modifying their cell distribution (Palozza et al. 2002). In line with this, it has been described that in rat colon cancer cells there is enhanced oxidative stress that leads to overproduction of ROS and even induction of endogenous antioxidants (SOD, CAT and GPx) which in turn contribute to pro-tumorigenic signaling pathways substantiating the maintenance of

redox homeostasis and survival advantage by transformed cancer cells (Sharma, Chellappan, Chinnaswamy & Nagarajan, 2017). Moreover, paradoxical effects of antioxidants in cancer have been related, with antioxidants decreasing ROS levels and strikingly increasing the proliferation of cancer cells which results in greater tumor burdens and reduced survival (Mendelsohn & Larrick, 2014). Therefore, the biological context in cytoprotection assays (use of differentiated cells as a more physiological *in vivo* situation instead of undifferentiated cancer cells) may be key and desirable in predicting the action of antioxidants in pre-clinical cell culture models.

4. Conclusions

The study of mechanisms involved in cell injury evoked by oxidative stress as well as the evaluation of biomarkers of cellular cytoprotection could help to identify functional foods or related bioactive compounds with the aim to establish potential strategies for the prevention or reduction of oxidative stress related diseases. In the present study, BF of citrus fruit pulps from Navel and Cara Cara oranges, and Clementine mandarin freshly harvested and postharvest stored, protected Caco-2 cells from H₂O₂-induced oxidative stress at luminal physiological concentrations by preserving cell viability and correct cell cycle progression. Also, BF of citrus pulps preserved partially mitochondrial membrane potential, diminished lipid peroxidation and partially reduced ROS production. The mechanism of action can be mainly related to the abolition of lipid peroxidation protecting the cell lipid bilayer, possibly through binding of specific bioactive components on both side of the bilayer and modulation of intracellular signaling cascades involved in cell apoptosis, rather than direct free radical scavenging activity. Remarkable differences in the concentration of bioactive compounds among samples (higher in Clementine mandarin postharvest stored 5 weeks at 12 °C and in Cara Cara orange than their respective counterparts), in general, did not

translate into any significant difference in their cytoprotective activity, supporting the opinion that total bioactive compounds are not essential for the antioxidant and cytoprotective activity of citrus fruit pulps.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Figure captions

Figure 1. Effects of BF of citrus fruit pulps from freshly harvested (M) and postharvest stored at 12°C (M12) Clementine mandarins and from Navel (N) and Cara Cara (CC) oranges, with/without oxidative stress on MTT conversion percentages in Caco-2 cells. The cells were incubated with BF (1:6 v/v in DMEM) for 24 hours and then treated or not with H₂O₂ 5 mM for 2 hours. Cell cultures exposed to culture media were considered as controls. Values are expressed as means ± standard deviation ($n=4$) from at least two independent experiments. Different superscript letters (a-c) denote statistical significant differences among treatments ($p<0.05$).

Figure 2. Effects of BF of citrus fruit pulps from freshly harvested (M) and postharvest stored at 12°C (M12) Clementine mandarins and from Navel (N) and Cara Cara (CC) oranges, with/without oxidative stress on intracellular MDA levels in Caco-2 cells. The cells were incubated with BF (1:6 v/v in DMEM) for 24 hours and then treated or not with H₂O₂ 5 mM for 2 hours. Cell cultures exposed to culture media were considered as controls. Values are expressed as means ± standard deviation ($n=4$) from at least two independent experiments. Different superscript letters (a-c) denote statistical significant differences among treatments ($p<0.05$).

Figure 3. Effects of BF of citrus fruit pulps from freshly harvested (M) and postharvest stored at 12°C (M12) Clementine mandarins and from Navel (N) and Cara Cara (CC) oranges, with/without oxidative stress on intracellular reactive oxygen species (ROS) production in Caco-2 cells. The cells were incubated with BF (1:6 v/v in DMEM) for 24 hours and then treated or not with H₂O₂ 5 mM for 2 hours. Cell cultures exposed to culture media were considered as controls. Values are expressed as means ± standard

deviation ($n=4$) from at least two independent experiments. Different superscript letters (a-c) denote statistical significant differences among treatments ($p<0.05$).

Figure 4. Effects of BF of citrus fruit pulps from freshly harvested (M) and postharvest stored at 12°C (M12) Clementine mandarins and from Navel (N) and Cara Cara (CC) oranges, with/without oxidative stress on changes in mitochondrial membrane potential ($\Delta\Psi_m$) in Caco-2 cells. The cells were incubated with BF (1:6 v/v in DMEM) for 24 hours and then treated or not with H_2O_2 5 mM for 2 hours. Cell cultures exposed to culture media were considered as controls. Values are expressed as means $\pm$ standard deviation ($n=4$) from at least two independent experiments. Different superscript letters (a-c) denote statistical significant differences among treatments ($p<0.05$).

Figure 5. Effects of BF of citrus fruit pulps from freshly harvested (M) and postharvest stored at 12°C (M12) Clementine mandarins and from Navel (N) and Cara Cara (CC) oranges, with/without oxidative stress on cell cycle phase distribution of Caco-2 cells. The cells were incubated with BF (1:6 v/v in DMEM) for 24 hours and then treated or not with H_2O_2 5 mM for 2 hours. Cell cultures exposed to culture media were considered as controls. (A) Values are expressed as means $\pm$ standard deviation ($n=4$) from at least two independent experiments. Different superscript letters (a-c) denote statistical significant differences among treatments ($p<0.05$). (B) Representative histograms of cell cycle analysis for each treatment.

Table 1. Concentration of major bioactive compounds (μM) and total antioxidant capacity (μM AAE/100 g) present in the bioaccessible fractions of citrus fruit pulps used for cytoprotective effects in Caco-2 cells (diluted 1:6 v/v in DMEM), calculated from previous data (Rodrigo et al. 2015^A; De Ancos et al. 2017^B).

Bioactive compound	M	M12	N	CC
<u>Carotenoids^A</u>				
Phytoene	62.7 $\pm$ 7.7 ^a	105.2 $\pm$ 28.5 ^a	17.1 $\pm$ 4.3 ^b	1011.7 $\pm$ 44.7 ^c
Phytofluene	n.d.	n.d.	traces	139.4 $\pm$ 32.9
β -carotene	traces	33.8 $\pm$ 0.9 ^a	n.d.	3.1 $\pm$ 1.3 ^b
Lycopene	n.d.	n.d.	n.d.	23.0 $\pm$ 1.6
β -cryptoxanthin	232.4 $\pm$ 71.8 ^a	564.4 $\pm$ 20.8 ^b	12.1 $\pm$ 2.1 ^c	6.3 $\pm$ 0.9 ^d
Zeaxanthin	10.0 $\pm$ 1.8 ^a	23.4 $\pm$ 1.8 ^b	traces	traces
Antheraxanthin	39.0 $\pm$ 8.3 ^a	73.5 $\pm$ 5.7 ^b	6.3 $\pm$ 1.4 ^c	8.3 $\pm$ 1.1 ^c
All-E-Violaxanthin	13.9 $\pm$ 2.5 ^a	30.0 $\pm$ 3.6 ^b	10.8 $\pm$ 1.7 ^c	6.9 $\pm$ 2.0 ^c
9-Z-Violaxanthin	60.5 $\pm$ 16.4 ^a	115.7 $\pm$ 3.3 ^b	42.7 $\pm$ 12.5 ^c	37.7 $\pm$ 1.4 ^c
Total carotenoids	418.5 $\pm$ 15.8 ^a	946.0 $\pm$ 9.2 ^b	89.0 $\pm$ 4.4 ^c	1236.4 $\pm$ 9.6 ^d
<u>Polyphenols^B</u>				
Apigenin 6,8-di-C-glucoside	n.d.	n.d.	5.0 $\pm$ 0.1 ^a	7.6 $\pm$ 1.3 ^b
Naringenin-7-O-glucoside	1.4 $\pm$ 0.03 ^a	3.2 $\pm$ 0.01 ^b	9.7 $\pm$ 0.3 ^c	12.1 $\pm$ 3.1 ^d
Eriocitrin	n.d.	n.d.	1.4 $\pm$ 0.02 ^a	2.7 $\pm$ 0.01 ^b
Rutin	0.28 $\pm$ 0.01 ^a	0.76 $\pm$ 0.04 ^b	0.78 $\pm$ 0.01 ^b	1.2 $\pm$ 0.02 ^c
Narirutin	1.0 $\pm$ 0.01 ^a	3.3 $\pm$ 0.01 ^b	17.5 $\pm$ 2.4 ^c	18.8 $\pm$ 3.4 ^c
Hesperidin	3.3 $\pm$ 0.1 ^a	8.6 $\pm$ 0.9 ^b	40.7 $\pm$ 7.2 ^c	70.0 $\pm$ 9.8 ^d
Dydimin	0.10 $\pm$ 0.02 ^a	0.90 $\pm$ 0.03 ^b	3.3 $\pm$ 0.03 ^c	2.8 $\pm$ 0.3 ^c
Total polyphenols	6.1 $\pm$ 0.1 ^a	16.8 $\pm$ 0.3 ^b	78.4 $\pm$ 2.0 ^c	115.2 $\pm$ 3.6 ^d
<u>Ascorbic acid^B</u>	314.0 $\pm$ 8.9 ^a	290.7 $\pm$ 2.6 ^a	366.2 $\pm$ 3.5 ^b	292.4 $\pm$ 0.6 ^a
<u>Total bioactive compounds</u>	738.6 $\pm$ 6.5 ^a	1253.5 $\pm$ 3.4 ^b	533.6 $\pm$ 5.1 ^c	1644.0 $\pm$ 5.3 ^d
<u>Total antioxidant capacity^B</u>				
ABTS ⁺	57.4 $\pm$ 0.6 ^a	60.0 $\pm$ 0.6 ^b	58.8 $\pm$ 1.3 ^{ab}	59.1 $\pm$ 0.2 ^{ab}
FRAP	23.5 $\pm$ 0.9 ^a	25.1 $\pm$ 1.8 ^a	31.2 $\pm$ 0.6 ^b	28.2 $\pm$ 2.3 ^b
DPPH [*]	11.6 $\pm$ 0.3 ^a	16.8 $\pm$ 1.1 ^b	18.4 $\pm$ 0.9 ^b	23.7 $\pm$ 0.4 ^c

769 M: freshly harvested Clementine mandarin. M12: Clementine mandarin postharvest stored at 12°C. N: Navel
770 orange. CC: CaraCara orange. n.d. not detected. Total bioactive compounds: sum of

771 carotenoids+polyphenols+ascorbic acid. Different superscript letters on the same line (a-d) indicate significant
772 differences among samples for a specific bioactive compound ($p<0.05$).

773

Figure 1. Cilla et al.

Figure 2. Cilla et al.

Figure 3. Cilla et al.

Figure 4. Cilla et al.

Figure 5. Cilla et al.

(A)

(B)