Impact of high-pressure processing on the stability and bioaccessibility of bioactive compounds of Clementine mandarin juice and its cytoprotective effect in Caco-2 cells

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ABSTRACT

Mandarin juice is a rich source of antioxidant bioactive compounds. While the content and profile of bioactives is known, the impact of high-pressure processing (HPP) on their stability and bioaccessibility (BA) is unknown, but may allow obtaining safe, nutritive fresh-tasting and with high extractability of bioactive compounds juices. Stability and BA of bioactive antioxidant compounds from Clementine mandarin juices untreated and HPP-treated (400 MPa/40° C/1 min), as well as the cytoprotective effect of its bioaccessible fractions (BF) obtained after simulated gastrointestinal digestion against H₂O₂-induced oxidative stress in differentiated Caco-2 cells was investigated. HPP-treatment provided BF with a better preservation of carotenoids, flavonoids, ascorbic acid, total polyphenols and FRAP value, and slightly higher cytoprotection (mitochondrial membrane potential and ROS) versus untreated juices. Therefore, HPP can be recommended as a suitable technology to retain or indeed increase antioxidant bioactives and its cytoprotective activity in mandarin juices after a gastrointestinal digestion.

Keywords: Mandarin juice, bioactive compounds, high-pressure processing, bioaccessibility, Caco-2 cells, cytoprotection.

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52 **1. Introduction**

53 Clementine mandarin (*Citrus clementina* Hort. ex Tanaka) is one of the main groups of
54 mandarin cultivars in the world, especially for the fresh-fruit market. Among the different
55 Clementine mandarins, Clemenules, a mutation of Clementine Fina identified in Castellón
56 (Spain), is the most important cultivar in terms of production in the Mediterranean Basin.¹
57 Clemenules is a midterm season mandarin, producing seedless, easy-peeling fruits of excellent
58 organoleptic quality by their juicy and sweet taste and attractive aromatic flavor, being highly
59 appreciated by the consumers for fresh consumption that increasingly claim more derivatives as
60 juices.^{2,3} Mandarin juices have been reported to confer health-benefits related to different
61 factors. Some examples refer to improvement of antioxidant status and reduction of oxidative
62 biomarkers in hypercholesterolemic and obese children,^{4,5} anticancer activity in preclinical
63 studies,⁶ visceral fat reduction in obese-diabetes mice,⁷ prevention of age-related cognitive
64 dysfunction and oxidative damage in senescence-accelerated mouse brain⁸, and anti-diabetic
65 action improving oxidative stress in streptozotocin-induced diabetic rat liver.⁹ The compounds
66 responsible of these health benefits in mandarin juices are mainly vitamin C (vitC), carotenoids
67 (primarily β -cryptoxanthin, but also zeaxanthin, β -carotene, phytoene and phytofluene) and
68 flavonoids (hesperidin, narirutin, eriocitrin, vicenin 2, rutin and dydimin). They may act in
69 concert (additively or synergistically), which can contribute to their notable antioxidant capacity
70 (AC).^{2,10-13} Moreover, it is important to remark that the concentration of these bioactive
71 compounds in citrus juices depends on many factors such as fruit cultivar, maturity stage, pre-
72 and postharvest conditions, and processing conditions.^{14,15}

73 The use of heat through thermal processing technologies (pasteurization, sterilization, etc.),
74 is still a common practice of the food industries in order to guarantee the microbial inactivation
75 but have a significant negative impact on sensory, nutritional and functional properties of plant
76 derived products. Non-thermal processing technologies such as high-pressure processing (HPP)
77 has been proposed as an alternative to the traditional thermal processing systems. With this

technology can be obtained safe, nutritive fresh-tasting plant-derived products, avoiding the degradation of nutrients and bioactive compounds, as well as inhibiting microbial growth and enzyme activity¹⁶. Moreover, the application of HPP on fruit juices, beverages, and other plant food derived products, could produce an increase of the extractability of bioactive compounds due to changes in the food matrix and in the cell wall permeability and in consequence their bioaccessibility could also be improved.^{17,18}

High-pressure processing (HPP) has been proposed to produce high-quality, safe and fresh-like mandarin juices by promoting microbiological and enzymatic inactivation and preserving the compounds responsible of its sensory quality and thus prolonging its stability and shelf-life.^{2,19} However, the effect of HPP on mandarin juice bioactive compounds and their bioaccessibility has been scarcely studied. In fact, only information about the effect of low-pressure homogenization (up to 100 MPa) on antioxidant characteristics and bioactive compounds of low-pulp mandarin juice have been found^{20,21}. The application of this processing system did not have negative effects on the antiradical activity (DPPH[•] scavenging capacity assay) or even slightly increased it at 20-30 MPa (ABTS^{•+} scavenging capacity assay).²⁰ In addition, Betoret et al.²¹ also reported that low homogenization pressure at 20 or 100 MPa has diverse impact on bioactive compounds in low-pulp mandarin juice. In fact, the lower treatment (20 MPa) may increase the levels of hesperidin and total AC (DPPH[•] and ABTS^{•+} methods), and maintain vitC, while at higher pressure (100 MPa) increased hesperidin content and ABTS^{•+} values, maintained DPPH[•] values and decreased vitC content. Sentandreu et al²² analyzed a minimally processed mandarin cv Ortanique juice pre-heated at 31 °C and processed by high-pressure homogenization (150 MPa) and observed that neither the flavonoid composition (flavones and flavanones) nor their bioaccessibilities were modified by the processing system employed. In addition, Di Nunzio et al²³ have also recently reported in mandarin cv Ortanique juice pasteurized (63°C/15 s) and processed by low-pressure homogenization (20 MPa) that some flavonoids (hesperidin, narirutin and didymin) decreased after processing but reached higher bioaccessibility compared to the only pasteurized juices.

Another important aspect when considering the nutritional quality and bioactivity in foods is to get information about its bioaccessibility (BA), defined as the amount of a food component released from the food matrix which is the maximum available for absorption . In the case of mandarins this information is very scarce (reviewed by Putnik et al.²). In this context, our research group previously showed that β -cryptoxanthin and phytoene were the main carotenoids in the bioaccessible fraction (BF) of both Clemenules mandarin pulp and fresh juice and that the former had 2-fold higher relative BA of total carotenoids than the corresponding juice.¹¹ Likewise, higher relative BA of ascorbic acid (AA), total polyphenols, total flavonoids and AC (FRAP), but lower AC (TEAC) and (DPPH[•]), were found for Clemenules mandarin pulp compared to fresh juice, respectively.¹² In addition, to obtain a better approach of the *in vivo* scenario, the bioactivity of the abovementioned BF of Clemenules mandarin pulps was tested for cytoprotection in differentiated Caco-2 intestinal-like cells against H₂O₂-induced oxidative stress. The BF of Clemenules mandarin pulps can preserve cell viability and correct cell cycle progression, diminish lipid peroxidation, and partially preserve mitochondrial membrane potential and reduce ROS production compared to the oxidative stress control cells.²⁴

Based on the previous background, the objective of the present study was to analyze the impact of a non-thermal food technology, as high hydrostatic pressure, on the stability and BA of main antioxidant bioactive compounds, such as carotenoids, flavonoids, total AC and vitC, in Clemenules mandarin juice. Moreover, the cytoprotective effect of its BF, obtained after simulated gastrointestinal digestion, against H₂O₂-induced oxidative stress in differentiated Caco-2 cells was investigated.

2. Materials and methods

2.1.Reagents

All reagents used were of analytical grade and were the same as employed in Cilla et al.²⁴

2.2.Samples

Clementine mandarin fruits (*Citrus clementina* cv Clemenules) were harvested at full maturity stage (pH, soluble solid, acidity and ripening index were 3.58, 9.26 °Brix, 0.50 % and 18.52, respectively) from adult trees from a commercial orchard located in Lliria (Valencia, Spain). Immediately after harvest, fruits were selected for uniformity and free of any external damage, and then delivered to ICTAN (Madrid, Spain) arriving within the next 24h and being stored at 2 °C during two days until processing.

Figure 1 shows the flow diagram of the experimental design. Clementine mandarin juices with low proportion of pulp (8%, w/v) were obtained from 20 whole peeled fruits using a domestic jar-blender (Oster 4655, USA) during 6 s, filtered using a 2 mm steel sieves and packed in replicate samples of 250 mL in high barrier flexible Doypack plastic bags (Polyskin XL, Amcor Flexiblehispania, Granollers, Spain) removing the air from the headspace.

2.3. High-pressure processing (HPP)

Processing design. The mandarin juice bags prepared as shown in the previous section were divided in two lots that were labelled as: Untreated (for freshly prepared mandarin juice) or HPP-400 (for mandarin juice processed at 400 MPa at 40 °C for 1 minute) (Figure 1).

Treatment conditions. HPP-400 samples were introduced in the pressure unit vessel (two bags for each cycle) filled with pressure medium (water) and treated at 400 MPa at 40 °C during 1 min. The hydrostatic pressure unit was formed by a vessel of 1925 mL capacity and it was able to work to a maximum pressure of 800 MPa and a maximum temperature of 70 °C (High Pressure Iso-Lab System, Model FPG7100:9/2C, Stansted Fluid Power LTD., Essex, UK). The compression and decompression rates were both 3 MPa/s. Because of adiabatic compression, the maximum temperature in the vessel was 40 °C at 400 MPa. Pressure, time and temperature were controlled by a computer program, being constantly monitored and recorded during the process. HPP at 400 MPa/40 °C/1 min has been employed in the present study based on the satisfactory previous results obtained in terms of microbial inactivation and increased bioactive compounds extraction from orange juice alone or as part of fruit-milk beverages.^{17,25}

2.4. In vitro gastrointestinal digestion

An *in vitro* gastrointestinal digestion procedure mimicking the physiological situation in the upper digestive tract including gastric and intestinal steps was performed. The BF obtained after centrifugation was used to evaluate the BA of carotenoids, vitC, flavonoids and hydrophilic antioxidant capacity according to the procedure described by Rodrigo et al.¹¹ BA was calculated as follows: 100 x (content in BF /content in non-digested sample).

2.5. Carotenoids determination

Carotenoids from Clementine mandarin juices were extracted as described.¹¹ Two ml were weighted in polypropylene tubes and 4 mL of MeOH plus 5 mL Tris-HCl (50 mM, pH 7.5) (containing 1 M NaCl) were added. The samples were sonicated 5 min in XUBA3 ultrasonic water bath at room temperature (35 ET AL; Grant Instruments, Cambridge, England). Ten ml of dichloromethane (DCM) were added, stirred for 5 min at 4°C and centrifuged at 3000 x g for 10 min at 4°C. The organic phase was recovered and the aqueous phase re-extracted with DCM until it was colorless. The pooled organic phases were dried on a rotary evaporator at 35°C. Samples were saponified in methanolic KOH (6%, w/v) overnight. Saponified carotenoids were re-extracted with petroleum ether:diethyl ether (9:1) and the pooled organic phase were dried and kept at -20°C until HPLC analysis. The carotenoid profile was analyzed by HPLC with a liquid chromatography system equipped with a 600E pump and a model 2998 DAD, and Empower3 software (Waters, Barcelona, Spain). A C30 carotenoid column (250 x 4.6 mm, 5 µm) coupled to a C30 guard column (20 x 4.0 mm, 5 µm) (YMC, Teknokroma, Spain) was used. Chromatographic conditions used, as well as identification and quantification of carotenoids are described in Rodrigo et al.¹¹ Briefly, the carotenoid peaks were integrated at their individual maximal wavelength (MaxPlot), and their contents were calculated using the appropriate calibration curves, as described elsewhere.¹¹ The carotenoids selected for quantification represented more than 90% of the peak area in the chromatograms.

2.6. Ascorbic acid determination

AA and vitC were extracted and quantified in 10 mL of mandarin juice by HPLC according to the procedure described by Cilla et al.¹⁸

2.7. Flavonoids determination

Flavonoids were extracted, separated, identified, and quantified by HPLC-DAD and HPLC-ESI-QTOF-MS from orange juice according to the procedure described by De Ancos et al.¹² with some modifications. Samples of 20 mL of mandarin juice with pulp were centrifuged at 9000×g for 15 min at 4 °C in a refrigerated centrifuge (Thermo Scientific Sorvall, mod. Evolution RC, Thermo Fisher Scientific Inc., USA) and the supernatant was separated. Then, 4 mL of this solution was loaded on a reversed phase C18 Sep-pack cartridge (200 mg of silica based bonded phase, 37x55 µm particle size) (Waters, USA), previously activated with 4 mL of methanol and 4 mL of water. Phenolic compounds were recovered from the cartridge by eluting with 2 mL of methanol and filtered through a 0.45µm syringe filter and stored at -80 °C until HPLC-DAD and HPLC-ESI-QTOF-MS analysis were carried out.

Results were expressed as mg of flavonoid per 100 mL of mandarin juice. Total flavonoids content was determined as the sum of individual compounds quantified by HPLC-DAD (TF-HPLC).

2.8. Antioxidant capacity determinations

Total AC was determined from 10 mL of mandarin juice according to the method of Re et al.²⁶ (for ABTS^{•+}), the method of Benzie & Strain²⁷ (for FRAP), the method of Sánchez-Moreno et al.²⁸ (for DPPH[•]), including an adaptation of the methods to 96-well microplate format as previously described by De Ancos et al.¹² Also, total phenolic compounds performed according to the Folin-Ciocalteu procedure (TP-FC) was used to quantify the sample's reducing capacity according to the procedure described by De Ancos et al.¹²

2.9. Cell culture cytoprotection assays

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 70 and 85. The cells were maintained and grown as previously described.²⁴ 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. Differentiated cells at seven days post-seeding (with transepithelial electrical resistance (TEER) values higher than $400 \Omega \times \text{cm}^2$ and formation of domes) were used in the cytoprotection assays. 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 BF of samples added in 1:6 proportion (v/v) with culture medium to preserve cell viability and ensure they were not cytotoxic. 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).

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 reported²⁴ by spectrophotometry.

Lipid peroxidation

Lipid peroxidation was measured as described by Cilla et al.²⁴ based on the cellular content of malondialdehyde (MDA) by spectrophotometry.

Intracellular reactive oxygen species (ROS)

The intracellular accumulation of ROS in Caco-2 cells was evaluated by flow cytometry using dihydrorhodamine (DHR) solution prepared in dimethyl sulfoxide, which is oxidized to fluorescent rhodamine, as previously described.²⁴

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

Mitochondrial membrane potential ($\Delta\psi_m$) changes were evaluated using DHR and propidium iodide (PI) double labeling by flow cytometry, as previously determined by Cilla et al.²⁴

DNA distribution in each phase of the cell cycle was determined by flow cytometry using RNase and labeling with PI according to Cilla et al.²⁴

2.10. Statistical analysis

The results shown represent mean values $\pm$ standard deviation of three replicates obtained in at least two separate experiments. One-way (type of sample) ANOVA was conducted followed by the Tukey *post hoc* test and Student's t test were used to compare pairs of means and determine statistical significance at the $P < 0.05$ level. The correlations within variables were examined by Pearson correlation. All analyses were performed by using the IBM SPSS Statistics 22 Core System (SPSS Inc, an IBM Company).

3. Results and discussion

3.1. Effect of HPP on the stability and BA of carotenoids in mandarin juices

The carotenoid profile in untreated non-digested juice of Clemenules mandarin (**Table 1**) is consistent with that reported in previous works.^{11,29} A total of 15 chromatographic peaks with a carotenoid-like spectra were detected in the juices and the most abundant or with relevance for nutrition and health were quantified.¹¹ The carotenoids analyzed in each sample represented more than 90% of the total area. The β -xanthophylls accounted for 615.7 ± 10.5 $\mu\text{g}/100$ ml which corresponds to 75% of the total content; being the β -cryptoxanthin with 374.5 ± 22.1 $\mu\text{g}/100$ ml the most abundant representing 45% of total carotenoids. The significant proportion of β -cryptoxanthin in mandarins has been previously pointed out since only a very limited number of fruits and vegetables accumulate this xanthophyll, and citrus juices are one of the main dietary sources.^{29,30} β -Carotene and zeaxanthin, carotenoids of nutritional and clinical relevance, accumulated in relative low amounts of approximately 20 $\mu\text{g}/100$ ml which represents about 2% of the total content. The linear carotenes, phytoene (70.8 ± 5.2 $\mu\text{g}/100$ ml) and phytofluene (42.3 ± 3.2 $\mu\text{g}/100$ ml) accounted together nearly 14% of the total amount. Other xanthophylls

present in the juice, antheraxanthin and violaxanthin (all-*E* and 9-*Z* isomers), with nearly 200 µg/100 ml, represented about 26% of the total (**Table 1**).

The HPP at 400 MPa/40°C/1 min (HPP-400) did not affect the qualitative carotenoid composition but reduced approximately 30% the total carotenoids content. This reduction is in line with the results obtained in a recent work with a high-pressure homogenization (HPH, 150 MPa) treatment in the mandarin Ortanique juice caused a reduction of 40% of total carotenoid levels compared to fresh juice.²²

Interestingly, the HPP-400 treatment did not affect the individual carotenoids in the same proportion (**Table 1**). Thus, phytoene, phytofluene and, particularly, β-carotene, were reduced between half and 10-times in HPP juice compared to untreated. The content of β,β-xanthophylls was less affected than carotenes by HPP-400 treatment and concentration of the main xanthophyll, β-cryptoxanthin, was 258±11.6 µg/100 ml in HPP-400 juice, about 31% reduction compared to untreated juice. Other xanthophylls experienced variable decreases between 10 and 30% (**Table 1**). This reduction in total and specific carotenoids is in good agreement with the effect reported of this treatment on the juice of sweet orange in which a decline of nearly 35% was observed for total carotenoids and most of the individual carotenoids, including the main sweet orange xanthophyll violaxanthin.³¹ However, due to the reduced content or absence of β-cryptoxanthin in sweet orange juices, the application of the HPP treatment to mandarin juice allowed us to explore the effect of this technology on this specific xanthophyll with a high relevance from a nutritional and health-related perspective. The different stability of carotenes and xanthophylls observed in this work upon HPP-treatment may be explained by their chemical structure and by the deposition form within the juice vesicle cells. It has been reported that carotenes, highly hydrophobic molecules, are accumulated in citrus fruit tissues in solid crystalloid structures or round lipophilic vesicles.^{32,33} However, xanthophylls are accumulated in plastoglobuli surrounded by a monolayer of phospholipids and proteins,³² which may protect them from degradation during HPP-treatment.

In contrast with our results, other works reported an increase of 54% on the total carotenoids concentration in sweet orange juice with pulp after an HPP treatment of 400 MPa/40 °C/1 min³⁴ and an increment between 53 and 99% in xanthophyll in beverages made mainly with orange juice and milk or soymilk treated by the same HPP conditions.¹⁸ The variable effects of HPP-400 treatment on carotenoid content in sweet orange or mandarin juices may be related not only to their specific carotenoid composition, mandarin is rich in b-cryptoxanthin while sweet oranges in epoxy-xanthophylls, but also to the juicing method used. It is hypothesized that juice obtained with a strong particle disrupting technology may contain higher levels of carotenoids but these compounds, released from the juice cells vesicle membranes to the aqueous matrix, would be more exposed and prone to degradation during the HPP-treatment.²²

Total and individual carotenoids contents were analyzed in the digested untreated and HPP-400 Clementine juices and their BA calculated (**Table 1**). Both untreated and HPP-400 juices showed a significant reduction in total carotenoid content in the BF, although more marked in the untreated juice. Total carotenoids in the BF of untreated juice was approximately 7-times lower compared to the non-digested juice, which is in the range previously described for fresh mandarin juices using the same digestion protocol.¹¹ Interestingly, the BA of total carotenoids in HPP-treated juice was only reduced by half which indicates that this technology may have a positive impact on carotenoid retention and micellar incorporation compared to untreated juice. A study in Ortanique mandarin also reported a significant retention of carotenoids in the micellar fraction of digested juice treated with HPH (150 MPa) compared to those of fresh juices or treated with different standard pasteurization conditions.²² The enhancement of carotenoid levels in the BA of HPH treated juices has been attributed to the reduction of juice particle size improving the action of digestive enzymes and incorporation in the micellar fraction.^{22, 33} Regarding BA of individual carotenoids important differences were observed between untreated and HPP-400 juices. In both juices, β -cryptoxanthin was the most abundant carotenoid in the digested fraction; however, its concentration in the BF was nearly 7-times higher in HPP-treated juice compared to untreated (240.0 ± 15.4 and $36.2 \pm 3.4 \mu\text{g}/100 \text{ ml}$, respectively), and its percentage of BA in HPP-400 juice reached 90% (**Table 1**). These results

suggest that HPP-400 treatment promotes the stability of β -cryptoxanthin in the digested juice. The preferential retention of β -cryptoxanthin in mandarin juices or pulps compared to other carotenoids, especially epoxy-xanthophylls, by using different juice treatments and/or digestion protocols have been already reported.^{35,36} This finding is of particular interest since β -cryptoxanthin is the main contributor to provitamin A content in citrus juices²⁹ and an important bioactive for health improvement and disease prevention.³⁷ The concentration of other carotenoids with nutritional and health-related benefits, such as β -carotene, zeaxanthin, phytoene and phytofluene,³⁸ were significantly reduced in the BF compared to non-digested juices (**Table 1**). However, in BF of HPP-400 juices, the concentration of zeaxanthin and phytoene were higher than in untreated BF while that of phytofluene and β -carotene were below the limit of detection. The epoxy-xanthophylls (antheraxanthin and violaxanthin) showed similar concentration and BA in both juices, but the presence of these xanthophylls may be not relevant for nutritional and functional studies since they have not been detected in the plasma and human tissues.³⁸

3.2. Effect of HPP on the stability and BA of flavonoids in mandarin juices

The qualitative and quantitative profile of flavonoids determined in untreated non-digested juice of Clementine mandarin (**Table 2**) is consistent with previous data reported for this mandarin cultivar grown under similar environmental and agronomical conditions.¹²

Seven main chromatographic peaks with a flavonoid-like spectra were detected and quantified in the mandarin juices according to a previous published study¹² where using similar protocols and materials, a full description of flavonoids identified in the chromatograms of Clementine mandarin samples juice has been included (**Table 2**). The major flavonoids in the mandarin juices were the flavanone glycosides, mainly hesperidin (hesperetin-7-*O*-rutinoside) (4.45 ± 0.23 mg/100 ml) and narirutin (naringenin-7-*O*-rutinoside) (1.81 ± 0.10 mg/100 ml) that accounted the 51% and 21% of the total flavonoid content analyzed, respectively (**Table 2**). The qualitative composition on flavonoids in the juice of Clementine mandarin is in agreement with published data.^{10,39} However, the concentration of hesperidin (4.45 ± 0.23 mg/100 ml) (found in

the present study was lower than that found by Abad-García et al.³⁹ for mandarin juices from different cultivars (between 16.6-87.9 mg/100 ml) but in the range of that reported by Milella et al.¹⁰ (between 6.3-16.5 mg/100 ml) and Hullun et al.⁴⁰ (0.55-8.08 mg/100 ml). Flavonoid composition in Citrus fruits varies greatly among cultivars, tissues, stages of ripening, growing region, agronomical and postharvest practices and differences in sample preparation and may then explain the discrepancies observed in our analysis respect to other studies.^{40,41}

The highest concentration of flavanones, as hesperidin, is found in the peel as compared to the flesh, and higher in the segment membrane than in the juice vesicles.⁴² Rutin is also mainly found in the citrus flavedo and its presence in the mandarin juice could be related with the strong particle disrupting technology (jar-blender) used in the present study to obtain the juice.

¹⁰ This juice extraction technology should have favored the extraction of polyphenols but also the release of enzymes as peroxidase that could have damaged the flavonoids as hesperidin. Other possibility is that the extraction procedure could decrease the portion of soluble flavanones by increasing the amounts of precipitated flavanones in the cloud forming crystals, which in turn may interact with proteins, pectins and other carbohydrates.⁴³

Unlike to carotenoids, HPP-400 slightly affected the qualitative and quantitative composition of flavonoid in the juice of Clementine mandarin. In fact, total flavonoid content in the untreated juice (8.79 ± 0.47 mg/100 ml) only showed a non-statistically decrease (2.6%) after HPP-400. This slight reduction of total flavonoids compared to the untreated juice was in line with the results obtained in mandarin Ortanique juice processed by HPH (150 MPa).²² Nevertheless, mandarin Ortanique juice subjected to pasteurization and low-pressure homogenization (20 MPa) showed a marked decrease in flavonoids (~35%) compared to only pasteurized juice.²³ The flavonoids more affected were narirutin and dydimin that decreased by 9% and 43%, respectively, and eriocitrin that increased 15% in comparison with the untreated juice (**Table 2**). This result is in good agreement with the effect of a similar HPP-treatment (400 MPa/40 °C/1 min) on sweet orange juice³¹ or in a beverage made mainly with sweet orange juice and milk or soymilk treated by HPP (400 MPa/40°C/5 min).¹⁷ One reason for this similar behavior of HPP on flavonoids of orange and mandarin juices could be due to *C. Clementina* is taxonomically

related to *C. Sinensis* and thus, both sweet orange and mandarin juices have similar flavanones composition, being hesperidin, the main component followed by narirutin. However, the total flavonoid concentration was significantly lower in mandarin juice than in orange juice.⁴⁰

Total and individual flavonoids content were analyzed before and after an *in vitro* gastrointestinal digestion of untreated and HPP-treated Clementine juices and their BA were calculated. All the individual flavonoids found in the non-digested mandarin juices (untreated and HPP-treated) were detected in their respective BF after the *in vitro* gastrointestinal digestion except dydimin (**Table 2**).

The total flavonoid content of the BF of both untreated (48.07 ± 2.56 mg/100 ml) and HPP-treated (56.21 ± 3.17 mg/100 ml) mandarin juices showed a significant reduction in the total of about 55% and 49%, respectively, compared to the corresponding non-digested juice but the concentration in the BF of HPP-treated mandarin juice was 12% higher than in the BF of untreated juice. Therefore, significant higher total flavonoid content was observed in the BF of HPP-treated juice in comparison with the untreated juice (**Table 2**). These results are similar to those of mandarin Ortanique juice pasteurized and low-pressure homogenized (20 MPa) compared to those only pasteurized with a 10% higher bioaccessibility of the flavonoids hesperidin, narirutin and didymin.²³ Regarding the BA of hesperidin and narirutin (42%) and total flavonoid (48%) in the untreated mandarin juice, our results were more in agreement with those found by Cilla et al.⁴⁴ who reported a 50% and 62% of BA for hesperidin and total flavonoid (flavone + flavanone), respectively, in a fruit beverage (with 4% of orange juice concentrate), and with Aschoff et al.⁴⁵ who found >90% of BA for hesperidin and narirutin in an orange juice. However, other studies carried out with mandarin juices obtained by a domestic squeezer and low maturity index (7.4) showed significant lower bioaccessibilities for hesperidin (6.4%), narirutin (2.6%) and total flavonoid (flavone + flavanone) (10 %).¹² These differences in the BA of total flavonoid obtained in the present study in comparison with others¹² may be explained by different harvested year and maturity index (7.4 vs. 18.2) and the extraction conditions for fruit juicing (squeezer vs. jar-blender). It is well known that BA of *Citrus* flavonoids varied with the food matrix composition due to: different cultivar or postharvest

conditions;¹² orange segments vs. orange juice;⁴⁵ orange juice alone or being part of a fruit-based beverages and also with the processing technology used such as HPP.^{17,44} The effect of juicing technologies (domestic squeezer vs. jar-blender) on total flavonoid of mandarin juices has been demonstrated to be statistically significant ($p < 0.05$)⁴⁶, so the BA could also be affected.

3.3. Effect of HPP on the stability and BA of AA and total vitC in mandarin juices

The AA content of untreated mandarin juice (32.47 ± 1.49 mg/100 ml) before gastrointestinal digestion (**Table 3**) was in line to those described by other authors as Pyo et al.⁴⁶ (28.35 mg/100 ml) but significantly lower ($P < 0.05$) than in other studies for the same mandarin cultivar (56.98 ± 1.00 mg/100 g fw).¹² This difference may be due to the higher maturity index of the mandarin fruit in the present study (18.2) than in De Ancos et al.¹² (7.4). The general trend in citrus fruits is to decrease the concentration of AA with fruit maturity.^{41,47} Also, the total vitC content of Clementine mandarin juice (35.98 ± 2.30 mg/100 ml) (**Table 3**) was in the range observed for Clementine cultivars by Milella et al.¹⁰ (ranging from 20.65 to 75.51 mg/100 ml).

As a consequence of processing and storage, AA could be oxidized to DHAA and thus increase the risk of losing the vitC efficacy due to the irreversible degradation of DHAA to 2,3-diketogulonic acid.⁴⁵ In general, HPP protects the thermo-labile molecule of ascorbate in vegetable products better than other thermal treatments.⁴⁸ In the present study, HPP-400 significantly ($P < 0.05$) decreased 18 % the AA and 15% the vitC content of fresh mandarin juice without modifying the DHAA concentration (4.62 ± 1.29 mg/100 ml). The *in vitro* gastrointestinal digestion produced a significant depletion of vitC in both juices, untreated (24%) and HPP-treated (10%), in comparison with the corresponding non-digested juices (**Table 3**). Therefore, a less marked decrease of total vitC was observed in the BF of HPP-treated juice in comparison with the untreated juice although the AA and VitC concentration was similar in both BF, untreated (20.86 ± 0.05 mg/ml and 27.29 ± 0.16 mg/ml, respectively) and HPP-treated (21.84 ± 0.05 mg/ml and 27.61 ± 0.07 mg/ml, respectively) (**Table 3**).

The vitC BA observed in the present study for mandarin juice (76.1%) (Table 3) was higher than in previous studies with a similar mandarin juice (46.3%) but obtained from fruits harvested in different year and with different maturity index.¹² However, vitC BA found in the present study for mandarin juice was similar to that described for sweet orange juice (74.8%)¹² or higher as in Aschoff et al.⁴⁵ (67%). In the present work, vitC BA in HPP-400 mandarin juice was 1.2 times higher than in the untreated juice. Changes in AA or vitC BA as a consequence of food matrix and processing technology have been well referenced.^{17,18}

3.4. Effect of HPP on the stability and BA of AC in mandarin juices

Different methods such as TP-FC and FRAP (sample's reducing capacity) and by DPPH• and ABTS•⁺ (sample's radical scavenging capacity) have been used to determine the antioxidant capacity (AC) of untreated and HPP-treated mandarin juice before and after an *in vitro* digestion (Table 4) due to the complex mechanism of action of the antioxidant compounds present in *Citrus* juices.⁴⁹

The AC values of untreated mandarin juice determined by FRAP (335.50±1.50 µmol AAE/100 ml), ABTS•⁺ (235±1.50 µmol AAE/100 ml), DPPH• (378.63±4.68 µmol AAE/100 ml) and TP-FC (18.89±0.72 mg GAE/100 ml) were modified after the HPP-400 treatment. Thus, AC values significant ($P<0.05$) decreased (~10%) when were determined by FRAP and ABTS•⁺. meanwhile increased up to 25% by TP-FC assay and DPPH• value was not modified (Table 4). In general, the decrease of AC (FRAP and ABTS•⁺) values after HPP-400 observed in the present study are in accordance with those previously published by Esteve and Frígola⁵⁰ in orange juice with a similar HPP-treatment (400 MPa/42 °C/5 min) (<10% decrease). However, De Ancos et al.³¹ found higher decrease of approximately 24% of the AC values in orange juices after applying a similar HPP (400 MPa/40 °C/1 min). The depletion of AC values (FRAP and ABTS•⁺) of mandarin juice in the present study could be attributed to the decrease of total carotenoids and total vitC after HPP (Tables 1 and 3). The decrease of AC in orange juices and orange juice beverage had been explained by other authors as a consequence of the

oxidation of bioactive compounds due to HPP-treatments.¹⁷ In general, HPP usually maintain or increase the AC values of fruit juices^{17,48} as in the present study happened with the DPPH[•] and TP-FC. Also, DPPH[•] results were consistent with those previously published for HPP-treated orange juice (400 MPa/40°C/1 min).⁵¹

In accordance with the the depletion of carotenoids, flavonoids and vitamin C concentration in the BF in comparison to the undigested samples, the *in vitro* gastrointestinal digestion also produced a significant decline of AC determined by TP-FC and FRAP (sample's reducing capacity) in untreated (42% and 54%, respectively) and HPP-treated (28% and 30%) juices, being these decreases less marked in the BF of HPP-treated than in the untreated juice (**Table 4**). However, the radical scavenging capacity determined by DPPH[•] (475.18±10.5 and 379.25±11.6 µmol AEE/100 ml) and ABTS^{•+} (355.42±2.42 and 250.21±2.53 µmol AEE/100 ml) of the mandarin juice (untreated and HPP-treated, respectively) was maintained or indeed increased after the gastrointestinal digestion. Thus, the AC in the BF of untreated juices determined by DPPH[•] and ABTS^{•+} was 25% and 51%, respectively, higher than in the non-digested juices (**Table 4**). The increase of AC determined by ABTS^{•+} assay after gastrointestinal digestion has been previously observed in a similar mandarin juice¹² and in fruits rich in polyphenolic compounds such as grapes.⁵² These increase in ABTS^{•+} values during digestion has been correlated with changes in polyphenols structure and pH value.⁵²

3.5. Cytoprotective effect of bioaccessible fractions of untreated and HPP-treated mandarin juices

Results regarding the effect of pre-incubation during 24 h with BF of Clementine mandarin juices (untreated and HPP-treated) upon cell viability (MTT test), intracellular ROS, changes in Δψ_m, intracellular MDA levels and cell cycle phase distribution in differentiated Caco-2 cells treated or not with H₂O₂ 5 mM for 2 h are shown in **Table 5**.

In the absence of an oxidative insult, there were no cytotoxic effects derived from the treatment with the BF (untreated or HPP-treated) of mandarin juices, since closely mirrored the behavior displayed by control cells in terms of viability, Δψ_m, MDA levels and cell cycle

progression and only a slight significant ($p<0.05$) increase in ROS production was detected for HPP-treated sample. In contrast, when compared to control cells, H_2O_2 significantly ($p<0.05$) decreased cell viability up to 53.3% and evoked a rise of 5-times in ROS production, 10-times in dissipation of mitochondrial membrane potential, and an increase of 22.3% in lipid peroxidation and 56.1% in sub- G_1 cell cycle phase (considered a marker of cell death by DNA fragmentation associated with apoptosis⁵³), indicating a clear alteration of the homeostasis and redox state of the cells. In addition, and as expected, there was an 8.8% decrease in the G_1 phase, a fact that is associated with alterations by peroxides of the G_1 checkpoint in the cell cycle progression linked with oxidation and damage in relevant biomolecules, such as DNA, proteins and lipids that may lead to mutagenesis and cell death as final outcomes.⁵⁴ Pretreatment with untreated and HPP-treated BF before H_2O_2 exposure prevented the decrease in cell viability, ROS overproduction, perturbation in mitochondrial membrane potential, lipid peroxidation, rise in sub- G_1 and descent of G_1 cell cycle phases compared to H_2O_2 treated cells, but without reaching control basal levels, except for lipid peroxidation and G_1 cell cycle phase distribution.

In general, a similar cytoprotective effect was observed when comparing both untreated and HPP-treated juices, but a higher significant ($p<0.05$) protection from alterations in mitochondrial membrane potential and a non-significant trend ($p>0.05$) in less ROS production was noted for HPP-treated samples. These results can be attributed to the significant higher ($p<0.05$) content and preservation of antioxidant bioactive compounds in the BF of HPP-treated juice. Specifically, higher levels of total flavonoids (mainly narirutin and naringenin-7-O-rutinoside-4'-O-glucoside), AA, AC in terms of reducing capacity (TP-FC and FRAP) and total carotenoids (mainly β -cryptoxanthin) were found in the BF of HPP-treated versus untreated Clementine mandarin juices (see **Tables 1-4**).

The results obtained in the present study were comparable to those reported by Tarozzi et al.⁵⁵ who indicated that pre-incubation during 24 h with blood orange extracts (6.25-50 mg/mL) dose-dependently inhibited cytotoxicity (10-70%) and increased ROS levels (20-90%) evoked

by 3 mM tert-butyl hydroperoxide in Caco-2 cells. In addition, our results are in tune with those of Di Nunzio et al.²³ where bioaccessible fractions from mandarin Ortanique juice (pasteurized and low-pressure processed (20 MPa)) pre-incubated 24 h at 100 μ L/mL showed a trend to decrease ROS induced by H₂O₂ (4 mM/1 h) in HepG2 cells, compared to only pasteurized juices. Similarly, Cilla et al.²⁴ showed that 24 h pre-incubation with BF of Clementine mandarin pulps were able to protect Caco-2 cells from H₂O₂ (5 mM/2 h)-induced oxidative stress by preserving cell viability and correct cell cycle progression, diminishing lipid peroxidation and partially reducing ROS and $\Delta\psi$ m. It can be then inferred a higher cytoprotective effect of Clementine mandarin pulps²⁴ compared with untreated or HPP-treated juices (present study), since the former completely recovered control basal levels in terms of viability, lipid peroxidation, sub-G₁ and G₁ cell cycle phases while the latter only in lipid peroxidation and G₁ cell cycle phase. As previously mentioned, this observation can be ascribed to the greater relative BA of total carotenoids¹¹, AA, total flavonoids and AC in terms of reducing capacity (TP-FC and FRAP)¹² for Clementine mandarin pulps compared to fresh juices. Nevertheless, in any case, both mandarin pulps and juices (untreated and HPP-treated) evidenced viable cell cultures with a more preserved redox homeostasis, mitochondrial integrity and metabolic response, suggesting cell adaptation to the oxidative stress induced injury.

4. Conclusions

Overall, after *in vitro* digestion, the concentration of total carotenoids, specifically β -cryptoxanthin, total flavonoids and narirutin, vitC, total polyphenols and FRAP value were higher in the BF of the HPP-treated juice compared with the untreated one. In addition, a slightly higher cytoprotection of HPP-400 samples, in terms of mitochondrial membrane potential and ROS production, was observed due to significantly improved stability and better preservation of antioxidant bioactive compounds in the BF of HPP-treated juice. Therefore, HPP-400 treatment can be recommended as a suitable technology to retain or indeed increase the main bioactive compounds and reducing capacity, and its cytoprotective activity in mandarin juices after a gastrointestinal digestion. However, more research is needed to optimize

the HPP-treatment conditions (pressure, treatment time and temperature) to improve these health-beneficial effects.

Abbreviations

AA, ascorbic acid; AC, antioxidant capacity; BA, bioaccessibility; BF, bioaccessible fraction; DHR, dihydrorhodamine; DMEM, Dulbecco's Modified Eagle Medium; HPP, high-pressure processing; MDA, malondialdehyde; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide; PI, propidium iodide; TEER, transepithelial electrical resistance; TP-FC, total polyphenols-Folin Ciocalteu; vitC, vitamin C; $\Delta\psi_m$, mitochondrial membrane potential changes

Author contributions

MJR, BdA, CS-M, PC, LZ, AA and RB conceived and designed the study. AC, MJR, LZ and BdA collected the data. AC, MJR, and BdA analyzed the data. AC, BdA and MJR wrote the draft of the manuscript, that was revised by the other authors. All authors approved the submitted version of the manuscript.

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Conflict of interest

There are no conflicts of interest to declare.

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Table 1. Major carotenoids in non-digested and digested Clemenules mandarin juices (untreated and HPP-treated)

Carotenoid	Non-digested juices ($\mu\text{g}/100\text{mL}$)		Digested juices (BF) ($\mu\text{g}/100\text{mL}$)		Bioaccessibility (%)	
	Untreated	HPP-400	Untreated	HPP-400	Untreated	HPP-400
Phytoene	70.8 ± 5.2^a	35.1 ± 6.2^b	25.4 ± 5.5^a	20.2 ± 3.8^a	34.9 ± 2.5^b	57.1 ± 3.9^a
Phytofluene	42.3 ± 3.2^a	10.1 ± 2.8^b	12.4 ± 3.5	n.d.	29.4 ± 1.8	-
β -carotene	22.1 ± 1.6^a	2.1 ± 0.5^b	5.3 ± 0.4	n.d.	23.9 ± 3.3	-
β -Cryptoxanthin	374.5 ± 22.1^a	258.0 ± 11.6^b	36.2 ± 3.4^b	240.0 ± 15.4^a	10.6 ± 1.4^b	93.2 ± 0.7^a
Zeaxanthin	19.5 ± 3.6^a	17.1 ± 2.4^a	n.d.	2.1 ± 0.2	-	14.6 ± 1.9
Antheraxanthin	61.9 ± 5.6^a	50.3 ± 3.3^a	2.9 ± 0.3^a	3.7 ± 0.3^a	4.7 ± 0.6^a	6.7 ± 0.9^a
All- <i>E</i> -Violaxanthin	34.6 ± 4.7^a	27.0 ± 3.7^a	2.2 ± 0.2^a	2.1 ± 0.1^a	6.8 ± 0.8^a	8.3 ± 1.1^a
9- <i>Z</i> -Violaxanthin	121.6 ± 11.8^a	96.4 ± 8.1^a	25.8 ± 1.3^a	22.1 ± 1.4^a	22.9 ± 3.3^a	24.1 ± 2.9^a
Total carotenoids	824.5 ± 25.2^a	589 ± 13.7^b	118.6 ± 12.6^b	285.4 ± 15.7^a	15.3 ± 1.9^b	47.7 ± 5.1^a

Data are mean $\pm$ SD (n=4). Different letters in the same row for each carotenoid and digestion phase indicate significant differences ($p \leq 0.05$) among treatments; HPP-400 = 400 MPa/40 °C/1 min; BF= Bioaccessible fraction. n.d.: not detected.

Table 2. Major Flavonoids in non-digested and digested Clemenules mandarin juices (untreated and HPP-treated)

Flavonoid	Non-digested juices (mg/100mL)		Digested juices (BF) (mg/100mL)		Bioaccessibility (%)	
	Untreated	HPP-400	Untreated	HPP-400	Untreated	HPP-400
Apigenin-6,8-di- <i>C</i> -glucoside (Vicenin 2)	0.47 ± 0.02 ^a	0.41 ± 0.06 ^a	0.18 ± 0.01 ^a	0.19 ± 0.01 ^a	37.51 ± 2.15 ^a	40.68 ± 2.09 ^a
Narigenin-7- <i>O</i> -rutinoside-4'- <i>O</i> -glucoside	0.77 ± 0.05 ^a	0.78 ± 0.03 ^a	0.60 ± 0.05 ^b	0.70 ± 0.04 ^a	77.09 ± 2.63 ^b	89.71 ± 3.45 ^a
Eriodyctiol-7- <i>O</i> -rutinoside (Eriocitrin)	0.59 ± 0.03 ^b	0.68 ± 0.06 ^a	0.29 ± 0.02 ^a	0.33 ± 0.02 ^a	49.34 ± 1.45 ^a	48.18 ± 3.42 ^a
Quercetin-3- <i>O</i> -rutinoside (Rutin)	0.49 ± 0.05 ^a	0.49 ± 0.06 ^a	0.20 ± 0.05 ^a	0.23 ± 0.01 ^a	39.80 ± 2.72 ^b	48.59 ± 1.34 ^a
Naringenin-7- <i>O</i> -rutinoside (Narirutin)	1.81 ± 0.10 ^a	1.65 ± 0.03 ^b	0.77 ± 0.05 ^b	0.97 ± 0.01 ^a	42.60 ± 2.98 ^b	58.72 ± 1.17 ^a
Hesperetin-7- <i>O</i> -rutinoside (Hesperidin)	4.45 ± 0.23 ^a	4.43 ± 0.08 ^a	1.87 ± 0.14 ^a	1.95 ± 0.13 ^a	42.06 ± 3.45 ^a	44.10 ± 2.17 ^a
Isosakunetin-7- <i>O</i> -Rutinoside (Dydimin)	0.21 ± 0.03 ^a	0.12 ± 0.00 ^b	n.d.	n.d.	-	-
Total flavonoids	8.79 ± 0.47^a	8.56 ± 0.47^a	3.91 ± 0.21^b	4.37 ± 0.23^a	48.07 ± 2.56^b	56.21 ± 3.17^a

Data are mean ± SD (n=4). Different letters in the same row for each flavonoid and digestion phase indicate significant differences ($p \leq 0.05$) among treatments; HPP-400 = 400 MPa/40 °C/1 min; BF= Bioaccessible fraction. n.d.: not detected.

Table 3. Ascorbic acid and total vitamin C in non-digested and digested Clemenules mandarin juices (untreated and HPP-treated)

	Non-digested juices (mg/100mL)		Digested juices (BF) (mg/100mL)		Bioaccessibility (%)	
	Untreated	HPP-400	Untreated	HPP-400	Untreated	HPP-400
Ascorbic acid	32.47 ± 1.49 ^a	26.63 ± 2.12 ^b	20.86 ± 0.15 ^b	21.84 ± 0.05 ^a	64.35 ± 3.07 ^b	81.58 ± 5.98 ^a
Total Vitamin C	35.98 ± 2.30 ^a	30.70 ± 2.82 ^b	27.29 ± 0.16 ^a	27.61 ± 0.07 ^a	76.09 ± 4.92 ^b	92.07 ± 8.63 ^a

Data are mean ± SD (n=4). Different letters in the row for each determination and digestion phase indicate significant differences ($p \leq 0.05$) among treatments; HPP-400 = 400 MPa/40 °C/1 min; BF= Bioaccessible fraction.

Table 4. Antioxidant activity in non-digested and digested Clemenules mandarin juices (untreated and HPP-treated)

	Non-digested juices		Digested juices (BF)	
	Untreated	HPP-400	Untreated	HPP-400
TP-FC (mg GAE/100 mL)	18.89 ± 0.72 ^b	23.67 ± 1.14 ^a	10.95 ± 0.22 ^b	17.02 ± 0.31 ^a
FRAP (μmol AAE/100 mL)	235.50 ± 1.50 ^a	212.68 ± 2.31 ^b	107.54 ± 8.09 ^b	148.65 ± 6.17 ^a
DPPH [•] (μmol AAE/100 mL)	378.63 ± 4.68 ^a	385.62 ± 4.92 ^a	475.18 ± 10.5 ^a	379.25 ± 11.6 ^b
ABTS (μmol AAE/100 mL)	235.50 ± 1.50 ^a	212.68 ± 2.31 ^b	355.42 ± 2.42 ^a	250.21 ± 2.53 ^b

Data are mean ± SD (n=4). Different letters in the same row for each determination and digestion phase indicate significant differences ($p \leq 0.05$) among treatments; HPP-400 = 400 MPa/40 °C/1 min; BF= Bioaccessible fraction; GAE = Gallic Acid Equivalents; AAE = Ascorbic Acid Equivalents.

Table 5. Effect of pre-incubation 24h with bioaccessible fractions (BF) of Clemenules mandarin juices (untreated and HPP-treated) diluted with DMEM 1:6 v/v upon cell viability (MTT test), intracellular reactive oxygen species (ROS), changes in mitochondrial membrane potential ($\Delta\psi_m$), intracellular MDA levels and cell cycle phase distribution in differentiated Caco-2 cells treated or not with H_2O_2 .

	Cell viability (% of control)	ROS (%)	$\Delta\psi_m$ (%)	MDA (nmol/mL cell homogenate)	Cell cycle distribution (%)			
					Sub-G ₁	G ₁	S	G ₂ /M
Control	100.0 ± 5.9 ^a	3.2 ± 0.7 ^a	4.0 ± 0.7 ^a	12.2 ± 1.0 ^a	1.8 ± 0.3 ^{ab}	62.9 ± 1.3 ^a	21.3 ± 1.0 ^a	14.4 ± 2.0 ^{ab}
Untreated	96.6 ± 1.8 ^a	4.5 ± 0.8 ^{ab}	8.3 ± 1.4 ^a	11.3 ± 1.0 ^a	1.4 ± 0.2 ^a	63.3 ± 1.4 ^a	20.8 ± 0.8 ^a	14.4 ± 1.0 ^{ab}
HPP-400	103.1 ± 15.1 ^a	6.9 ± 1.5 ^{bd}	4.8 ± 0.9 ^a	11.8 ± 0.5 ^a	1.3 ± 0.4 ^a	62.4 ± 1.7 ^a	21.1 ± 1.3 ^a	14.9 ± 1.5 ^a
H_2O_2	53.3 ± 6.7 ^b	15.7 ± 1.1 ^c	43.5 ± 7.0 ^b	15.7 ± 0.8 ^b	4.1 ± 0.6 ^c	57.4 ± 2.4 ^b	20.1 ± 1.2 ^a	12.6 ± 1.6 ^{bc}
Untreated + H_2O_2	69.7 ± 5.8 ^c	11.2 ± 2.6 ^c	30.1 ± 5.4 ^c	12.2 ± 1.1 ^a	2.3 ± 0.3 ^{bd}	65.8 ± 1.8 ^c	18.0 ± 1.5 ^b	10.9 ± 0.7 ^{cd}
HPP-400 + H_2O_2	67.8 ± 6.8 ^c	9.1 ± 0.8 ^{de}	22.6 ± 5.9 ^d	12.6 ± 1.4 ^a	2.5 ± 0.2 ^d	66.8 ± 1.0 ^c	18.0 ± 1.0 ^b	10.0 ± 1.0 ^d

Data are mean ± standard deviation (n=4). Different superscript letters in the same column for each determination indicate statistically significant differences ($p \leq 0.05$). Cell cultures exposed to culture media were considered as controls. HPP-400 = 400 MPa/40 °C/1 min. H_2O_2 5mM for 2h.

Figure 1. Flow diagram of the experimental design.