



# Exploring the potential of phenolic compounds from the coffee pulp in preventing cellular oxidative stress after *in vitro* digestion

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## ABSTRACT

The coffee pulp, a by-product of the coffee industry, contains a high concentration of phenolic compounds and caffeine. Simulated gastrointestinal digestion may influence these active compounds' bioaccessibility, bioavailability, and bioactivity. Understanding the impact of the digestive metabolism on the coffee pulp's phenolic composition and its effect on cellular oxidative stress biomarkers is essential. In this study, we evaluated the influence of *in vitro* gastrointestinal digestion of the coffee pulp flour (CPF) and extract (CPE) on their phenolic profile, radical scavenging capacity, cellular antioxidant activity, and cytoprotective properties in intestinal epithelial (IEC-6) and hepatic (HepG2) cells. The CPF and the CPE contained a high amount of caffeine and phenolic compounds, predominantly phenolic acids (3',4'-dihydroxycinnamoylquinic and 3,4-dihydroxybenzoic acids) and flavonoids (3,3',4',5,7-pentahydroxyflavone derivatives). Simulated digestion resulted in increased antioxidant capacity, and both the CPF and the CPE demonstrated free radical scavenging abilities even after *in vitro* digestion. The CPF and the CPE did not induce cytotoxicity in intestinal and hepatic cells, and both matrices exhibited the ability to scavenge intracellular reactive oxygen species. The coffee pulp treatments prevented the decrease of glutathione, thiol groups, and superoxide dismutase and catalase enzymatic activities evoked by *tert*-butyl hydroperoxide elicitation in IEC-6 and HepG2 cells. Our findings suggest that the coffee pulp could be used as a potent food ingredient for preventing cellular oxidative stress due to its high content of antioxidant compounds.

## 1. Introduction

Oxidative stress refers to the condition where reactive oxygen species (ROS) synthesis exceeds the antioxidant defenses, resulting in an imbalance in favor of oxidants. Normal cellular metabolism produces ROS such as superoxide anion radical ( $O_2^{\cdot-}$ ), hydrogen peroxide ( $H_2O_2$ ), and hydroxyl radical ( $\cdot OH$ ) by the use of molecular oxygen (Collin, 2019). Some radicals, especially  $O_2^{\cdot-}$  combined with nitric oxide (NO), can generate reactive nitrogen species (RNS) such as peroxynitrite ( $ONOO^-$ ) (Mandal et al., 2022). ROS can adversely damage vital biomolecules such as carbohydrates, lipids, proteins, and DNA and alter their functions. The effects caused by ROS can trigger the development of chronic diseases such as diabetes, neurological disorders, hypertension, atherosclerosis, or cancer (Sharifi-Rad et al., 2020). A diet rich in antioxidant compounds, such as vitamins and phytochemicals, appears

to be related to an increase in endogenous antioxidants, thus, maintaining the balance between ROS and antioxidant levels (García-Sánchez, Miranda-Díaz, & Cardona-Muñoz, 2020). Phenolic compounds are one of the most important natural antioxidants that can be ingested through the diet, as they are widely present in fruits, vegetables, beverages, pulses, and cereals and can neutralize ROS, among other free radicals. Phenolic compounds can promote the endogenous cellular antioxidant defense systems based on the activities of the antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) to restore cellular redox homeostasis and regulate cytokine-induced inflammation (Zhang & Tsao, 2016; Espinosa-Diez et al. 2015).

Foods rich in phenolic compounds include coffee, which stands out as one of the most commonly consumed beverages globally. It is well known that coffee can promote health and prevent numerous disorders

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(del Castillo et al., 2019). However, coffee's high production generates a considerable amount of agri-food waste that could be harmful to the environment. Coffee by-products obtained during processing could be recovered as new value-added products, as they are a source of phytochemicals, including phenolic compounds, which could have health-promoting benefits (Andrade et al., 2022). Phenolic compounds found in coffee by-products can effectively prevent diseases related to oxidative stress, such as diabetes mellitus type 2, obesity, and disorders related to inflammation (Rebollo-Hernanz et al., 2019, 2022). The coffee pulp (CP), which is the primary by-product of coffee's wet processing, contains a high concentration of antioxidant compounds and dietary fiber (Sangta et al., 2021). Then, the CP could play an important role in preventing oxidative stress, as well as supporting proper digestive function and contributing to glycemic control. Consequently, the CP can be valorized into a bioactive food ingredient, providing health benefits supported by its bioactive compounds (Rebollo-Hernanz et al., 2023). However, large amounts of antioxidant compounds do not guarantee their bioavailability since phenolic compounds are unstable in the gastrointestinal tract and could exhibit low bioavailability (Kawabata, Yoshioka, & Terao, 2019). The bioaccessibility of antioxidant compounds can be compromised by various factors, including the food matrix, food processing, and interactions during digestion (e.g., dietary fiber may hinder the release and absorption of these compounds) (Shahidi & Pan, 2022). Previously, we studied the bioaccessibility and bioavailability of the phytochemicals found in the CP through the use of simulated *in vitro* gastrointestinal digestion conditions and *in silico* models. The results indicated that a significant bioaccessible fraction was available after the digestive process (Cañas et al., 2022a). Hence, the CP could be a source of bioaccessible and, presumably, bioavailable caffeine and phenolic compounds. Studying the antioxidant activity of the bioaccessible phenolic compounds from the CP using cell culture models could be a valuable tool for screening their bioactive potential in relation to oxidative stress. In this context, the intestine, the central organ of food digestion, is exposed to numerous toxic substances and microbial pathogens, which can cause oxidative stress and inflammation (Wang et al., 2020). Besides, the liver, which performs vital biological functions, including drug metabolism, is more vulnerable to oxidative conditions than other organs (Sadasiyam, Kim, Radhakrishnan, & Kim, 2022). Therefore, controlling oxidative stress in the organs mentioned above is essential. The goal of the present study was to investigate the effect of simulated *in vitro* gastrointestinal and colonic digestion on the antioxidant capacity of the CP flour (CPF) and aqueous extract (CPE) and the evaluation of their cytoprotective effects against oxidative damage in intestinal epithelial (IEC-6) and hepatic cells (HepG2).

## 2. Material and methods

### 2.1. Materials

Acetonitrile, ethylenediaminetetraacetic acid (EDTA), ferric chloride hexahydrate, Folin-Ciocalteu reagent, formic acid, hydrochloride acid, methanol, nitric acid, potassium chloride, sodium bicarbonate, sodium carbonate, sodium chloride, sodium hydroxide, sodium nitrite, sodium nitroprusside and were provided by Panreac Química S.L.U. (Barcelona, Spain). Reference phytochemicals (purity  $\geq 96\%$ ), including 1,3,7-trimethylxanthine, 3,3',4',5,7-pentahydroxyflavone 3- $\beta$ -glucoside, 3,4,5-trihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 3',4'-dihydroxycinnamic acid, 4-hydroxy-3-methoxybenzoic acid, 4'-hydroxycinnamic acid, and 4',5,7-trihydroxyflavone-6-C-glucoside were acquired from Sigma Aldrich (Sigma-Aldrich, Alcobendas, Spain) and Extrasynthese (Genay, France). 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), 2',7'-dichlorofluorescein diacetate (DCFDA), 5,5-dithiobis-2-nitrobenzoic acid (DTNB), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), *o*-phthalaldehyde (OPT), ammonium carbonate, ammonium hydrogen carbonate, ascorbic acid, calcium chloride dihydrate, Griess reagent,

hydrogen peroxide, magnesium chloride hexahydrate, manganese oxide, nitrotriazolium blue chloride (NBT), porcine pancreatin, porcine pepsin, potassium persulfate, potassium phosphate monobasic, pronase E, pyrogallol, pyrogallol red, reduced glutathione (GSH), *tert*-butyl hydroperoxide (*t*-BHP) and Viscozyme were provided by Sigma Aldrich (Sigma-Aldrich, Alcobendas, Spain).

### 2.2. CPF and CPE preparation

The CP from the Arabica species was given by "Las Morenitas" (Nicaragua). For obtaining the CPF, the CP was milled and stored in closed flasks protected from light at  $-20^{\circ}\text{C}$  until use. Following a previously optimized extraction protocol of coffee by-products, an aqueous CPE was prepared (Rebollo-Hernanz et al., 2021). The CPF was boiled in water ( $100^{\circ}\text{C}$ ,  $0.02\text{ g mL}^{-1}$  flour-to-water ratio) and agitated for 90 min. The aqueous CPE obtained required filtration, freezing at  $-20^{\circ}\text{C}$ , and freeze-drying to be finally prepared. Similarly, the CPE was stored in closed flasks protected from light at  $-20^{\circ}\text{C}$  until further use.

### 2.3. CPF and CPE *in vitro* gastrointestinal digestion

The *in vitro* simulated static digestion of the CPF and the CPE was carried out following the INFOGEST procedure with minor adjustments (Brodkorb et al., 2019). In brief, the CPF (1 g) or the freeze-dried CPE (0.1 g) were combined with a simulated oral fluid and stirred (2 min,  $37^{\circ}\text{C}$ ) to mimic the oral fraction. Due to the lack of starch in the samples, no amylase was added. To complete the gastric phase, the resulting oral phase was merged with the simulated gastric fluid and pepsin ( $2000\text{ U mL}^{-1}$ ) and incubated under continuous stirring (2 h,  $37^{\circ}\text{C}$ ). To model the intestinal phase, the gastric phase was combined with the simulated intestinal fluid that included pancreatin ( $100\text{ U trypsin mL}^{-1}$ ) and incubated (2 h,  $37^{\circ}\text{C}$ ). The colonic phase was performed as previously described (Cañas et al., 2022b). The intestinal phase was mixed with 5 mL of Pronase E ( $1\text{ mg mL}^{-1}$ ), the pH was adjusted to 8.0, and the digested CP was stirred at  $37^{\circ}\text{C}$  for 1 h. After that, the pH in the digestion media was adjusted to 4.0, Viscozyme ( $150\text{ }\mu\text{L}$ ,  $0.08\text{ U }\mu\text{L}^{-1}$ ) was added, and colonic digestion media were further incubated under stirring ( $37^{\circ}\text{C}$ , 16 h). All digestions were performed in duplicate. An empty digestion blank was run for each digestion phase comprising the mixture of enzymes and reagents found in the simulated digestion fluids. The bioaccessible (supernatants) and non-digested fractions (residues) gathered from each digestion phase were freeze-dried and stored at  $-20^{\circ}\text{C}$  until utilization.

### 2.4. Phytochemical profile analysis by HPLC-PDA-ESI/MS<sup>n</sup>

The analysis of phenolic compounds and caffeine in the bioaccessible digestion fractions from the CP was performed following a previously described methodology (Cañas et al., 2022b). The CP's phytochemicals were separated using an HPLC coupled with a photodiode array detector (PDA) (Hewlett-Packard-1100, Agilent Technologies, Palo Alto, CA, USA) using a Spherisorb S3 ODS-2 C8 ( $3\text{ }\mu\text{m}$ ,  $150\text{ mm} \times 4.6\text{ mm i.d.}$ ) column from Waters (Milford, MA, USA). The elution gradient was achieved through a stepwise increase in solvent B concentration: 15% for 5 min, 15–20% for 5 min, 20–25% for 10 min, 25–35% for 10 min, 35–50% for 10 min, followed by column re-equilibration. The mobile phases were 0.1% formic acid in water (solvent A), and 100% acetonitrile (solvent B), and the flow rate was set to  $0.5\text{ mL min}^{-1}$  at  $35^{\circ}\text{C}$ . The concentration of each compound was expressed as  $\mu\text{g } 100\text{ g}^{-1}$  of the digested CP.

### 2.5. Extraction of free and bound phenolic compounds

The extraction of free and bound phenolic compounds was performed as previously detailed (Benítez et al., 2023). Free phenolic compounds were extracted from the non-digested CPF fractions

obtained after the *in vitro* digestion of the CPF using acidified (0.1% HCl) methanol/water (80:20, v/v). The residues from the previous step were hydrolyzed in an alkali medium (4 mol L<sup>-1</sup> NaOH), and bound phenolic compounds were obtained from the hydrolyzed non-digested fractions.

## 2.6. Assessment of the total phenolic content

The total phenolic content (TPC) was determined using the Folin-Ciocalteu colorimetric method (Singleton et al., 1999). As previously described, the experiment was adapted to a micromethod format in 96-well plates (Cañas et al., 2022b). The TPC values were reported as mg 3,4,5-trihydroxybenzoic (gallic) acid equivalents per gram (mg GAE g<sup>-1</sup>).

## 2.7. Evaluation of the antioxidant capacity

### 2.7.1. ABTS antioxidant capacity

Antioxidant capacity was determined by the ABTS method, as reported before (Cañas et al., 2022b). To obtain ABTS<sup>•+</sup> radical cations, a 7 mmol L<sup>-1</sup> ABTS was combined with 2.45 mmol L<sup>-1</sup> K<sub>2</sub>S<sub>2</sub>O<sub>8</sub> and incubated at room temperature for 16 h under stirring protected from light. The obtained ABTS<sup>•+</sup> solution was adjusted to an absorbance of 0.70 at 734 nm by diluting in 5 mmol L<sup>-1</sup> PBS (Phosphate Buffer Saline), pH 7.4. The assay was conducted in a 96-well plate by mixing 30 µL of the digested CP or standard with 270 µL of the diluted ABTS<sup>•+</sup> solution and letting it react for 10 min in the dark. Finally, the absorbance was recorded at 734 nm. Trolox (0–0.06 mg mL<sup>-1</sup>) served as the standard for preparing a calibration curve. The antioxidant capacity was calculated as mg Trolox equivalent per gram (mg TE g<sup>-1</sup>).

### 2.7.2. Ferric reducing antioxidant power (FRAP)

The FRAP assay was performed following the already explained method (Cañas et al., 2022b). FRAP reagent was produced by combining 0.3 mol L<sup>-1</sup> acetate buffer pH 3.6 with 10 mmol L<sup>-1</sup> TPTZ, 40 mmol L<sup>-1</sup> HCl, and 20 mmol L<sup>-1</sup> FeCl<sub>3</sub>·6H<sub>2</sub>O (10:1:1, v/v/v). The assay was performed by reacting 10 µL of the digested CP or standard and 300 µL of the FRAP solution in a 96-well plate and incubating at 37 °C for 10 min. Finally, the absorbance was recorded at 593 nm. Trolox (25–800 µmol L<sup>-1</sup>) served as the standard for preparing a calibration curve. The antioxidant capacity was expressed as mmol Trolox equivalent per gram (mmol TE g<sup>-1</sup>).

## 2.8. Evaluation of the radical scavenging capacity

### 2.8.1. Superoxide anion radical (O<sub>2</sub><sup>•-</sup>) scavenging capacity

O<sub>2</sub><sup>•-</sup> scavenging was determined using a described protocol with slight modifications (Panzella et al., 2016). The method is based on the inhibition of the formation of blue diformazan resulting from the reduction of NBT by the superoxide generated from pyrogallol autooxidation. Briefly, 100 µL of the digested CP were combined with 100 µL of 50 mmol L<sup>-1</sup> ammonium hydrogen carbonate buffer (pH 9.3, 0.33 mmol L<sup>-1</sup> EDTA), in a 96-well plate. Then, 30 µL of NBT (50 µmol L<sup>-1</sup>) were added, and the mixture was agitated vigorously. Finally, 30 µL of pyrogallol (16.5 mmol L<sup>-1</sup>) were added, and the plate was shaken. Blanks were prepared for each sample. Ascorbic acid (0–70 mg mL<sup>-1</sup>) was used as standard. The radical scavenging capacity was expressed as g ascorbic acid equivalent per gram (g AAE g<sup>-1</sup>).

### 2.8.2. Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) scavenging capacity

The ability of the digested CP to scavenge H<sub>2</sub>O<sub>2</sub> was analyzed according to Cañas et al. (2023) with slight modifications. The digested CP (120 µL) was combined with 70 µL of H<sub>2</sub>O<sub>2</sub> (40 mmol L<sup>-1</sup>). The mix was stirred and incubated for 10 min at room temperature. The optical density was measured at 230 nm using a microplate reader. Blanks were prepared for each sample. Ascorbic acid (0–1250 µg mL<sup>-1</sup>) was used as standard. The radical scavenging capacity was expressed as mg ascorbic

acid equivalent per g (mg AAE g<sup>-1</sup>).

### 2.8.3. Nitric oxide (NO) scavenging capacity

The NO scavenging capacity was evaluated following the method described by Cañas et al. (2023) with slight modifications. The NO generated from sodium nitroprusside is decomposed in the presence of oxygen, generating nitrite (NO<sub>2</sub>), which can be detected spectrophotometrically by forming a magenta chromophore with the Griess reagent. Briefly, 50 µL of the digested CP were added to a 96-well plate and combined with 50 µL of sodium nitroprusside (20 mmol L<sup>-1</sup>). The plate was kept in agitation for 5 min at room temperature, and 50 µL of Griess reagent were added to each well. The optical density was recorded at 550 nm in a microplate reader. Blanks were prepared for each sample. A curve of ascorbic acid (0–2500 µg mL<sup>-1</sup>) was prepared. The radical scavenging capacity was expressed as mg ascorbic acid equivalent per g (mg AAE g<sup>-1</sup>).

### 2.8.4. Peroxynitrite (ONOO<sup>-</sup>) scavenging capacity

ONOO<sup>-</sup> was synthesized following the method of Robinson and Beckman with slight modifications (Robinson & Beckman, 2005). 75 mL of acidified 2 mol L<sup>-1</sup> H<sub>2</sub>O<sub>2</sub> (2 mol L<sup>-1</sup> HNO<sub>3</sub>) were mixed with 75 mL of NaNO<sub>2</sub> (2 mol L<sup>-1</sup>) on an ice bath under vigorous stirring. The reaction was stopped after 1 s by adding 150 mL of NaOH (4 mol L<sup>-1</sup>). The yellow peroxynitrite solution was kept on ice. The residual H<sub>2</sub>O<sub>2</sub> was removed by adding MnO<sub>2</sub> (7 mg mL<sup>-1</sup>) and stirring for 1 h. The solution was centrifuged for 5 min at 1600 × g, and the supernatant obtained was filtered. The peroxynitrite obtained was frozen at –80 °C until use, and its concentration was defined spectrophotometrically at 302 nm (ε = 1670 M<sup>-1</sup> cm<sup>-1</sup>). The pyrogallol red bleaching assay was employed to quantify the scavenging of ONOO<sup>-</sup> through competition kinetics. Briefly, 70 µL of the digested CP were mixed with 140 µL of pyrogallol red (100 µmol L<sup>-1</sup>) in a 96-well plate. The mix was incubated under agitation for 15 min, and 40 µL of peroxynitrite (500 µmol L<sup>-1</sup>) were added. After 10 min, the optical density was measured spectrophotometrically at 540 nm. Blanks were prepared for each sample. Ascorbic acid was used as standard (0–500 µg mL<sup>-1</sup>). The radical scavenging capacity was expressed as mg ascorbic acid equivalent per g (mg AAE g<sup>-1</sup>).

## 2.9. Evaluation of the cellular antioxidant capacity

### 2.9.1. Cell culture materials and cell growth conditions

Rat intestinal epithelial IEC-6 and human hepatoma HepG2 cell lines were acquired from the American Type Culture Collection (ATCC, Rockville, MD, USA). IEC-6 cells were grown in Dulbecco's Modified Eagle Medium (DMEM), containing 4.5 g L<sup>-1</sup> glucose, and supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin/streptomycin, and 0.1 U mL<sup>-1</sup> bovine insulin. HepG2 cells were cultured in DMEM, supplemented with FBS (10%), L-glutamine (1%), and penicillin/streptomycin (1%). Cell cultures were incubated at 37 °C and 100% humidity in a 5% CO<sub>2</sub> atmosphere using a humidified incubator (BINDER CB series 2010, Tuttlingen, Germany).

### 2.9.2. Cell viability

To determine viability, IEC-6 and HepG2 cells were grown in 96-well plates at 5.0 × 10<sup>5</sup> cells mL<sup>-1</sup>. After 24 h, the cells were treated with the digested CPF and the CPE (200 µg mL<sup>-1</sup>) and incubated for 24 h. Cell viability was determined using the CellTiter® 96 Aqueous One Solution Proliferation assay (Promega) as indicated by the manufacturer.

### 2.9.3. Intracellular reactive oxygen species (ROS) measurement

Cellular ROS were quantified in IEC-6 and HepG2 cells by the 2,7-dichlorofluorescein (DCFH) assay (Wang & Joseph, 1999) with slight modifications. Cells were seeded into a 96-well culture plate (1.0 × 10<sup>4</sup> cells per well) and incubated for 24 h. After incubation, cells were pre-treated with the digested extracts from CPF and CPE at 200 µg mL<sup>-1</sup>

for 24 h. Cells were rinsed with PBS and incubated with  $12.5 \mu\text{mol L}^{-1}$  (final concentration) of DCFDA for 40 min. After incubation, the cells were washed again with PBS and co-treated with the digested CPF or CPE at  $200 \mu\text{g mL}^{-1}$  in the presence of *t*-BHP ( $1 \text{ mmol L}^{-1}$ ) for 1 h. Cells were ultimately washed with PBS, and ROS production was examined using a fluorescence reader. The signal was measured at excitation/emission wavelengths of 485/530 nm, respectively. Results were normalized against cell viability values.

#### 2.9.4. Cell lysates preparation

To determine GSH, thiol groups, CAT and SOD, IEC-6 and HepG2 cells were seeded into a 6-well culture plate ( $1.0 \times 10^6$  cells per well). After 48 h of incubation, cells were washed with PBS and treated with non-digested and digested extracts from CPF and CPE at  $200 \mu\text{g mL}^{-1}$  in the presence of  $1 \text{ mmol L}^{-1}$  *t*-BHP for 2 h (IEC-6) and 3 h (HepG2). Cells treated with different conditions were lysed at  $4^\circ\text{C}$  in PBS by ultrasonication after removing the culture medium. The cells were then centrifuged at  $1200 \times g$  for 30 min. Supernatants were collected, the protein concentration was determined using the Bradford reagent, and all lysates were aliquoted and stored at  $-80^\circ\text{C}$  until used for further analysis.

#### 2.9.5. Reduced glutathione (GSH)

GSH was determined using a fluorometric assay by means of the OPT reaction (Hissin & Hilf, 1976). Briefly,  $10 \mu\text{L}$  of each cell lysate were mixed with  $12.5 \mu\text{L}$  of  $\text{HPO}_3$  (25% w/v) and  $37 \mu\text{L}$  of phosphate-EDTA buffer, pH 8.0 ( $0.1 \text{ mol L}^{-1}$  sodium phosphate in  $5 \text{ mmol L}^{-1}$  EDTA). The mixture was preserved on ice for 10 min and centrifuged at  $10,000 g$  for 15 min at  $4^\circ\text{C}$ . After centrifugation,  $10 \mu\text{L}$  of each supernatant,  $180 \mu\text{L}$  of phosphate-EDTA buffer, and  $10 \mu\text{L}$  of OPT ( $0.1\%$  w/v methanol) were added to a 96-well plate. The plate was shaken for 1 min and incubated at room temperature in the dark for 15 min. The fluorescent signal was analyzed in a microplate spectrophotometer at 360 nm excitation and 460 nm emission. Blanks were performed, adding  $10 \mu\text{L}$  of each cell lysate and  $190 \mu\text{L}$  of phosphate-EDTA buffer. A calibration curve of GSH ( $0\text{--}10 \mu\text{g mL}^{-1}$ ) was performed. The GSH level of the cell lysates was expressed as  $\text{nmol GSH mg}^{-1}$  protein.

#### 2.9.6. Thiol groups

The thiol groups method is based on the ability of the thiol groups to react with Ellman's reagent (DTNB). Briefly,  $10 \mu\text{L}$  of supernatants were combined with  $200 \mu\text{L}$  of  $0.5 \text{ mmol L}^{-1}$  DTNB in phosphate-potassium-saline buffer, pH 7.4, in a 96-well plate. The plate was shaken and incubated for 30 min at room temperature in the dark. The optical density was read at 412 nm. Blanks were prepared by mixing  $10 \mu\text{L}$  of each cell lysate with  $200 \mu\text{L}$  of phosphate-potassium-saline buffer. A standard curve was prepared using pure GSH ( $0\text{--}0.5 \text{ mmol L}^{-1}$ ). Thiol levels were expressed as  $\text{mol GSH mg}^{-1}$  protein.

#### 2.9.7. Catalase (CAT) and superoxide dismutase (SOD) activity

CAT and SOD activities were assessed using commercial kits (KB-03-012 and KB-03-011, respectively, Bioquochem, Gijón, Spain) following the indication provided by the manufacturer. CAT and SOD activity was reported as  $\text{U mg}^{-1}$  protein.

#### 2.10. Statistical analysis

The results were presented as the mean  $\pm$  standard deviation (SD) of at least three independent experiments ( $n = 3$ ). Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by post-hoc Tukey's test, to compare the different digestive phases. When only two digestion phases were assayed, statistical comparisons were made using *T*-tests between the oral and intestinal phases of the digestion of the CP. To compare against the control group, which consisted of oxidized cells or non-treated cells in cell culture studies, a one-way ANOVA and post-hoc Dunnett's test were conducted. Differences were

considered significant at a  $p < 0.05$ . To account for positive and negative effects in the comparison and allow for the observation of the effects of the digestion phase, the percentage of protection against oxidative damage in cell culture experiments was calculated using the following equation:

$$\% \text{ protection} = 1 - \frac{(NT - V_i)}{(NT - OX)}$$

Where *NT* is the parameter value in non-treated cells, *OX* in cells stimulated with *t*-BHP, and *V<sub>i</sub>* the parameter value in each digestion phase (Rebollo-Hernanz et al., 2023). Pearson correlations were calculated to examine the associations between the digested CPF and CPE phenolic profile and their antioxidant properties. Principal component analysis (PCA) and hierarchical cluster analysis were used to classify the CP based on their phenolic composition and antioxidant properties. GraphPad Prism 8.0 (San Diego, CA, US) was used to univariate a bivariate statistical analysis and produce the bar graphs. XLSTAT2021 was employed to run multivariate analysis (PCA and hierarchical clustering).

### 3. Results and discussion

#### 3.1. The concentration of phenolic compounds and caffeine in the coffee pulp decreased throughout gastrointestinal digestion

Phenolic compounds were determined in the non-digested CPF and CPE and their different digestion fractions. As shown in Table 1, the non-digested CPF was mainly composed of caffeine ( $473.1 \text{ mg } 100 \text{ g}^{-1}$ ) and phenolic acids, including hydroxybenzoic (62.5% respect to the CPF total phenolics) and hydroxycinnamic (31.1% respect to the CPF total phenolics) acids. Flavonoids are also notably present, highlighting flavonols (4.7% with respect to the total phenolics) and, to a lesser extent, flavones. Regarding hydroxybenzoic acids, the primary compound was 3,4-dihydroxybenzoic (protocatechuic) acid, which was released 92.0% during the intestinal phase. 3,4,5-Trihydroxybenzoic (gallic) acid was liberated over the oral and gastric phases (more than 50%); however, a decrease was observed across the intestinal and colonic phases. This decrease may be related to the dehydroxylation of gallic acid (Jara-Palacios et al., 2018). 4-Hydroxy-3-methoxybenzoic (vanillic) acid, detected exclusively during the colonic phase, was probably tightly bound to the fiber matrix, being released by cell wall hydrolyzing enzymes (Mosele et al., 2018). Concerning hydroxycinnamic acids, 4-(3',4'-dihydroxycinnamoyl)quinic acid (*trans*; 4-CQA) was present in high concentrations in the gastric and intestinal phases ( $63.5$  and  $67.7 \text{ mg } 100 \text{ g}^{-1}$ , respectively), showing a significant loss in the colonic phase (73.4%). 3-(3',4'-Dihydroxycinnamoyl)quinic (3-CQA), 5-(3',4'-dihydroxycinnamoyl)quinic (5-CQA), 3,5-bis(3',4'-dihydroxycinnamoyl)quinic (3,5-diCQA), and 5-(4'-hydroxycinnamoyl)quinic (5-CoQA) acids, reached their maximum concentration in the intestinal phase and 4-CQA (*cis*) in the colonic phase. 5-(4'-Hydroxy-3'-methoxycinnamoyl)quinic acid (5-FQA) was only present in the gastric phase ( $1.4 \text{ mg } 100 \text{ g}^{-1}$ ), and 4'-dihydroxycinnamic (caffeic) and 4'-hydroxycinnamic (*p*-coumaric) acids were identified only in the colonic phase. 1,3,7-Trimethylxanthine (caffeine) was the main compound released from the fiber matrix throughout the digestive process, reaching 76.5% of release in the colonic stage.

The CPE also contained a high concentration of phenolic acids (Table 1), highlighting the hydroxybenzoic acids (63.8% with respect to the CPE total phenolics) and hydroxycinnamic acids (31.0% with respect to the CPE total phenolics). Among flavonoids, flavones and flavonols showed a low concentration. Caffeine ( $787.9 \text{ mg } 100 \text{ g}^{-1}$ ) was the main compound identified. During gastrointestinal digestion, protocatechuic acid was detected in high concentration in all phases, although a slight decrease (28.4%) was observed at the digestion's conclusion. However, gallic acid, which decreased during the intestinal stage, experienced an increase (3.0-fold) from the intestinal to the colonic stage. Regarding

**Table 1**

Concentration of individual phenolic compounds and caffeine (mg 100 g<sup>-1</sup>) in non-digested and digested coffee pulp flour (CPF) and extract (CPE) throughout the different phases of the *in vitro* digestion.

Compounds	ND	OP	GP	IP	CP
<b>Coffee pulp flour</b>					
<i>Hydroxybenzoic acids</i>					
3,4,5-Trihydroxybenzoic acid (Gallic acid)	46.9 ± 2.0 <sup>a</sup>	28.2 ± 2.4 <sup>b</sup>	25.0 ± 1.2 <sup>b</sup>	14.4 ± 2.0 <sup>c</sup>	10.4 ± 1.3 <sup>c</sup>
3,4-Dihydroxybenzoic acid (Protocatechuic acid)	175.7 ± 0.7 <sup>a</sup>	107.9 ± 2.0 <sup>b</sup>	131.6 ± 8.2 <sup>b</sup>	161.6 ± 24.0 <sup>a</sup>	108.9 ± 11.7 <sup>b</sup>
4-Hydroxy-3-methoxybenzoic acid (Vanillic acid)	n.d.	n.d.	n.d.	n.d.	0.8 ± 0.0
Total	222.7 ± 2.7 <sup>a</sup>	136.1 ± 4.4 <sup>cd</sup>	156.5 ± 9.4 <sup>bc</sup>	176.0 ± 26.0 <sup>b</sup>	120.1 ± 13.0 <sup>d</sup>
<i>Hydroxycinnamic acids</i>					
3-(3',4'-Dihydroxycinnamoyl) quinic acid (3-Caffeoylquinic acid, 3-CQA)	5.3 ± 0.6 <sup>b</sup>	3.0 ± 0.2 <sup>d</sup>	4.0 ± 0.5 <sup>c</sup>	6.5 ± 0.4 <sup>a</sup>	5.8 ± 0.5 <sup>ab</sup>
4-(3',4'-Dihydroxycinnamoyl) quinic acid (4-Caffeoylquinic acid, 4-CQA) ( <i>cis</i> )	6.6 ± 0.5 <sup>bc</sup>	4.1 ± 0.2 <sup>d</sup>	5.9 ± 0.5 <sup>cd</sup>	8.1 ± 1.2 <sup>ab</sup>	9.4 ± 1.3 <sup>a</sup>
4-(3',4'-Dihydroxycinnamoyl) quinic acid (4-Caffeoylquinic acid, 4-CQA) ( <i>trans</i> )	85.3 ± 1.4 <sup>a</sup>	45.9 ± 0.6 <sup>c</sup>	63.5 ± 4.2 <sup>b</sup>	67.7 ± 7.3 <sup>b</sup>	18.0 ± 2.1 <sup>d</sup>
5-(3',4'-Dihydroxycinnamoyl) quinic acid (5-Caffeoylquinic acid, 5-CQA)	3.9 ± 0.3 <sup>a</sup>	1.5 ± 0.1 <sup>c</sup>	1.3 ± 0.2 <sup>c</sup>	2.8 ± 0.4 <sup>b</sup>	n.d.
3',4'-Dihydroxycinnamic acid (Caffeic acid)	n.d.	n.d.	n.d.	n.d.	11.0 ± 1.5
5-(4'-Hydroxy-3'-methoxycinnamoyl) quinic acid (5-Feruloylquinic acid, 5-FQA)	2.0 ± 0.1	n.d.	1.4 ± 0.0	n.d.	n.d.
4'-Hydroxycinnamic acid ( <i>p</i> -Coumaric acid)	n.d.	n.d.	n.d.	n.d.	5.1 ± 0.8
3,5-bis(3',4'-Dihydroxycinnamoyl) quinic acid (3,5-Dicaffeoylquinic acid, 3,5-diCQA)	4.9 ± 0.1 <sup>a</sup>	1.4 ± 0.1 <sup>d</sup>	2.2 ± 0.2 <sup>c</sup>	3.9 ± 0.4 <sup>b</sup>	2.0 ± 0.3 <sup>c</sup>
5-(4'-Hydroxycinnamoyl) quinic acid (5- <i>p</i> -Coumaroylquinic acid, 5-CoQA)	2.8 ± 0.0 <sup>a</sup>	0.2 ± 0.0 <sup>d</sup>	0.6 ± 0.1 <sup>c</sup>	2.3 ± 0.3 <sup>b</sup>	0.4 ± 0.0 <sup>cd</sup>
Total	110.9 ± 3.1 <sup>a</sup>	56.2 ± 1.3 <sup>c</sup>	78.8 ± 5.8 <sup>b</sup>	91.2 ± 10.1 <sup>b</sup>	51.7 ± 6.5 <sup>c</sup>
<i>Flavones</i>					
5,7,4'-Trihydroxyflavone-6,8-di-C-glucoside (Apigenin-6,8-di-C-glucoside)	6.4 ± 0.2 <sup>a</sup>	1.8 ± 0.0 <sup>d</sup>	3.4 ± 0.3 <sup>b</sup>	2.7 ± 0.4 <sup>c</sup>	2.0 ± 0.2 <sup>d</sup>
<i>Flavonols</i>					
3,3',4',5,7-Pentahydroxyflavone 3,7-di-β-glucoside (Quercetin-3,7-di-O-glucoside)	2.6 ± 0.1 <sup>a</sup>	1.2 ± 0.1 <sup>c</sup>	1.7 ± 0.0 <sup>b</sup>	1.6 ± 0.1 <sup>b</sup>	n.d.
3,3',4',5,7-Pentahydroxyflavone-3-rutinoside (Quercetin-3-O-rutinoside)	7.9 ± 0.2 <sup>a</sup>	3.4 ± 0.1 <sup>d</sup>	5.5 ± 0.6 <sup>b</sup>	4.6 ± 0.5 <sup>c</sup>	n.d.
3,3',4',5,7-Pentahydroxyflavone-3-	6.1 ± 0.3 <sup>a</sup>	1.9 ± 0.1 <sup>c</sup>	3.1 ± 0.4 <sup>b</sup>	3.5 ± 0.5 <sup>b</sup>	2.1 ± 0.2 <sup>c</sup>

**Table 1 (continued)**

Compounds	ND	OP	GP	IP	CP
<i>β-glucoside (Quercetin-3-O-glucoside)</i>					
Total	16.6 ± 0.6 <sup>a</sup>	6.5 ± 0.3 <sup>c</sup>	10.3 ± 1.0 <sup>b</sup>	9.7 ± 1.1 <sup>b</sup>	2.1 ± 0.2 <sup>d</sup>
<i>Methylxanthines</i>					
1,3,7-Trimethylxanthine (Caffeine)	473.1 ± 6.1 <sup>a</sup>	201.3 ± 0.0 <sup>d</sup>	300.9 ± 31.5 <sup>c</sup>	388.7 ± 22.1 <sup>b</sup>	361.9 ± 3.0 <sup>b</sup>
<b>Coffee pulp extract</b>					
<i>Hydroxybenzoic acids</i>					
3,4,5-Trihydroxybenzoic acid (Gallic acid)	68.5 ± 4.0 <sup>a</sup>	76.0 ± 7.8 <sup>a</sup>	46.6 ± 2.8 <sup>b</sup>	22.3 ± 2.3 <sup>c</sup>	66.6 ± 7.4 <sup>a</sup>
3,4-Dihydroxybenzoic acid (Protocatechuic acid)	312.2 ± 7.5 <sup>a</sup>	326.7 ± 11.2 <sup>a</sup>	303.7 ± 28.2 <sup>a</sup>	221.3 ± 25.8 <sup>b</sup>	233.8 ± 29.4 <sup>b</sup>
Total	381.7 ± 11.6 <sup>a</sup>	402.6 ± 19.0 <sup>a</sup>	350.3 ± 31.0 <sup>ab</sup>	243.6 ± 28.1 <sup>c</sup>	300.5 ± 36.8 <sup>bc</sup>
<i>Hydroxycinnamic acids</i>					
3-(3',4'-Dihydroxycinnamoyl) quinic acid (3-Caffeoylquinic acid, 3-CQA)	12.1 ± 1.0 <sup>a</sup>	12.2 ± 2.3 <sup>a</sup>	12.4 ± 1.4 <sup>a</sup>	10.5 ± 1.4 <sup>a</sup>	n.d.
4-(3',4'-Dihydroxycinnamoyl) quinic acid (4-Caffeoylquinic acid, 4-CQA) ( <i>cis</i> )	12.7 ± 0.6 <sup>b</sup>	15.5 ± 1.9 <sup>a</sup>	13.7 ± 0.9 <sup>ab</sup>	14.5 ± 0.4 <sup>ab</sup>	n.d.
4-(3',4'-Dihydroxycinnamoyl) quinic acid (4-Caffeoylquinic acid, 4-CQA) ( <i>trans</i> )	145.0 ± 0.4 <sup>a</sup>	150.8 ± 2.1 <sup>a</sup>	113.8 ± 9.7 <sup>b</sup>	65.2 ± 0.8 <sup>c</sup>	23.1 ± 0.8 <sup>d</sup>
3',4'-Dihydroxycinnamic acid (Caffeic acid)	n.d.	n.d.	n.d.	n.d.	168.4 ± 20.2
5-(4'-Hydroxy-3'-methoxycinnamoyl) quinic acid (5-Feruloylquinic acid, 5-FQA)	8.7 ± 0.1 <sup>ab</sup>	8.1 ± 0.4 <sup>b</sup>	9.7 ± 0.8 <sup>a</sup>	n.d.	n.d.
4'-Hydroxycinnamic acid ( <i>p</i> -Coumaric acid)	n.d.	n.d.	n.d.	n.d.	2.3 ± 0.2
3,5-bis(3',4'-Dihydroxycinnamoyl) quinic acid (3,5-Dicaffeoylquinic acid, 3,5-diCQA)	7.0 ± 0.1 <sup>a</sup>	7.4 ± 0.8 <sup>a</sup>	6.7 ± 0.5 <sup>a</sup>	6.9 ± 0.8 <sup>a</sup>	n.d.
5-(3',4'-Dihydroxycinnamoyl) quinic acid (5-Caffeoylquinic acid, 5-CQA)	n.d.	n.d.	n.d.	n.d.	n.d.
Total	185.5 ± 2.2 <sup>a</sup>	194.0 ± 7.5 <sup>a</sup>	156.4 ± 13.3 <sup>b</sup>	97.0 ± 3.4 <sup>c</sup>	193.8 ± 21.2 <sup>a</sup>
<i>Flavones</i>					
5,7,4'-Trihydroxyflavone-6,8-di-C-glucoside (Apigenin-6,8-di-C-glucoside)	8.0 ± 1.1 <sup>a</sup>	7.0 ± 1.1 <sup>a</sup>	6.9 ± 0.8 <sup>a</sup>	n.d.	n.d.
<i>Flavonols</i>					
3,3',4',5,7-Pentahydroxyflavone-3-rutinoside (Quercetin-3-O-rutinoside)	14.5 ± 0.2 <sup>a</sup>	14.9 ± 0.9 <sup>a</sup>	13.8 ± 1.3 <sup>a</sup>	n.d.	n.d.
3,3',4',5,7-Pentahydroxyflavone 3-β-glucoside (Quercetin-3-O-glucoside)	8.9 ± 0.2 <sup>ab</sup>	9.9 ± 0.7 <sup>a</sup>	8.5 ± 0.8 <sup>b</sup>	n.d.	n.d.
Total	23.5 ± 0.4 <sup>a</sup>	24.8 ± 1.5 <sup>a</sup>	22.4 ± 2.1 <sup>a</sup>	n.d.	n.d.
<i>Methylxanthines</i>					
1,3,7-Trimethylxanthine (Caffeine)	787.9 ± 34.3 <sup>abc</sup>	833.9 ± 29.8 <sup>a</sup>	813.7 ± 85.2 <sup>ab</sup>	668.9 ± 70.1 <sup>bc</sup>	653.9 ± 93.3 <sup>c</sup>

Results are reported as mean  $\pm$  SD ( $n = 3$ ). Mean values within rows followed by different superscript letters (a, b, c, d) are significantly different when subjected to Tukey's test ( $p < 0.05$ ). ND: Non-Digested; OP: Oral Phase; GP: Gastric Phase; IP: Intestinal Phase; CP: Colonic Phase; nd: non-detected.

hydroxycinnamic acids, 4-CQA (*trans*), the primary compound identified, suffered a reduction of 84.7% at the end of digestion. 3-CQA, 4-CQA (*cis*), and *p*-coumaric acids were completely liberated during all stages of digestion until the intestinal phase. However, they were not detected at the end of the digestive process.

The absence of some phenolic compounds from the CPE in the colonic phase may be due to the transformation or degradation of phenolics during digestion (Qin et al., 2022). As found in the CPF, caffeic and *p*-coumaric acids were only identified during the colonic phase, concurring with the absence of 3-CQA and 4-CQA (*cis*), which can be hydrolyzed into caffeic acid through the ester bond cleavage between caffeic and quinic acid. Meanwhile, *p*-coumaric acid can be derived from the dehydroxylation of caffeic acid (Kishida & Matsumoto, 2019). The complete release of all the flavonoids was observed over the course of the oral and gastric phases, although they did not reach the intestinal and colonic stages. Caffeine was released throughout digestion, reaching its maximum content at the beginning of the gastrointestinal process. The digestion process caused the delivery of caffeine and phenolic compounds, including phenolics not detected in the non-digested CP, and a partial reduction in some phenolic compounds' concentration, mainly from the CPE. These bioactive compounds from the CPF were generally progressively released until the intestinal phase, suffering a decrease during the colonic phase. During the simulated digestion, the phytochemicals present in the CPE showed a decrease from the oral to the intestinal phase, followed by a slight increase from the intestinal to the colonic phase. Phenolic compounds not assimilated during the intestinal phase can reach the colon, where the gut microbiota can transform their chemical structures, explaining the changes observed (Cañas et al., 2022a).

In brief, this section provides comprehensive information on the composition, concentration, and changes in phenolic compounds throughout the digestion of the CPF and the CPE. The findings suggest that they are rich sources of bioactive compounds, including caffeine, phenolic acids, and flavonoids. Results also highlight the importance of considering the entire digestive process when evaluating the biological activity of these compounds, as the concentration and composition of phenolic compounds changed significantly during digestion.

### 3.2. The antioxidant capacity of the digested coffee pulp flour and extract increased throughout gastrointestinal digestion

The TPC, determined throughout the *in vitro* digestion in the digested fractions from the CPF and the CPE, increased by 45.1 and 25.1%, respectively, from the oral to the colonic phase (Table 2). Conversely, the TPC in the free, bound, and total phenolic fractions extracted from the CPF's non-digested fraction decreased by 67.1, 38.7, and 58.8%, respectively, throughout digestion, with respect to the raw material, due to an increased release of phenolic compounds to the digested fraction. The TPC in the CPF was associated with the concentration of 3-CQA and 4-CQA (*cis*) ( $r = 0.968$  and  $0.972$ ,  $p < 0.05$ , respectively), and caffeine ( $r = 0.921$ ,  $p < 0.05$ ) (Supplementary Table 1).

The antioxidant capacity, as measured by the ABTS method, experienced a significant increase ( $p < 0.05$ ) throughout the digestion in the digested fractions of both matrices, representing an increase of 72.0% for the CPF and 65.4% for the CPE (Table 2). Similarly, the non-digested phase of the CPF also experienced a reduction of 43.8%. The same pattern was observed for the CPE when antioxidant capacity was analyzed by the FRAP method (Table 2). The antioxidant capacity in the digested fraction increased (11.4-fold), whereas, in the non-digested fraction, it decreased (62.9%). However, a decrease of 48.2% in antioxidant capacity was observed during the CPE's digestion. The

**Table 2**

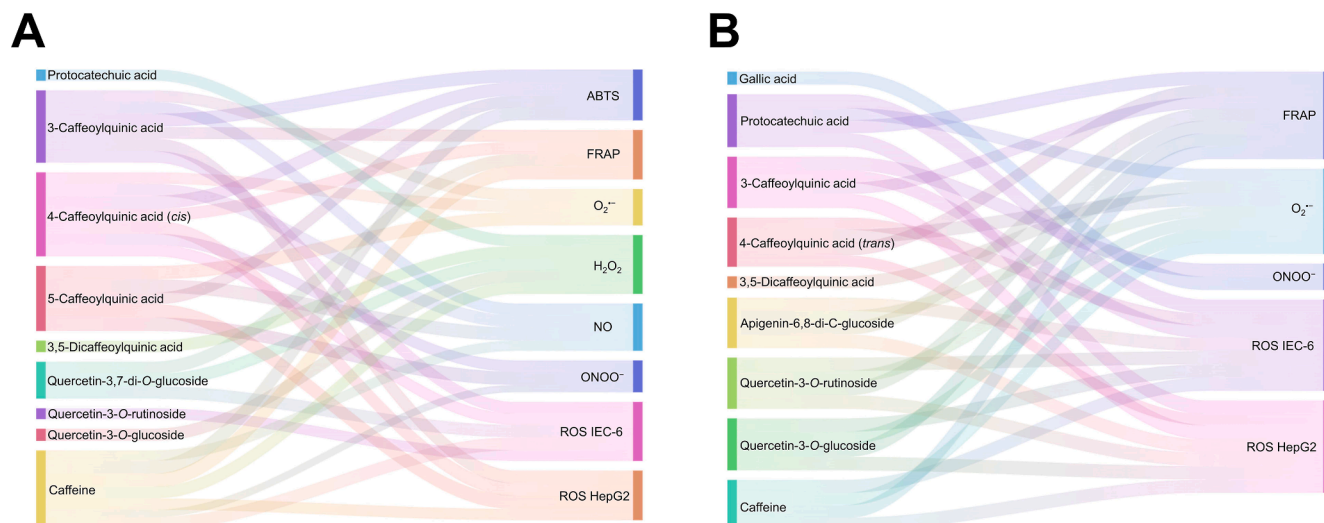
Total phenolic content and antioxidant capacity in the digested and non-digested coffee pulp flour (CPF) and coffee pulp extract (CPE) throughout the different phases of the *in vitro* digestion.

	Digestion phase	Digested fraction	CPF			CPE
			Non-digested fraction			Digested fraction
			Free	Bound	Total	
TPC (mg GAE g <sup>-1</sup> )	Raw	–	28.9	11.9	40.8	46.5 $\pm$ 1.5 <sup>d</sup>
	Material	–	$\pm 1.1^a$	$\pm 0.7^a$	$\pm 1.8^a$	
	Oral Phase	20.1 $\pm$ 1.9 <sup>c</sup>	25.0	10.7	35.7	50.0 $\pm$ 1.0 <sup>c</sup>
			$\pm 0.9^b$	$\pm 0.4^b$	$\pm 1.3^b$	
	Gastric Phase	23.4 $\pm$ 0.7 <sup>b</sup>	14.2	9.7 $\pm$ 0.7 <sup>c</sup>	23.9	61.2 $\pm$ 2.0 <sup>b</sup>
			$\pm 1.4^c$		$\pm 2.1^c$	
	Intestinal Phase	35.5 $\pm$ 0.4 <sup>a</sup>	7.9 $\pm$ 0.6 <sup>e</sup>	8.6 $\pm$ 0.4 <sup>d</sup>	16.6	68.7 $\pm$ 3.2 <sup>a</sup>
					$\pm 1.0^d$	
	Colonic Phase	36.6 $\pm$ 0.7 <sup>a</sup>	9.5 $\pm$ 0.5 <sup>d</sup>	7.3 $\pm$ 0.5 <sup>e</sup>	16.8	66.8 $\pm$ 3.0 <sup>a</sup>
					$\pm 1.0^d$	
	Raw	–	70.3	31.7	102.0	90.0 $\pm$ 1.0 <sup>e</sup>
	Material	–	$\pm 1.8^a$	$\pm 0.9^a$	$\pm 2.7^a$	
ABTS (mg TE g <sup>-1</sup> )	Oral Phase	27.0 $\pm$ 1.0 <sup>c</sup>	50.0	28.0	78.0	100.0 $\pm$ 1.0 <sup>d</sup>
			$\pm 1.0^b$	$\pm 1.0^b$	$\pm 2.0^b$	
	Gastric Phase	54.4 $\pm$ 0.7 <sup>b</sup>	32.2	25.9	58.1	138.1 $\pm$ 5.1 <sup>c</sup>
			$\pm 2.2^c$	$\pm 1.8^c$	$\pm 4.0^c$	
	Intestinal Phase	95.2 $\pm$ 0.8 <sup>a</sup>	20.0	21.7	41.7	276.5 $\pm$ 10.2 <sup>b</sup>
			$\pm 0.9^e$	$\pm 2.0^d$	$\pm 3.0^d$	
	Colonic Phase	96.5 $\pm$ 0.2 <sup>a</sup>	24.2	19.6	43.8	289.3 $\pm$ 11.6 <sup>a</sup>
			$\pm 1.0^d$	$\pm 2.0^c$	$\pm 3.0^d$	
	Raw	–	120.2	39.9	160.1	303.1 $\pm$ 30.7 <sup>a</sup>
	Material	–	$\pm 4.8^a$	$\pm 1.3^a$	$\pm 6.1^a$	
	Oral Phase	8.9 $\pm$ 1.0 <sup>c</sup>	100.0	37.0	137.0	290.0 $\pm$ 10.0 <sup>a</sup>
			$\pm 1.0^b$	$\pm 1.0^b$	$\pm 2.0^b$	
FRAP (mmol TE g <sup>-1</sup> )	Gastric Phase	76.8 $\pm$ 4.7 <sup>b</sup>	50.5	34.8	85.2	208.2 $\pm$ 13.7 <sup>b</sup>
			$\pm 4.0^c$	$\pm 2.8^b$	$\pm 6.9^c$	
	Intestinal Phase	100.2 $\pm$ 6.2 <sup>a</sup>	26.9	27.2	54.1	128.0 $\pm$ 10.0 <sup>c</sup>
			$\pm 2.9^d$	$\pm 2.6^c$	$\pm 5.5^d$	
	Colonic Phase	101.7 $\pm$ 5.2 <sup>a</sup>	30.0	20.7	50.8	150.1 $\pm$ 12.5 <sup>c</sup>
			$\pm 1.8^d$	$\pm 2.2^d$	$\pm 4.0^d$	

Results are reported as mean  $\pm$  SD ( $n = 3$ ). Mean values within rows followed by different superscript letters (a, b, c, d, e) are significantly different when subjected to Tukey's test ( $p < 0.05$ ). TPC: Total Phenolic Content; GAE: Gallic acid equivalent; TE: Trolox equivalent.

antioxidant capacity (ABTS method) in the CPF was mainly associated with the concentration of 3-CQA and 4-CQA (*cis*) ( $r = 0.978$  and  $0.977$ , respectively,  $p < 0.05$ ) and caffeine ( $r = 0.973$ ,  $p < 0.05$ ). The antioxidant capacity measured by the FRAP method correlated with the content of 4-CQA (*cis*) and caffeine ( $r = 0.914$  and  $0.974$ , respectively,  $p < 0.05$ ) (Fig. 1A, Supplementary Table 1). Regarding the antioxidant activity (FRAP method) in the CPE, it was associated significantly ( $p < 0.05$ ) with the concentration of protocatechuic acid and the total content of hydroxybenzoic acids ( $r = 0.956$  and  $0.966$ , respectively), with the total content of flavonols ( $r = 0.910$ ), including quercetin-3-*O*-rutinoside ( $r = 0.903$ ) and quercetin-3-*O*-glucoside ( $r = 0.924$ ), and with the concentration of caffeine ( $r = 0.907$ ) (Fig. 1B, Supplementary Table 1).

During the *in vitro* simulated digestion, both the TPC and the ABTS antioxidant capacity augmented in the CPF and the CPE digested fractions, whereas a decrease was observed in the CPF's non-digested fraction. The simulated gastrointestinal digestion may facilitate the liberation of phenolic compounds from the CPF's non-digested insoluble fiber fraction, consistent with Folin-Ciocalteu and ABTS results throughout digestion. Contrariwise, the individual phenolic compounds experienced a decrease in the CPE's gastric and intestinal phases. These differences between the individual phenolic compounds and the results obtained for TPC and their antioxidant capacity are potentially attributable to the low specificity of the Folin-Ciocalteu method. It is less reliable for total phenolic quantification as it interferes with other biomolecules liberated in the course of the simulated digestion, which may either lead to overestimated results or hinder the reaction with any of the individual phenolic compounds which were evaluated chromatographically. Nonetheless, these techniques can be used to estimate *in*

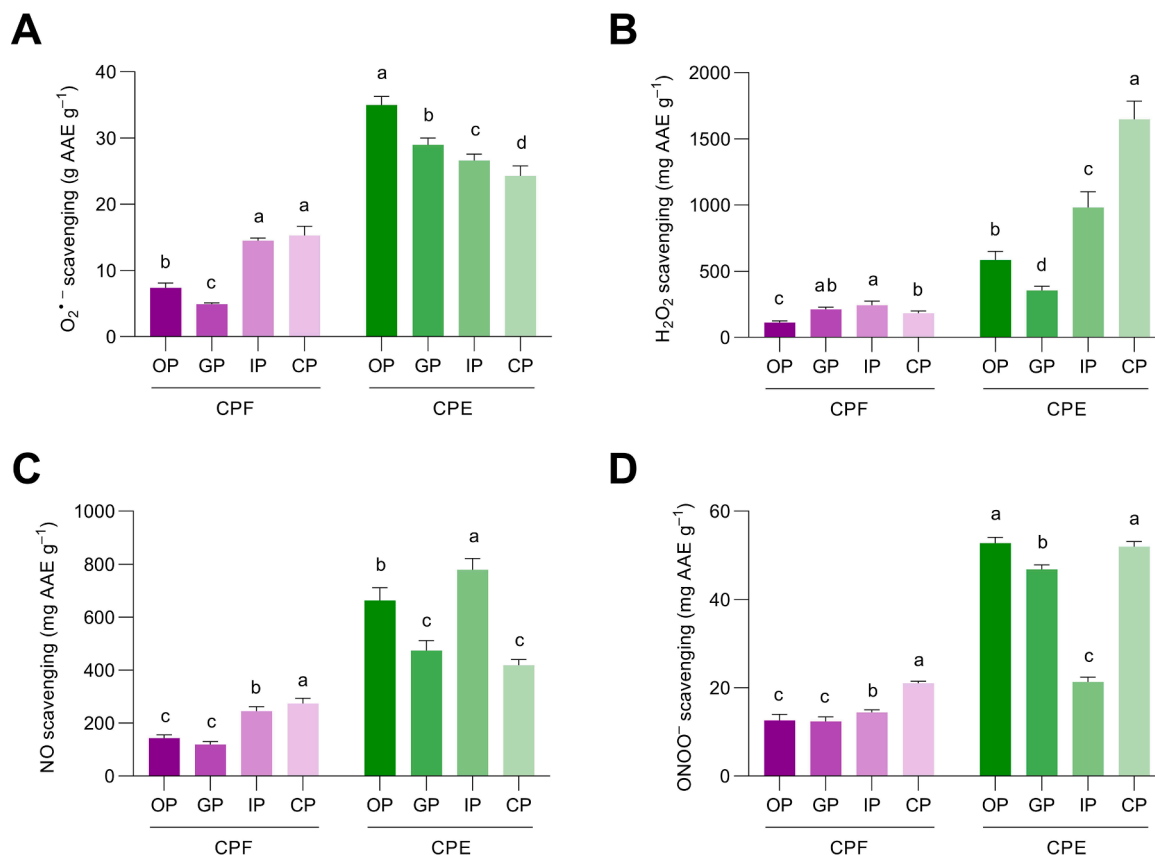


**Fig. 1.** Sankey diagram depicting the significant ( $p < 0.05$ ) Pearson correlations ( $\geq 0.8$ ) among the antioxidant properties and the phenolic compounds of coffee pulp flour (CPF, **A**) and coffee pulp extract (CPE, **B**). 3-Caffeoylquinic acid, 3-CQA: 3-(3',4'-dihydroxycinnamoyl)quinic acid; 3,5-dicaffeoylquinic acid: 3,5-bis(3',4'-dihydroxycinnamoyl)quinic acid; 4-caffeoylquinic acid, 4-CQA (*cis*): 4-(3',4'-dihydroxycinnamoyl)quinic acid; 4-caffeoylquinic acid, 4-CQA (*trans*): 4-(3',4'-dihydroxycinnamoyl)quinic acid; 5-caffeoylquinic acid, 5-CQA: 5-(3',4'-dihydroxycinnamoyl)quinic acid; apigenin-6,8-di-C-glucoside: 5,7,4'-trihydroxyflavone-6,8-di-C-glucoside; caffeine: 1,3,7-trimethylxanthine; gallic acid: 3,4,5-trihydroxybenzoic acid; protocatechuic acid: 3,4-dihydroxybenzoic acid; quercetin 3-O-rutinoside: 3,3',4',5,7-pentahydroxyflavone-3-rutinoside; quercetin 3-O-glucoside: 3,3',4',5,7-pentahydroxyflavone 3- $\beta$ -glucoside.

*vitro* antioxidant capacity because of the great reducing power of phenolic compounds (Everette et al., 2010).

To sum up, the *in vitro* simulated digestion of the CPF and the CPE,

which showed a progressive release of phenolic compounds, increased the TPC and the antioxidant activity in the digested fractions. Interestingly, the non-digested fraction of the CPF showed a reduction in the



**Fig. 2.** Radical scavenging capacity of the digested fractions from the coffee pulp flour (CPF) and the coffee pulp extract (CPE) for superoxide anion ( $O_2^{\bullet -}$ ) (**A**), hydrogen peroxide ( $H_2O_2$ ) (**B**), nitric oxide (NO) (**C**), and peroxynitrite ( $ONOO^-$ ) (**D**). Bars with different letters denote significant differences between the digested phases of the CPF or the CPE according to ANOVA and Tukey's multiple range test ( $p < 0.05$ ). OP: Oral Phase; GP: Gastric Phase; IP: Intestinal Phase; CP: Colonic Phase.

TPC and antioxidant capacity. Our results suggest that the gastrointestinal digestion process may facilitate the liberation of phenolic compounds from the non-digested insoluble fiber fraction, contributing to the overall antioxidant activity. Multiple antioxidant capacity assays are required to evaluate phenolic compounds' potential to scavenge free radicals comprehensively (Sadeer, Montesano, Albrizio, Zengin, & Mahomoodally, 2020). Hence, we further examined the *in vitro* radical scavenging activity of the digested CPF against physiological radicals such as  $O_2^{\bullet -}$ ,  $H_2O_2$ , NO, and ONOO $^-$ .

### 3.3. Phenolic compounds from the coffee pulp may scavenge reactive oxygen and nitrogen species

The  $O_2^{\bullet -}$  is one of the main ROS produced from the reaction between molecular oxygen and electrons. The reaction between the  $O_2^{\bullet -}$  and NO can generate  $\cdot OH$  or ONOO $^-$ , a highly toxic molecule (Fuloria et al., 2021). Phenolic compounds, particularly flavonoids, can eliminate the  $O_2^{\bullet -}$  by transferring protons and hydrogen atoms (Bendary et al., 2013). The CPF-digested fractions showed a higher capacity to scavenge  $O_2^{\bullet -}$  at the end of digestion, increasing by 2.1-fold from the oral to the colonic phase (Fig. 2A). On the contrary, simulated gastrointestinal digestion triggered a decrease in the CPE's  $O_2^{\bullet -}$  scavenging capacity (30.6% from the oral to the colonic phase). Phenolic compounds in the CP's digestive fractions could be responsible for the  $O_2^{\bullet -}$  scavenging. Particularly, 5-CQA in the CPF correlated significantly with the  $O_2^{\bullet -}$  scavenging capacity ( $r = 0.991$ ,  $p < 0.05$ ) (Fig. 1A; Supplementary Table 1), whereas 4-CQA (*trans*) in the CPE ( $r = 0.956$ ,  $p < 0.05$ ) (Fig. 1B; Supplementary Table 1).

$H_2O_2$ , generated from the clearance of  $O_2^{\bullet -}$  by SOD, can also cause cell toxicity (Fuloria et al., 2021). The  $H_2O_2$  scavenging capacity of phenolic compounds depends mainly on their hydrogen-donating capacity, which relies on the number of hydroxyl groups linked to the aromatic ring and their positions (Bendary et al., 2013). The CPF's hydrogen peroxide scavenging activity increased 2.2-fold from the oral to the intestinal phase (Fig. 2B). In contrast, a significant decrease (24.8%) was observed from the intestinal to the colonic phase. The differences observed are probably affected by the phenolic composition of each digestive phase. The gastric and intestinal phases, which reached the highest  $H_2O_2$  scavenging activity, also contained the highest concentration of hydroxybenzoic acids compared to the other digestive phases. This may suggest that phenolic acids, particularly hydroxybenzoic acids, could be associated with higher  $H_2O_2$  scavenging activity because of the large number of hydroxyl groups in their structures. Nonetheless, quercetin-3-O-glucoside was significantly correlated with the  $H_2O_2$  scavenging capacity ( $r = 0.906$ ,  $p < 0.05$ ) in the CPF (Fig. 1A; Supplementary Table 1). In this line, this compound previously showed a high  $H_2O_2$  scavenging capacity, protecting cells from  $H_2O_2$ -triggered oxidative stress (Warnakulasuriya, Ziaullah, & Rupasinghe, 2016). In the CPE, the strongest  $H_2O_2$  scavenging activity was reported in the colonic phase. The  $H_2O_2$  scavenging capacity increased by 2.8-fold from the oral to the colonic phase, probably by the appearance of new compounds derived from phenolic transformations, such as caffeic acid, which contains hydroxyl groups in its structure (Bendary et al., 2013). Previous studies have shown that the transformation of phenolic compounds during digestion can lead to the formation of new metabolites with enhanced bioactivity (Wojtunik-Kulesza et al., 2020). Therefore, the appearance of these new phenolic metabolites could be a potential explanation for the increased  $H_2O_2$  scavenging activity observed during the digestion of the CPF and the CPE. These findings suggest that not only the release of phenolic compounds but also the transformation of phenolic compounds during digestion is an important factor contributing to the overall antioxidant potential of CPF and CPE.

NO plays a crucial role in various physiological processes. It is synthesized through L-arginine metabolism by nitric oxide synthases. However, excess NO and the consequent generation of more reactive species, such as ONOO $^-$ , are related to several cardiovascular diseases

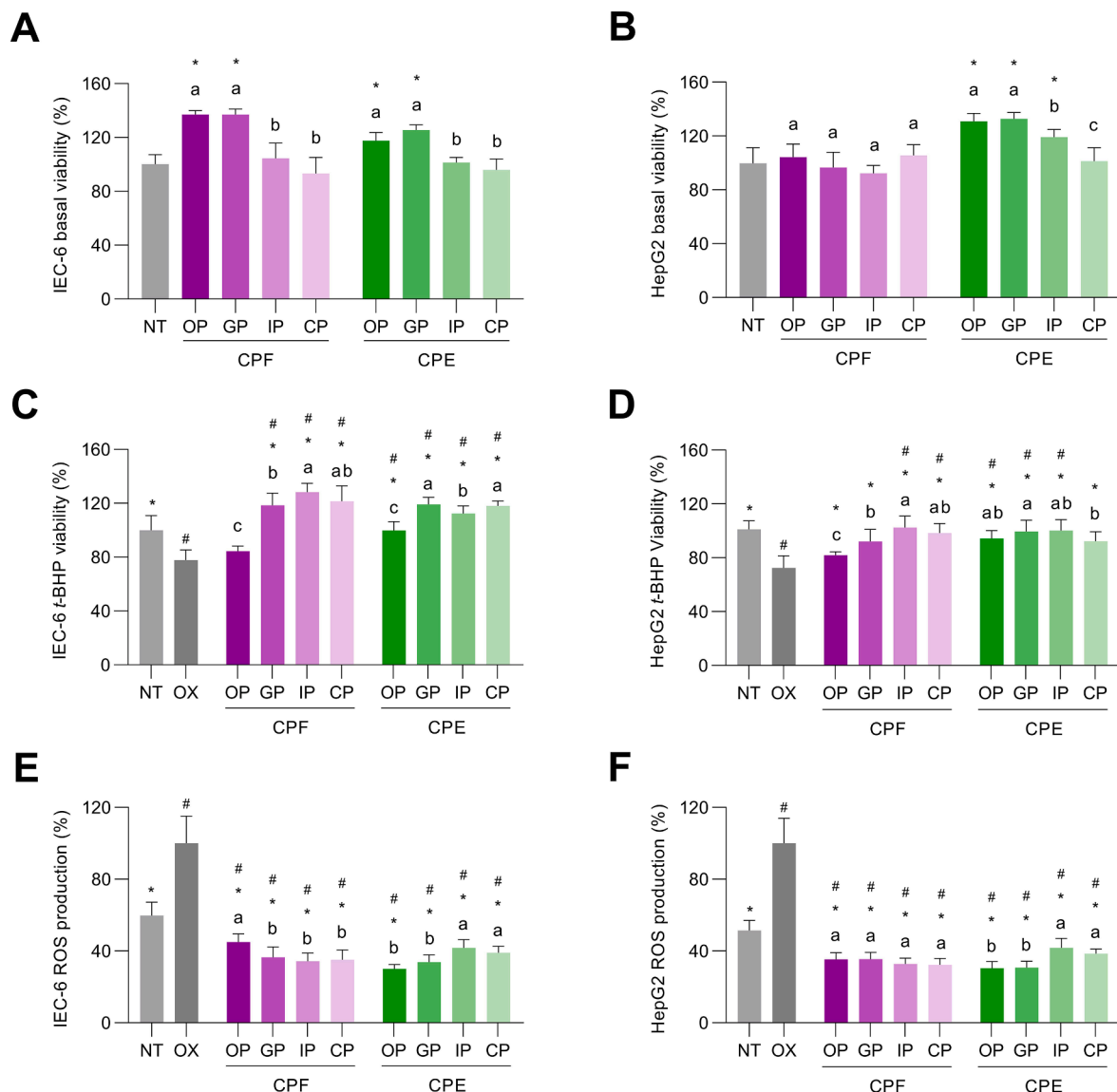
(Di Meo, Reed, Venditti, & Victor, 2016). NO scavenging capacity increased by 1.9-fold during digestion in the CPF (Fig. 2C), correlating with the concentration of 5-CQA ( $r = 0.998$ ,  $p < 0.05$ ) (Fig. 1A; Supplementary Table 1). In contrast, the NO scavenging capacity decreased by 28.5% from the oral to the gastric phase in the CPE digestion, but during the intestinal phase, the highest scavenging value was obtained (778.5 mg g $^{-1}$ ).

The reaction between NO and  $O_2^{\bullet -}$  generates ONOO $^-$ , a highly oxidative compound that oxidizes amino acids and DNA and induces lipid peroxidation (Shaw et al., 2022). Phenolic compounds can eliminate ONOO $^-$  and their structure influences the mechanism of ONOO $^-$  scavenging, possibly by nitration or electron donation (Rodrigues et al., 2013). Regarding the CPF, the ONOO $^-$  scavenging activity did not show significant differences between the oral and gastric phases (12.6 and 12.4 mg AAE g $^{-1}$ , respectively) (Fig. 2D). However, the digestive process enhanced the ONOO $^-$  scavenging capacity, with a remarkable increase (40.0%) from the oral to the colonic phase, probably due to the release of phenolic compounds from the CPF matrix. The ONOO $^-$  scavenging capacity in the CPF was significantly associated with the content of 5-CQA ( $r = 0.999$ ,  $p < 0.05$ ) (Fig. 1A; Supplementary Table 1). Regarding the CPE, the ONOO $^-$  scavenging activity suffered a drastic decrease of 59.7% until the intestinal phase. However, the ONOO $^-$  scavenging capacity was recovered during the colonic phase, exhibiting a 2.4-fold higher scavenging capacity than in the intestinal phase. These results correlated significantly ( $p < 0.05$ ) with the concentration of gallic acid ( $r = 0.934$ ) and the total concentration of hydroxycinnamic acids ( $r = 0.976$ ) (Fig. 1B; Supplementary Table 1). ONOO $^-$  nitrates tyrosine residues of proteins forming nitrotyrosine. Phenolic compounds, and especially hydroxycinnamic acids, can suppress this tyrosine nitration. Hydroxycinnamic acids can be nitrated due to their structural similarity to tyrosine. Conversely, phenolic compounds containing catechol structures can eliminate ONOO $^-$  by electron donation, yielding the subsequent quinone. Therefore, the ability to scavenge ONOO $^-$  increases with the number of hydroxyl groups in the phenolic structures (Rodrigues et al., 2013).

Results further highlight the role of digestion in releasing and transforming phenolic compounds, leading to enhanced bioactivity, particularly in  $H_2O_2$  and ONOO $^-$  scavenging. Then, our research findings indicate that phenolic compounds present in the CP possess potent scavenging activity against reactive oxygen and nitrogen species, such as  $O_2^{\bullet -}$ ,  $H_2O_2$ , NO, and ONOO $^-$  even after *in vitro* digestion. To gain insights into the antioxidant activity of the CPF and the CPE, we investigated if the effects observed *in vitro* could be translated to cell culture. This will help to understand the potential biological relevance of the observed antioxidant effects.

### 3.4. Gastrointestinal digestion preserved the coffee pulp's ability to reduce ROS production in intestinal and hepatic cells

The digested bioaccessible fractions of the CPF and the CPE did not exert cytotoxicity when incubated at 200  $\mu$ g mL $^{-1}$  for 24 h in intestinal (Fig. 3A) and hepatic cells (Fig. 3B). IEC-6 cells stimulated with *t*-BHP exhibited reduced viability (22.2%) (Fig. 3C). The cells exposed to the different digested bioaccessible fractions of the CPF and the CPE and challenged with *t*-BHP were able to preserve their viability, excluding those cells treated with the oral fraction of the CPF. The preservation of viability correlated ( $p < 0.05$ ) with caffeine ( $r = 0.960$ ) in the CPF (Fig. 1A; Supplementary Table 1). Caffeine has been shown to have potential protective effects against oxidative stress-induced cell damage. Studies have reported that caffeine can scavenge ROS, reduce lipid peroxidation, and increase antioxidant enzyme activity in various cell types (del Castillo et al., 2019). In addition, caffeine has been shown to inhibit apoptosis and promote cell survival under oxidative stress conditions (Li et al., 2018). In other research, caffeine did not provide protection against oxidative stress triggered by *t*-BHP in HepG2 cells (Baeza et al., 2014). Likewise, this methylxanthine within a coffee



**Fig. 3.** Effect of the digested fractions from the coffee pulp flour (CPF) and the coffee pulp extract (CPE) on the basal cell viability in intestinal (A) and hepatic cells (B). Protective effect of the digested CPF and CPE against *tert*-butyl hydroperoxide (*t*-BHP, 1 mmol L<sup>-1</sup>) cell viability reduction in intestinal (C) and hepatic cells (D), and ROS production in intestinal (E) and hepatic cells (F). Bars with different letters denote significant differences between the digested phases of the CPF or the CPE according to ANOVA and Tukey's multiple range test ( $p < 0.05$ ). Asterisks (\*) and hashes (#) indicate differences between samples and the oxidised cells (OX) and non-treated cells control (NT) groups, respectively, according to Dunnett's test ( $p < 0.05$ ). OP: Oral Phase; GP: Gastric Phase; IP: Intestinal Phase; CP: Colonic Phase.

silverskin extract has shown no protective consequence in streptozotocin-induced damage in pancreatic INS-1E beta cells (Fernandez-Gomez et al., 2016). The oxidative elicitation with *t*-BHP also induced a loss of cell viability in HepG2 cells (28.6%) (Fig. 3D). However, the hepatic cells treated with the digested fractions of the CPF and the CPE preserved their viability. The protective effect of the digested bioaccessible fractions of the CPF and the CPE may be attributed to their high content of phenolic compounds. Preservation of viability in the CPF correlated with the content of 3-CQA ( $r = 0.974$ ,  $p < 0.05$ ), 4-CQA, (*cis*) ( $r = 0.905$ ,  $p < 0.05$ ), and caffeine ( $r = 0.999$ ,  $p < 0.01$ ) (Fig. 1A; Supplementary Table 1).

Phenolic compounds have been shown to have potential cytoprotective effects against oxidative stress-induced cytotoxicity since they can modulate cellular signaling pathways and gene expression to promote cell survival and prevent cell death (Mehta, Rayalam, & Wang, 2018). For the first time, these findings propose that the digested bioaccessible fractions of CPF and CPE have protective effects against *t*-BHP-evoked oxidative stress-induced cell damage in both intestinal and

hepatic cells. Then, the CP may prevent the development of oxidative stress-related diseases and disorders, such as inflammatory bowel disease, liver dysfunction, and metabolic syndrome (Manzoor et al., 2022).

In this regard, *t*-BHP-treated intestinal cells experienced a stressful situation compared to non-treated cells (Fig. 3E). The significant increase in ROS production (1.7-fold) in IEC-6 cells exposed to *t*-BHP was entirely overcome by the pre- and co-treatment (24 h + 1 h) with the digested fractions from both CP matrices. The *in vitro* digestion of the CPF favored ROS reduction as cells treated with CPF's intestinal phase demonstrated a decrease in ROS production by 34.3%, correlating with the content of caffeine ( $r = 0.959$ ,  $p < 0.05$ ) (Fig. 1A; Supplementary Table 1). In relation to the CPE, IEC-6 cells treated with the oral fraction reduced ROS production by up to 30.5%, while these cells treated with the intestinal and colonic phases exhibited a lower ROS production (up to 41.7 and 39.1%, respectively). In this case, ROS scavenging correlated with the concentration of protocatechuic acid ( $r = 0.986$ ,  $p < 0.01$ ), the total concentration of hydroxybenzoic acids ( $r = 0.987$ ,  $p < 0.01$ ), quercetin 3-O-rutinoside ( $r = 0.946$ ,  $p < 0.05$ ), quercetin 3-O-glucoside

( $r = 0.959$ ,  $p < 0.05$ ), the total concentration of flavonols ( $r = 0.951$ ,  $p < 0.05$ ), and caffeine ( $r = 0.940$ ,  $p < 0.05$ ) (Fig. 1 B; Supplementary Table 1). The treatment with *t*-BHP also induced oxidative stress in hepatic cells (1.9-fold). The pre- and co-treatment with the digested bioaccessible fractions from the CPF and the CPE effectively inhibited ROS production ( $p < 0.05$ ) (Fig. 3F). ROS production in cells treated with the digested phases of the CPF was reduced up to 32.3%, with no significant differences between the different digestive fractions. These results correlated ( $p < 0.05$ ) with the concentration of 3-CQA ( $r = 0.926$ ), 4-CQA, (*cis*) ( $r = 0.941$ ), and 5-CQA ( $r = 0.992$ ) (Fig. 1 A; Supplementary Table 1). Contrarywise, hepatocytes treated with the CPE exhibited lower ROS production when incubated with the oral (30.5%) and gastric (30.8%) CPE's digested fractions. In this instance, ROS scavenging correlated ( $p < 0.05$ ) with the concentration of protocatechuic acid ( $r = 0.973$ ), the total concentration of hydroxybenzoic acids ( $r = 0.943$ ), 3-CQA ( $r = 0.995$ ), apigenin-6,8-di-C-glucoside ( $r = 0.969$ ), and the flavonols ( $r = 0.935$ ), quercetin-3-O-rutinoside, and quercetin-3-O-glucoside ( $r = 0.968$  and  $r = 0.964$ , respectively), and the caffeine ( $r = 0.947$ ) (Fig. 1B; Supplementary Table 1). *t*-BHP mimics the effect of lipid hydroperoxides as it is a short-chain organic hydroperoxide analog. Upon cell penetration, *t*-BHP can interact with ferrous iron and generate peroxy radicals in the cytosol, which imitates the toxic effect of peroxidized fatty acids. Furthermore, *t*-BHP can induce mitochondrial permeability changes, resulting in cell death (Juan, de la Lastra, Plou, & Pérez-Lebeña, 2021). Our experimental results demonstrated that a pre-treatment for 24 h and a co-treatment for 1 h with all the digestion phases of the CPF and the CPE could effectively prevent oxidative stress, inhibiting the generation of ROS, mainly due to its high content of antioxidant compounds. Phenolic compounds, especially flavonoids, can scavenge peroxy radicals and chelate metals with redox activity, preventing the decomposition of the hydrogen peroxide and its subsequent conversion into the hydroxyl radical (Kurutas, 2015).

In summary, our results demonstrate that the digested bioaccessible fractions of CPF and CPE exhibit potent antioxidant activity and protect against *t*-BHP-induced oxidative stress in intestinal and hepatic cells. These beneficial effects could be attributed to the high content of phenolic compounds such as 3-CQA, 4-CQA (*cis*), 5-CQA, and flavonoids, as well as caffeine. These findings suggest that the consumption of CPF and CPE could have potential health benefits in preventing oxidative stress-related diseases.

### 3.5. The digested coffee pulp activated the cellular antioxidant defense system in intestinal and hepatic cells

The organism comprises a complex antioxidant defense system composed of endogenous enzymatic and non-enzymatic antioxidants (Demirci-Çekiç et al., 2022). The levels of the non-enzymatic antioxidant molecules (GSH and thiols groups) and the activities of the cellular antioxidant enzymes (SOD and CAT) were evaluated in intestinal and hepatic cells.

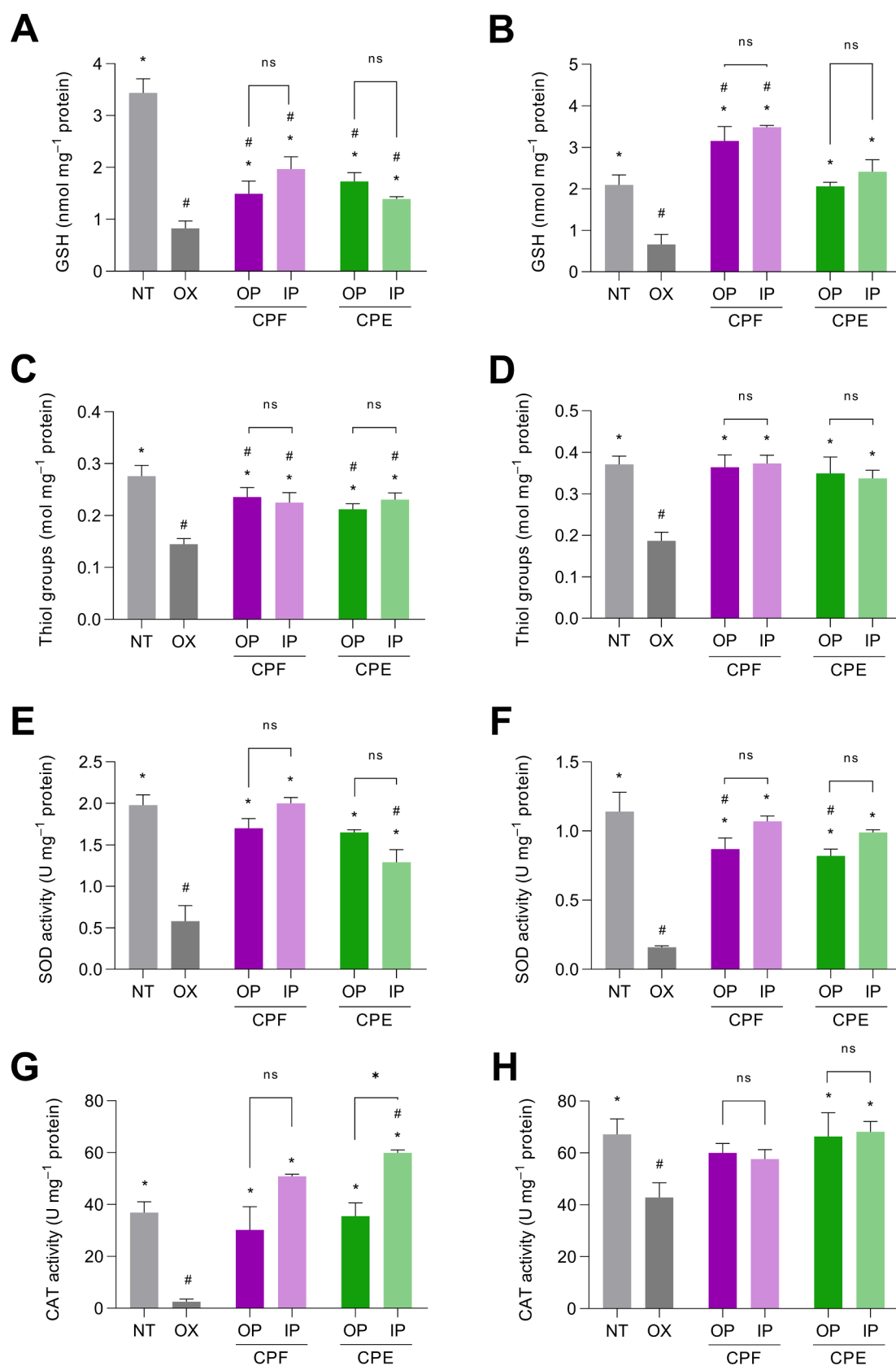
GSH is the cell's primary non-enzymatic antioxidant defense, which can reduce different peroxides, hydroperoxides, and radicals such as alkyl, alkoxy, and peroxy radicals. GSH can donate its electron to  $H_2O_2$  to catalyze its reduction to  $H_2O$  and  $O_2$ . Hence, GSH depletion is considered an oxidative stress index (Pisoschi et al., 2021). As shown in Fig. 4A, GSH levels in non-treated intestinal cells were 75.9% higher than in *t*-BHP-stimulated cells. Intestinal cells co-treated with the oxidant and the oral and intestinal fractions of the CPF exhibited higher GSH levels (44.7 and 57.9%, respectively) than cells stimulated with *t*-BHP. Similarly, IEC-6 cells treated with the oral and the intestinal fractions of the CPE also presented a higher GSH level (2.1- and 1.7-fold, respectively) than those treated only with the oxidant. GSH concentration in HepG2 cells stimulated with *t*-BHP decreased by 68.4% compared to non-treated cells (Fig. 4B). The cells treated with the digested fractions from the CPF showed an increased GSH level of 1.5-fold (oral fraction) and 1.7-fold (intestinal fraction) compared to their basal level.

However, GSH concentration in hepatocytes treated with the digested CPE did not significantly differ ( $p < 0.05$ ) from non-treated cells. Accordingly, IEC-6 and HepG2 cells stimulated with *t*-BHP showed a remarkable decrease in GSH levels, circumvented by pre- and co-treatment with CPF and CPE. *t*-BHP can be detoxified by cytosolic GSH peroxidases through its reduction to *tert*-butyl alcohol, provoking a diminution in GSH levels by its conversion to glutathione disulfide (GSSG) (Kučera et al., 2014). The treatment with the digested CP rich in phenolic compounds probably increased the GSH concentration. Phenolic compounds, especially flavonoids, can boost the expression of the enzyme  $\gamma$ -glutamylcysteine synthetase, which is involved in GSH synthesis. Thus, the production of GSH can prepare the cells against oxidative stress, preventing its decrease to the levels of the cells treated only with *t*-BHP (Rudrapal et al., 2022).

Oxidative stress can also lead to the oxidation of protein cysteine thiol groups. Hence, the depletion of thiol groups is related to increased oxidative damage (Ulrich & Jakob, 2019). Thiol concentration decreased by 47.5% in IEC-6 cells stimulated with *t*-BHP compared to the non-treated cells (Fig. 4C). Cells treated with the digested fractions, including the oral and the intestinal phases of the CPF, exhibited 1.6- and 1.5-fold higher thiol levels, respectively, than *t*-BHP-stimulated cells. The same behavior was observed in cells treated with the oral and intestinal fractions of the CPE, showing 1.5- and 1.6-fold higher thiol concentrations, respectively, when compared with cells stimulated with the oxidant. There was no significant difference ( $p < 0.05$ ) between both matrices' oral and intestinal fractions. *t*-BHP induced a noticeable decline (49.6%) in the thiol concentration in HepG2 cells (Fig. 4D). The CPF and the CPE administration to hepatic cells effectively prevented the loss of thiol groups, maintaining the thiol concentration at levels comparable with non-treated cells. No significant differences ( $p < 0.05$ ) were appreciated between the oral and intestinal fractions within the same matrix. According to our results, cells stimulated with *t*-BHP showed a decreased concentration of thiol groups. Probably reactive oxygen, nitrogen, or sulfur species generated by *t*-BHP stimulation reacted with the thiol group of cysteines, producing modifications, mainly nitrosylation, sulfonylation, and persulfidation reactions (Ulrich & Jakob, 2019). However, the antioxidant-rich digested CP fractions protected cells, especially hepatocytes, against thiol degradation, which may contribute to modulating cell death, considering the significant role of thiol redox switches in regulating cell death pathways (apoptosis, necroptosis, and pyroptosis) (Benhar, 2020).

Cells have developed a defense system composed of several antioxidant enzymes to prevent cell injury and damage to molecules such as proteins, lipids, and DNA, including SOD and CAT (Ulrich & Jakob, 2019). IEC-6 cells stimulated with *t*-BHP presented a loss of SOD enzyme activity (70.7%) compared to the non-treated cells (Fig. 4E). Cells co-treated with *t*-BHP and both digestive fractions of the CPF and the oral fraction of the CPE did not exhibit a decrease in their SOD activity compared to the non-treated cells. No significant differences were found between the digestive phases of the CPF and the CPE. Hepatic cells also exhibited lower SOD enzymatic activity (86.0%) than at the basal level when stimulated by *t*-BHP (Fig. 4F). SOD activity in hepatocytes co-treated with the oxidant and the CP was up to 6.7-fold higher than in cells stimulated only with the oxidant, reflecting the protection exerted by phenolic compounds. Furthermore, SOD activity in hepatic cells treated with the intestinal fractions of both by-products did not show significant differences compared to their basal state. SOD is the first detoxifying enzyme, catalyzing the conversion of  $O_2^{\bullet -}$  to  $H_2O_2$  and  $O_2$ , so the loss of SOD activity is associated with increased oxidative damage (Wang et al., 2018). Treatments rich in phenolic compounds exhibited protection against oxidative stress in both cell lines. Phenolic compounds may act as antioxidants as they can donate electrons to scavenge free radicals, chelation metals to prevent the formation of ROS, as well as regulate the gene expression of antioxidant enzymes (Zeb, 2020).

CAT also forms part of the first line of defense against oxidative stress, as it catalyzes the reduction of  $H_2O_2$  to  $H_2O$  and  $O_2$  (Demirci-



**Fig. 4.** Effect of the digested coffee pulp flour (CPF) and coffee pulp extract (CPE) (oral and intestinal fractions) on *tert*-butyl hydroperoxide (*t*-BHP, 1 mmol L<sup>-1</sup>) derived dysregulation of the cellular antioxidant response including glutathione levels in IEC-6 (A) and HepG2 cells (B), thiol levels in IEC-6 (C) and HepG2 cells (D), superoxide dismutase (SOD) activity (U mg<sup>-1</sup>) in IEC-6 (E) and HepG2 cells (F), and catalase (CAT) activity (U mg<sup>-1</sup>) in IEC-6 (G) and HepG2 cells (H). Asterisks (\*) and hashes (#) indicate differences between samples and the oxidised cells (OX) and non-treated cells control (NT) groups, respectively, according to Dunnett's test ( $p < 0.05$ ). OP: Oral Phase; IP: Intestinal Phase.

Çekiç et al., 2022). According to our findings, CAT activity in IEC-6 decreased by 93.2% in cells stimulated with *t*-BHP compared to the non-treated cells (Fig. 4G). The enzymatic activity of CAT in cells treated with the CPF or the CPE did not decrease and was comparable to that of non-treated cells. In addition, the enzymatic activity of cells treated with the intestinal fraction of CPE was found to be 1.6-fold higher than that of non-treated cells. The observed increase in catalase gene expression is likely due to the ability of phenolic compounds to activate the nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor that regulates cellular response against oxidative stress. Nrf2 activates genes involved in detoxification, antioxidant defense, and DNA repair. This increase was probably caused by the ability of phenolic compounds to regulate the expression of the catalase gene through the activation of Nrf2 (Lee, Song, & Kim, 2021). Likewise, a significant decrease in the CAT activity (36.2%) was evident in HepG2 cells when they were exposed to oxidative stress with *t*-BHP (Fig. 4H). There was no significant difference ( $p < 0.05$ ) in CAT activity among hepatocytes treated with the CPF (oral and intestinal fractions), non-treated cells, and *t*-BHP-treated cells. However, cells treated with oral and intestinal phases from the CPE showed higher CAT activity than *t*-BHP-treated cells, reaching the level of non-treated cells. No significant differences were observed between oral and intestinal phases within the same matrix. The stimulation with *t*-BHP in intestinal and hepatic cells decreased the CAT activity, suggesting that the cell experienced oxidative stress. However, the digested fractions of the CPE achieved effectively prevent oxidative stress due to the protection of phenolic compounds (Zeb, 2020).

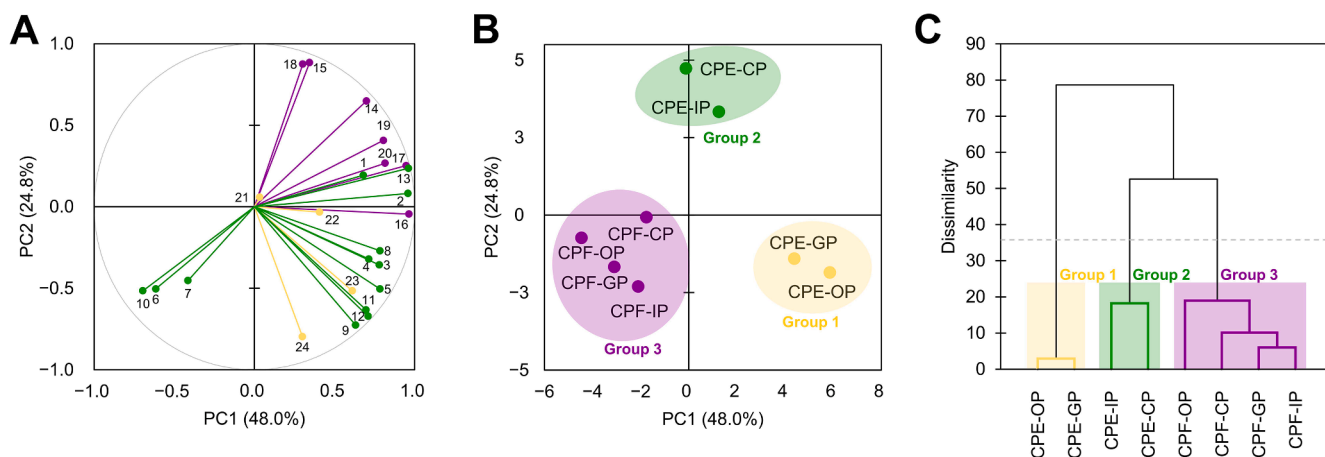
In summary, results showed that the digested bioaccessible fractions of the CPF and the CPE could prevent oxidative stress-induced cell damage by maintaining the levels of GSH and thiol groups and the activities of SOD and CAT. The protection provided by the phenolic compounds found in the digested fractions of the CPF and the CPE is likely due to their ability to activate transcription factors involved in the cellular response against oxidative stress.

### 3.6. The antioxidant and free radical scavenging capacity of the coffee pulp was influenced by its phenolic composition

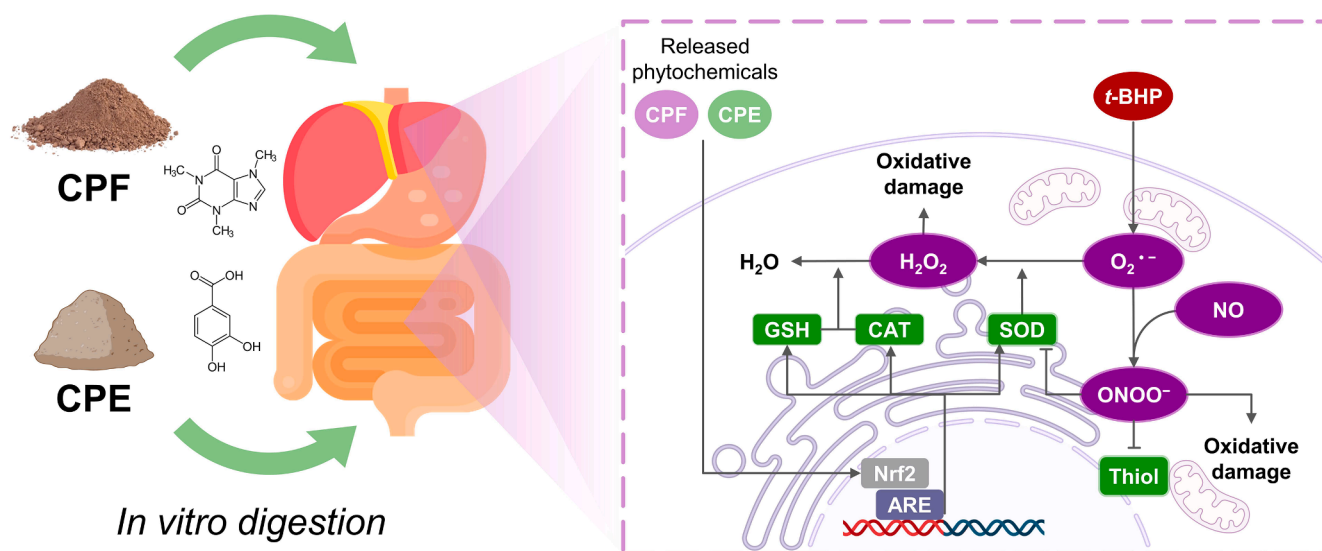
PCA and hierarchical cluster showed in Fig. 5A–C classified the digested bioaccessible fractions from the CP according to their phenolic composition and antioxidant properties. The two principal components (out of seven) could explain 72.8% of the whole variability (Fig. 5A). The first component (PC1), representing 48.0% of the total variance,

was positively linked to the concentration of caffeine and protocatechuic acid, FRAP antioxidant capacity, and  $O_2^{\cdot-}$ , NO, and ONOO<sup>-</sup> scavenging activity. PC2 accounted for 24.8% of the total variance. It was positively correlated with the concentration of apigenin-6,8-di-C-glucoside and quercetin-3-O-glucoside, TPC and ABTS antioxidant capacity,  $H_2O_2$  scavenging activity, and ROS scavenging in HepG2 cells. PC3 accounting for 12.1% of the variability, was associated with IEC-6 and HepG2 cell viability protection against oxidative stress. Principal components' scores (Fig. 5B), and the dendrogram (Fig. 5C), classified the digested CP into three differentiated groups. The first group clustered the digested oral and gastric fractions of the CPE, characterized by the highest caffeine, protocatechuic acid, and caffeoylquinic acids (3-CQA and 4-CQA, both *cis* and *trans* isomers). Then, the second group clustered the digested intestinal and colonic fraction of the CPE. This cluster was characterized by a high TPC, ABTS antioxidant capacity, and  $H_2O_2$  scavenging. Finally, the third group, including all digested fractions from the CPF, was characterized by the highest 5-CQA, 5-CoQA, and quercetin-3,7-O-diglucoside, but with lower antioxidant and radical scavenging properties. Overall, results suggest that the different digestive fractions of the CPF and the CPE may have distinct antioxidant properties depending on the digestion phase and the CP matrix. These findings demonstrate that the phenolic composition of the digested CP samples is an important factor in determining their antioxidant and radical scavenging properties.

Oxidative stress plays a pivotal role in the pathogenesis of multiple diseases. The intestine and liver are particularly susceptible to oxidative stress due to their constant exposure to environmental toxins, xenobiotics, and a diverse array of metabolites (Manzoor et al., 2022). The potential of natural antioxidants, particularly phenolic compounds derived from plant sources, in mitigating oxidative stress and its associated adverse effects has gained significant interest in recent years (Rudrapal et al., 2022). In this context, the present study explored the potential of the CP phenolic compounds in protecting cells against oxidative stress-induced damage. Fig. 6 summarizes the effects of the phytochemicals released from the CPF and the CPE on the course of gastrointestinal digestion on the prevention of oxidative stress in hepatic and intestinal cells. Under basal conditions, Nrf2 is bound to Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm. Nrf2 is released from Keap1 and translocates to the nucleus, where it binds to the antioxidant response element (ARE) in the promoter regions of target genes in response to oxidative stress or electrophilic chemicals (Thiruvengadam et al., 2021). Upon exposure to *t*-BHP, cellular mitochondria



**Fig. 5.** Principal component analysis loadings (A) and scores (B). Agglomerative hierarchical cluster analysis (C) illustrating the behaviour of phenolic compounds and methylxanthines from the coffee pulp flour (CPF) and extract (CPE) during simulated gastrointestinal digestion. Number identification: 1: gallic acid; 2: protocatechuic acid; 3: 3-CQA; 4: 4-CQA (*cis*); 5: 4-CQA (*trans*); 6: 5-CQA; 7: 5-CoQA; 8: 3,5-diCQA; 9: apigenin-6,8-di-C-glucoside; 10: quercetin-3,7-O-diglucoside; 11: quercetin-3-O-rutinoside; 12: quercetin-3-O-glucoside; 13: caffeine; 14: total phenolic content (TPC); 15: ABTS antioxidant capacity; 16: FRAP antioxidant capacity; 17:  $O_2^{\cdot-}$  scavenging; 18:  $H_2O_2$  scavenging; 19: NO scavenging; 20: ONOO<sup>-</sup> scavenging; 21: cytoprotection of IEC-6 cells; 22: cytoprotection of HepG2 cells; 23: ROS scavenging in IEC-6 cells; 24: ROS scavenging in HepG2 cells. OP: Oral Phase; GP: Gastric Phase; IP: Intestinal Phase; CP: Colonic Phase.



**Fig. 6.** A schematic illustration of the effects of the phytochemicals released from the coffee pulp flour (CPF) and the coffee pulp extract (CPE) during simulated gastrointestinal digestion on the prevention of oxidative stress in intestinal and hepatic cells.

may undergo damage, resulting in elevated production of mitochondrial ROS, particularly  $O_2^{\bullet -}$ . The CP's phenolic compounds, as other phytochemicals, have the potential to activate the Nrf2 pathway, subsequently boosting the activity of downstream antioxidant enzymes such as SOD, CAT, and the expression of GSH (Thiruvengadam et al., 2021). Consequently, these phenolic compounds may facilitate the SOD-driven conversion of  $O_2^{\bullet -}$  into  $H_2O_2$ , which can subsequently be broken down into  $H_2O$  through the action of CAT and GSH. ROS can activate inflammation-associated transcription factors like nuclear factor kappa B (NF- $\kappa$ B) and activator protein 1 (AP-1). However, GSH can also modulate the activity of NF- $\kappa$ B and other pro-inflammatory signaling pathways, thereby reducing the production of cytokines and chemokines that contribute to inflammation (Saha, Buttari, Panieri, Profumo, & Saso, 2020). Simultaneously, NO may rapidly interact with  $O_2^{\bullet -}$ , producing  $ONOO^-$ , which, due to its highly reactive nature, can react with thiols, disrupting the function of proteins and cellular signaling cascades, can induce cellular damage and trigger apoptosis (programmed cell death) or necrosis (cell death resulting from injury or disease) by provoking oxidative stress and initiating cell death signaling pathways, and can inactivate SOD, which results in a decrease in SOD activity and an increase in  $O_2^{\bullet -}$  (Pérez-Torres et al., 2020).

The CP's antioxidant and radical scavenging properties are significantly influenced by its phytochemical composition, gastrointestinal digestion, and food matrix. This study demonstrated that the gastrointestinal digestion process and the food matrix played a crucial role in determining the phytochemical composition and, therefore, the associated antioxidant and radical scavenging capacities of the CP. Additional research is required to fully comprehend the bioavailability and bioactivity of these compounds *in vivo*, as well as to investigate their potential applications in bioactive food ingredients and nutraceuticals. Future studies should also focus on the synergistic effects of the CP's phenolic compounds in combination with other nutrients and bioactive molecules, as this may further enhance their antioxidant properties. Moreover, understanding the interactions between these compounds and the human gut microbiota could provide insights into the potential prebiotic effects of the CP, which could contribute to improved gut health and overall well-being.

#### 4. Conclusions

In conclusion, the CP has proven to be a potent source of phytochemicals, such as caffeine and phenolic compounds, which potentially

exert antioxidant effects on intestinal and liver cells. The present study suggests that the CP's phenolic composition can influence the *in vitro* antioxidant capacity, the radicals' scavenging activity, and the antioxidant activity in intestinal and hepatic cells after an *in vitro* digestion. The CP was mainly composed of protocatechuic, gallic, and caffeoylquinic acids and flavonoids, which decreased during the simulated digestion. Caffeine was found in higher concentrations than phenolic compounds. The digestive process increased the scavenging capacity of reactive oxygen and nitrogen species in CPF, while in the CPE, radical scavenging decreased. Both CP matrices, the CPF and the CPE, prevented oxidative stress in intestinal and hepatic cells by activating antioxidant responses and inhibiting cell death. In addition, the CP-digested fractions increased the concentration of the antioxidant proteins (GSH and thiol groups) and the activity of enzymes (SOD and CAT). Consequently, the CP's phenolic compounds may counteract inflammation and cellular damage by neutralizing harmful ROS and RNS. Collectively, these findings support the potential of the CP as a valuable antioxidant food ingredient that could help regulate oxidative stress in both intestinal and liver cells. Future trends based on this research could involve the exploration of coffee by-products for health benefits, their integration into functional foods and dietary supplements, and the promotion of sustainable practices in the coffee industry. This would potentially lead to personalized nutrition strategies, changes in food regulation, technological advancements for efficient compound extraction, and initiatives in public health designed to mitigate the prevalence of diseases associated with oxidative stress.

#### CRediT authorship contribution statement

**Silvia Cañas:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Miguel Rebollo-Hernanz:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – review & editing, Visualization. **María Martín-Trueba:** Formal analysis, Investigation. **Cheyenne Braojos:** Writing – review & editing. **Alicia Gil-Ramírez:** Writing – review & editing. **Vanessa Benítez:** Writing – review & editing. **María A. Martín-Cabrejas:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision, Project administration. **Yolanda Aguilera:** Conceptualization, Writing – review & editing, Supervision.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodres.2023.113116>.

## References

- Andrade, C., Perestrelo, R., & Câmara, J. S. (2022). Bioactive compounds and antioxidant activity from spent coffee grounds as a powerful approach for its valorization. *Molecules*, 27(21). <https://doi.org/10.3390/molecules27217504>
- Baeza, G., Amigo-Benavent, M., Sarriá, B., Goya, L., Mateos, R., & Bravo, L. (2014). Green coffee hydroxycinnamic acids but not caffeine protect human HepG2 cells against oxidative stress. *Food Research International*, 62, 1038–1046. <https://doi.org/10.1016/j.foodres.2014.05.035>
- Bendary, E., Francis, R. R., Ali, H. M. G., Sarwat, M. I., & El Hady, S. (2013). Antioxidant and structure-activity relationships (SARs) of some phenolic and anilines compounds. *Annals of Agricultural Sciences*, 58(2). <https://doi.org/10.1016/j.aos.2013.07.002>
- Benhar, M. (2020). Oxidants, antioxidants and thiol redox switches in the control of regulated cell death pathways. *Antioxidants*, 9(4), 309. <https://doi.org/10.3390/antiox9040309>
- Benítez, V., Rebollo-Hernanz, M., Braojos, C., Cañas, S., Gil-Ramírez, A., Aguilera, Y., & Martín-Cabrejas, M. A. (2023). Changes in the cocoa shell dietary fiber and phenolic compounds after extrusion determine its functional and physiological properties. *Current Research in Food Science*, 6, 100516. <https://doi.org/10.1016/j.crf.2023.100516>
- Brodtkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., ... Recio, I. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols*, 14(4), 991–1014. <https://doi.org/10.1038/s41596-018-0119-1>
- Cañas, S., Rebollo-Hernanz, M., Braojos, C., Benítez, V., Ferreras-Charro, R., Dueñas, M., Aguilera, Y., & Martín-Cabrejas, M. A. (2022a). Understanding the Gastrointestinal Behavior of the Coffee Pulp Phenolic Compounds under Simulated Conditions. *Antioxidants*, 11(9), 1818. <https://doi.org/10.3390/ANTIOX11091818>
- Cañas, S., Rebollo-Hernanz, M., Braojos, C., Benítez, V., Ferreras-Charro, R., Dueñas, M., Aguilera, Y., & Martín-Cabrejas, M. A. (2022b). Gastrointestinal fate of phenolic compounds and amino derivatives from the cocoa shell: An in vitro and in silico approach. *Food Research International*, 162, Article 112117. <https://doi.org/10.1016/j.foodres.2022.112117>
- Collin, F. (2019). Chemical basis of reactive oxygen species reactivity and involvement in neurodegenerative diseases. *International Journal of Molecular Sciences*, 20(10), 2407. <https://doi.org/10.3390/ijms20102407>
- del Castillo, M. D., Iriondo-DeHond, A., Fernandez-Gomez, B., Martinez-Saez, N., Rebollo-Hernanz, M., Martín-Cabrejas, M. A., & Farah, A. (2019). Coffee Antioxidants in Chronic Diseases. In A. Farah (Ed.), *Coffee: Consumption and Health Implications* (1st ed., pp. 20–56). Royal Society of Chemistry. <https://doi.org/10.1039/9781788015028-00020>
- Demirci-Çekiç, S., Özkan, G., Avan, A. N., Uzunboy, S., Çapanoğlu, E., & Apak, R. (2022). Biomarkers of oxidative stress and antioxidant defense. *Journal of Pharmaceutical and Biomedical Analysis*, 209, Article 114477. <https://doi.org/10.1016/j.jpba.2021.114477>
- Espinosa-Diez, C., Miguel, V., Mennerich, D., Kietzmann, T., Sánchez-Pérez, P., Cadenas, S., & Lamas, S. (2015). Antioxidant responses and cellular adjustments to oxidative stress. *Redox Biology*, 6, 183–197. <https://doi.org/10.1016/j.redox.2015.07.008>
- Everette, J. D., Bryant, Q. M., Green, A. M., Abbey, Y. A., Wangila, G. W., & Walker, R. B. (2010). Thorough study of reactivity of various compound classes toward the Folin-Ciocalteu reagent. *Journal of Agricultural and Food Chemistry*, 58(14). <https://doi.org/10.1021/jf1005935>
- Fernandez-Gomez, B., Ramos, S., Goya, L., Mesa, M. D., del Castillo, M. D., & Martín, M. A. (2016). Coffee silverskin extract improves glucose-stimulated insulin secretion and protects against streptozotocin-induced damage in pancreatic INS-1E beta cells. *Food Research International*, 89, 1015–1022. <https://doi.org/10.1016/j.foodres.2016.03.006>
- Fuloria, S., Subramaniam, V., Karupiah, S., Kumari, U., Sathasivam, K., Meenakshi, D. U., ... Fuloria, N. K. (2021). Comprehensive review of methodology to detect reactive oxygen species (ROS) in mammalian species and establish its relationship with antioxidants and cancer. *Antioxidants*, 10(1), 1–35. <https://doi.org/10.3390/antiox10010128>
- Cañas, S., Rebollo-Hernanz, M., Bermúdez-Gómez, P., Rodríguez-Rodríguez, P., Braojos, C., Gil-Ramírez, A., ... Martín-Cabrejas, M. A. (2023). Radical scavenging and cellular antioxidant activity of the cocoa shell phenolic compounds after simulated digestion. *Antioxidants*, 289(12), 1007. <https://doi.org/10.3390/antiox12051007>
- García-Sánchez, A., Miranda-Díaz, A. G., & Cardona-Muñoz, E. G. (2020). The role of oxidative stress in physiopathology and pharmacological treatment with pro- and antioxidant properties in chronic diseases. *Oxidative Medicine and Cellular Longevity*, 2020, 2082145. <https://doi.org/10.1155/2020/2082145>
- Hissin, P. J., & Hilf, R. (1976). A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Analytical Biochemistry*, 74(1). [https://doi.org/10.1016/0003-2697\(76\)90326-2](https://doi.org/10.1016/0003-2697(76)90326-2)
- Di Meo, S., Reed, T. T., Venditti, P., & Victor, V. M. (2016). Role of ROS and RNS Sources in Physiological and Pathological Conditions. *Oxidative Medicine and Cellular Longevity*, 2016, 1245049. doi: 10.1155/2016/1245049.
- Jara-Palacios, M. J., Gonçalves, S., Hernanz, D., Heredia, F. J., & Romano, A. (2018). Effects of in vitro gastrointestinal digestion on phenolic compounds and antioxidant activity of different white winemaking byproducts extracts. *Food Research International*, 109, 433–439. <https://doi.org/10.1016/j.foodres.2018.04.060>
- Juan, C. A., de la Lastra, Plou, F. J., & Pérez-Lebeña, E. (2021). The chemistry of reactive oxygen species (ROS) revisited: Outlining their role in biological macromolecules (DNA, lipids and proteins) and induced pathologies. *International Journal of Molecular Sciences*, 22(9), 4642. <https://doi.org/10.3390/ijms22094642>
- Kawabata, K., Yoshioka, Y., & Terao, J. (2019). Role of intestinal microbiota in the bioavailability and physiological functions of dietary polyphenols. *Molecules*, 24(2), 370. <https://doi.org/10.3390/molecules24020370>
- Kishida, K., & Matsumoto, H. (2019). Urinary excretion rate and bioavailability of chlorogenic acid, caffeic acid, p-coumaric acid, and ferulic acid in non-fasted rats maintained under physiological conditions. *Heliyon*, 5(10), Article e02708. <https://doi.org/10.1016/j.heliyon.2019.e02708>
- Kučera, O., Endlicher, R., Rousar, T., Lotková, H., Garnol, T., Drahota, Z., & Červinková, Z. (2014). The effect of tert-butyl hydroperoxide-induced oxidative stress on lean and steatotic rat hepatocytes in vitro. *Oxidative Medicine and Cellular Longevity*, 2014, 752506. doi:10.1155/2014/752506.
- Kurutas, E. B. (2015). The importance of antioxidants which play the role in cellular response against oxidative/nitrosative stress: Current state. *Nutrition Journal*, 15(1), 71. <https://doi.org/10.1186/s12937-016-0186-5>
- Lee, D. Y., Song, M. Y., & Kim, E. H. (2021). Role of oxidative stress and Nrf2/Keap1 signaling in colorectal cancer: Mechanisms and therapeutic perspectives with phytochemicals. *Antioxidants*, 10(5), 743. <https://doi.org/10.3390/antiox10050743>
- Li, Y. F., Ouyang, S. H., Tu, L. F., Wang, X., Yuan, W. L., Wang, G. E., ... He, R. R. (2018). Caffeine protects skin from oxidative stress-induced senescence through the activation of autophagy. *Theranostics*, 8(20), 5713. <https://doi.org/10.7150/thno.28778>
- Mandal, M., Sarkar, M., Khan, A., Biswas, M., Masi, A., Rakwal, R., Agrawal, G. K., Srivastava, A., & Sarkar, A. (2022). Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) in plants— maintenance of structural individuality and functional blend. *Advances in Redox Research*, 5, Article 100039. <https://doi.org/10.1016/j.arres.2022.100039>
- Manzoor, M. F., Arif, Z., Kabir, A., Mehmood, I., Munir, D., Razzaq, A., ... Rusu, A. (2022). Oxidative stress and metabolic diseases: Relevance and therapeutic strategies. *Frontiers in Nutrition*, 9, 2150. <https://doi.org/10.3389/fnut.2022.994309>
- Mehta, J., Rayalam, S., & Wang, X. (2018). Cytoprotective effects of natural compounds against oxidative stress. *Antioxidants*, 7(10), 147. <https://doi.org/10.3390/antiox7100147>
- Mosele, J. I., Motilva, M. J., & Ludwig, I. A. (2018). Beta-glucan and phenolic compounds: Their concentration and behavior during in vitro gastrointestinal digestion and colonic fermentation of different barley-based food products. *Journal of Agricultural and Food Chemistry*, 66(34), 8966–8975. <https://doi.org/10.1021/acs.jafc.8b02240>
- Panzella, L., Cerruti, P., Ambrogio, V., Agustin-Salazar, S., D'Errico, G., Carfagna, C., ... D'Ischia, M. (2016). A superior all-natural antioxidant biomaterial from spent coffee grounds for polymer stabilization, cell protection, and food lipid preservation. *ACS Sustainable Chemistry and Engineering*, 4(3), 1169–1179. <https://doi.org/10.1021/acsschemeng.5b01234>
- Pérez-Torres, I., Manzano-Pech, L., Rubio-Ruiz, M. E., Soto, M. E., & Guamer-Lans, V. (2020). Nitrosative stress and its association with cardiometabolic disorders. *Molecules*, 25(11), 2555. <https://doi.org/10.3390/molecules25112555>
- Pisoschi, A. M., Pop, A., Iordache, F., Stanca, L., Predoi, G., & Serban, A. I. (2021). Oxidative stress mitigation by antioxidants - An overview on their chemistry and influences on health status. *European Journal of Medicinal Chemistry*, 209, Article 112891. <https://doi.org/10.1016/j.ejmech.2020.112891>

- Qin, W., Ketnawa, S., & Ogawa, Y. (2022). Effect of digestive enzymes and pH on variation of bioavailability of green tea during simulated in vitro gastrointestinal digestion. *Food Science and Human Wellness*, 11(3), 669–675. <https://doi.org/10.1016/j.fshw.2021.12.024>
- Rebollo-Hernanz, M., Aguilera, Y., Gil-Ramírez, A., Benítez, V., Cañas, S., Braojos, C., & Martín-Cabrejas, M. A. (2023). Biorefinery and stepwise strategies for valorizing coffee by-products as bioactive food ingredients and nutraceuticals. *Applied Sciences*, 2023060188. <https://doi.org/10.20944/preprints202306.0188.v1>
- Rebollo-Hernanz, M., Aguilera, Y., Martín-Cabrejas, M. A., & Gonzalez de Mejia, E. (2022). Activating effects of the bioactive compounds from coffee by-products on FGF21 signaling modulate hepatic mitochondrial bioenergetics and energy metabolism in vitro. *Frontiers in Nutrition*, 9, Article 866233. <https://doi.org/10.3389/fnut.2022.866233>
- Rebollo-Hernanz, M., Cañas, S., Taladrí, D., Benítez, V., Bartolomé, B., Aguilera, Y., & Martín-Cabrejas, M. A. (2021). Revalorization of coffee husk: Modeling and optimizing the green sustainable extraction of phenolic compounds. *Foods*, 10(3), 653. <https://doi.org/10.3390/foods10030653>
- Rebollo-Hernanz, M., Kusumah, J., Bringe, N. A., Shen, Y., & Gonzalez de Mejia, E. (2023). Peptide release, radical scavenging capacity, and antioxidant responses in intestinal cells are determined by soybean variety and gastrointestinal digestion under simulated conditions. *Food Chemistry*, 405, Article 134929. <https://doi.org/10.1016/j.foodchem.2022.134929>
- Rebollo-Hernanz, M., Zhang, Q., Aguilera, Y., Martín-Cabrejas, M. A., & de Mejia, E. G. (2019). Relationship of the phytochemicals from coffee and cocoa by-products with their potential to modulate biomarkers of metabolic syndrome in vitro. *Antioxidants*, 8(8), 279. <https://doi.org/10.3390/antiox8080279>
- Robinson, K. M., & Beckman, J. S. (2005). Synthesis of peroxynitrite from nitrite and hydrogen peroxide. *Methods in Enzymology*, 396, 207–214. [https://doi.org/10.1016/S0076-6879\(05\)96019-9](https://doi.org/10.1016/S0076-6879(05)96019-9)
- Rodrigues, E., Mariutti, L. R. B., & Mercadante, A. Z. (2013). Carotenoids and phenolic compounds from *Solanum sessiliflorum*, an unexploited amazonian fruit, and their scavenging capacities against reactive oxygen and nitrogen species. *Journal of Agricultural and Food Chemistry*, 61(12). <https://doi.org/10.1021/jf3054214>
- Rudrapal, M., Khairnar, S. J., Khan, J., Dukhyil, A. B., Ansari, M. A., Alomary, M. N., ... Devi, R. (2022). Dietary polyphenols and their role in oxidative stress-induced human diseases: Insights into protective effects, antioxidant potentials and mechanism(s) of action. *Frontiers in Pharmacology*, 13, 283. <https://doi.org/10.3389/fphar.2022.806470>
- Sadasivam, N., Kim, Y. J., Radhakrishnan, K., & Kim, D. K. (2022). Oxidative stress, genomic integrity, and liver diseases. *Molecules*, 27(10), 3159. <https://doi.org/10.3390/molecules27103159>
- Sadeer, N. B., Montesano, D., Albrizio, S., Zengin, G., & Mahomoodally, M. F. (2020). The versatility of antioxidant assays in food science and safety—chemistry, applications, strengths, and limitations. *Antioxidants*, 9(8), 709. <https://doi.org/10.3390/antiox9080709>
- Saha, S., Buttari, B., Panieri, E., Profumo, E., & Saso, L. (2020). An overview of Nrf2 signaling pathway and its role in inflammation. *Molecules*, 25(22), 5474. <https://doi.org/10.3390/molecules25225474>
- Sangta, J., Wongkaew, M., Tangpao, T., Withee, P., Haituk, S., Arjin, C., ... Cheewangkoon, R. (2021). Recovery of polyphenolic fraction from arabica coffee pulp and its antifungal applications. *Plants*, 10(7), 1422. <https://doi.org/10.3390/plants10071422>
- Shahidi, F., & Pan, Y. (2022). Influence of food matrix and food processing on the chemical interaction and bioaccessibility of dietary phytochemicals: A review. *Critical Reviews in Food Science and Nutrition*, 62(23), 6421–6445. <https://doi.org/10.1080/10408398.2021.1901650>
- Sharifi-Rad, M., Anil Kumar, N. V., Zucca, P., Varoni, E. M., Dini, L., Panzarini, E., Rajkovic, J., Tsouh Fokou, P. V., Azzini, E., Peluso, I., Prakash Mishra, A., Nigam, M., El Rayess, Y., Beyrouthy, M. El, Polito, L., Iriti, M., Martins, N., Martorell, M., Docea, A. O., ... Sharifi-Rad, J. (2020). Lifestyle, oxidative stress, and antioxidants: Back and forth in the pathophysiology of chronic diseases. *Frontiers in Physiology*, 11, 694. doi:10.3389/fphys.2020.00694.
- Shaw, P., Kumar, N., Sahun, M., Smits, E., Bogaerts, A., & Privat-Maldonado, A. (2022). Modulating the antioxidant response for better oxidative stress-inducing therapies: How to Take advantage of two sides of the same medal? *Biomedicines*, 10(4), 823. <https://doi.org/10.3390/biomedicines10040823>
- Singleton, V. L., Orthofer, R., & Lamuela-Raventós, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods in Enzymology*, 299, 152–178. [https://doi.org/10.1016/S0076-6879\(99\)90017-1](https://doi.org/10.1016/S0076-6879(99)90017-1)
- Thiruvengadam, M., Venkidasamy, B., Subramanian, U., Samynathan, R., Shariati, M. A., Rebezov, M., ... Chung, I. M. (2021). Bioactive compounds in oxidative stress-mediated diseases: Targeting the Nrf2/ARE signaling pathway and epigenetic regulation. *Antioxidants*, 10(12), 1859. <https://doi.org/10.3390/antiox10121859>
- Ulrich, K., & Jakob, U. (2019). The role of thiols in antioxidant systems. *Free Radical Biology and Medicine*, 140, 14–27. <https://doi.org/10.1016/j.freeradbiomed.2019.05.035>
- Wang, H., & Joseph, J. A. (1999). Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. *Free Radical Biology and Medicine*, 27(5–6), 612–616. [https://doi.org/10.1016/S0891-5849\(99\)00107-0](https://doi.org/10.1016/S0891-5849(99)00107-0)
- Wang, Y., Branicky, R., Noë, A., & Hekimi, S. (2018). Superoxide dismutases: Dual roles in controlling ROS damage and regulating ROS signaling. *In Journal of Cell Biology*, 217(6), 1915–1928. <https://doi.org/10.1083/jcb.201708007>
- Wang, Y., Chen, Y., Zhang, X., Lu, Y., & Chen, H. (2020). New insights in intestinal oxidative stress damage and the health intervention effects of nutrients: A review. *Journal of Functional Foods*, 75, Article 104248. <https://doi.org/10.1016/j.jff.2020.104248>
- Warnakulasuriya, S. N., Ziaullah, & Rupasinghe, H. P. V. (2016). Long chain fatty acid esters of quercetin-3-O-glucoside attenuate H<sub>2</sub>O<sub>2</sub>-induced acute cytotoxicity in human lung fibroblasts and primary hepatocytes. *Molecules*, 21(4), 452. <https://doi.org/10.3390/molecules21040452>
- Wojtunik-Kulesza, K., Oniszczuk, A., Oniszczuk, T., Combrzyński, M., Nowakowska, D., & Matwijczuk, A. (2020). Influence of in vitro digestion on composition, bioaccessibility and antioxidant activity of food polyphenols-A non-systematic review. *Nutrients*, 12(5), 1401. <https://doi.org/10.3390/nu12051401>
- Zhang, H., & Tsao, R. (2016). Dietary polyphenols, oxidative stress and antioxidant and anti-inflammatory effects. *Current Opinion in Food Science*, 8, 33–42. <https://doi.org/10.1016/j.cofs.2016.02.002>
- Zeb, A. (2020). Concept, mechanism, and applications of phenolic antioxidants in foods. *Journal of Food Biochemistry*, 44(9), Article e13394. <https://doi.org/10.1111/jfbc.13394>