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Valorization of local regional apple (*Malus domestica* Borkh.) cultivars versus commercial samples from Spain: Phenolic compounds by HPLC-MS/MS, cytotoxicity and biological potential on nitric oxide radicals and lipoxygenase inhibition

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ABSTRACT

Sustainability in the fruit industry and protection of local cultivars are nowadays important tasks for consumers and authorities. Phenolic compounds are interesting to explore because they play an important role in fruit-related health benefits. The phenolic profile of the regional and commercial Spanish apple cultivars was performed by high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). For a deeper understanding of their anti-inflammatory and antioxidant potential, the 5-lipoxigenase inhibition and NO^- scavenging were evaluated. Moreover, cellular viability was determined to perform MTT assay in HepG2 and HeLa cell lines to discard cytotoxicity. The results showed that procyanidin B2 (PB2), 3-caffeoylquinic acid, epicatechin and 4-hydroxy benzoic acid were the most abundant phenolic compounds. Autochthonous apple cultivars showed better 5-LOX/NO^- inhibition and nontoxic effects in the tested cell lines with an interesting profile of phenolic compounds. Local autochthonous apples can be considered a better source of bioactive compounds than the commercial samples.

1. Introduction

Consuming specific individual nutrients naturally present in plant foods may be linked to the prevention of many human diseases. However, this association cannot account for the overall protective impact of plant-based foods on health. Consequently, there is speculation that other non-nutritive components found in plants, such as polyphenolic compounds, might contribute to the observed beneficial effects of fruits and vegetables intake (Hyson, 2011).

The most abundant (poly)phenolic compounds, also known as polyphenols, subclasses in human diet are flavonoids and phenolic acids. Flavonoids share a basic C6-C3-C6 common carbon skeleton and they are divided into different classes based on molecular structure (Hyson, 2011; Ramos, 2007). Particularly in apples, significant quantities of flavanols, flavonols, and anthocyanidins as well as dihydrochalcones but also hydroxycinnamic acids are present (Tsao, Yang, Xie, Sockovie, & Khanizadeh, 2005).

Antioxidant potential is one of the main properties of phenolic compounds. They may play an important role in human diet scavenging free radicals and reactive oxygen species (ROS), reducing their negative effect over a wide range of biomolecules (Wojdyło & Oszmiański, 2020).

However, inflammatory mediators may also become a critical issue in the development of certain chronic and metabolic disorders such as obesity, type-2 diabetes or cardiovascular risk. Chronic inflammation related gene expression, such as cyclo-oxygenase-2, 5-LOX (5-lipoxygenase) suggests their link with cancer (Sethi, Shanmugam, Ramachandran, Kumar, & Tergaonkar, 2012). 5-LOX is involved with proinflammatory leukotrienes metabolism and the overexpression of the enzyme has been related to renal cell carcinoma (Faronato et al., 2007), visceral fat depots or insulin resistant adipokines (Martínez-Clemente, Clària, & Titos, 2011).

Bioavailability of those phytochemicals modulates the in vivo

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effects. For example, the anti-inflammatory potential is focused on tissues exposed to higher phenolics concentrations such as digestive system (Fraga & Oteiza, 2011) and even gut microbiota (Wu et al., 2021). Phenolic compounds from apples have been related to the regulation of inflammation mediated by molecules such as cytokines (TNF-α and IL-6), and prostaglandin E2 (M. C. Denis et al., 2013). Moreover, they seem down regulate pro inflammatory cytokines and even improve mitochondrial dysfunction in some intestinal diseases (M.-C. Denis et al., 2016).

Regarding those approaches, the phenolic profile should play a key role in explaining potential health benefits. The availability of phenolics compounds in apple fruit varies significantly among cultivars (Bondonno et al., 2020) and their presence is influenced by the growing season and environmental factors such as their harvest point or climate (Tsao et al., 2005) (Serra, Anthony, Boscolo Sesillo, Masia, & Musacchi, 2021). Although this variability, the most common phenolic compounds found in apples are generally flavanols, dihydrochalcones, flavan-3-ols and phenolic acids (Rana & Bhushan, 2016). Moreover, accumulation of phenolics varies on the apple tissues, obtaining big differences between peel, pulp, or leaves. This may suggest the direct influence of genetic factors for individual phenolic compounds and their total polyphenol content (TPC) accumulation (Volz & McGhie, 2011).

Despite widespread data resources linking beneficial properties of phenolic compounds intake, they represent huge chemical heterogeneity and their potential toxicity should be evaluated and supported to reveal any harmful cellular effects (Boncler et al., 2017). Elevated dietary phenolics consumption has been linked to the alteration of physiological conditions for the transport of thiamin, folic acid, the activity of certain drugs, and even their transporters (Sinha, Sachan, Bhattacharya, Singh, & Parthasarathi, 2022).

The aim of this research is to determine the phenolic profile of 15 commercial and autochthonous apple pulp extracts. Additionally, cytotoxicity tests were performed on two human cell lines to identify possible alterations in cell viability. We also evaluated their effects on enzymatic and non-enzymatic systems to gain a deeper understanding of their influence in anti-inflammatory-related experiments. These results could enhance the value of certain apple cultivars and their potential health benefits, promoting the consumption and preservation of autochthonous accessions due to their bioactivity.

2. Material and methods

2.1. Apple samples

The selection of samples was based on a project in which local and commercial samples are compared to establish significant differences in terms of composition and bioactivity. Apples were obtained/collected from different areas of Aragón (for the commercial samples Pinova, Verde Doncella, Royal Gala, and the local cultivars Pomera del País, Pomera de Pomes agrias, Doncella de Martin, Esperiega, Helada, Borau 01 and Amarilla de Octubre) and from Navarra (local cultivars known as Arraiza, De Mine, Gordoncha, M. Tomate and Ziordia), Spain (Supplementary 1). Selected samples were collected between August and October 2021 at the ripening period of each cultivar. Pinova, Verde Doncella and Royal Gala were the commercial samples whereas the rest of them were considered local and autochthonous accessions. Phenolic compounds were extracted by using **acidic methanol** in a 33:1 (*v/w*) mixture of solvent:lyophilized pulp tissue and ultrasonication. The solvent was eliminated in a rotatory evaporator as described in previous work (Millán-Laleona et al., 2023).

2.2. Determination of phenolic compounds by HPLC-MS/MS

2.2.1. Reagents and standards

Cyanidin-3-glucoside chloride, delphinidin-3,5-diglucoside chloride, delphinidin-3-galactoside chloride, petunidin-3-glucoside chloride, malvidin-3-galactoside chloride, quercetin-3-glucoside and kaempferol-3-glucoside were purchased from PhytoLab (Vestenbergsgreuth, Germany). The remaining 31 analytical standards of the 38 phenolic compounds were supplied by Sigma-Aldrich (Milan, Italy). Individual stock solutions of each analyte, at a concentration of 1000 mg L^{-1}, were prepared by dissolving pure standards in HPLC-grade methanol. All solvents and solutions were filtered through a 0.2 μm polyamide filter from Sartorius Stedim (Goettingen, Germany). Before HPLC analysis, all samples were filtered with Phenex™ RC 4 mm 0.2 μm syringeless filter, Phenomenex (Castel Maggiore, BO, Italy).

2.2.2. HPLC-ESI-MS/MS analysis

HPLC-MS/MS studies were performed using an Agilent 1290 Infinity series and a Triple Quadrupole 6420 from Agilent Technology (Santa Clara, CA) equipped with an electrospray ionization (ESI) source operating in negative and positive ionization modes following a previous developed method in our laboratory (Mustafa et al., 2022). The separation of target compounds was achieved on a Synergi Polar–RP C18 analytical column (250 mm × 4.6 mm, 4 μm) from Phenomenex (Chesire, UK). The column was preceded by a Polar RP security guard cartridge (4 mm × 3 mm ID). The mobile phase was a mixture of (A) water and (B) methanol, both with formic acid 0.1 %, at a flow rate of 0.8 mL min^{-1} in gradient elution mode. The composition of the mobile phase varied as follows: 0–1 min, isocratic condition, 20 % B; 1–25 min, 20–85 % B; 25–26 min, isocratic condition, 85 % B; 26–32 min, 85–20 % B. All solvents and solutions were filtered through a 0.2 μm polyamide filter from Sartorius Stedim (Goettingen, Germany). The injection volume was 2 μL. The temperature of the column was 30 °C, and the temperature of the drying gas in the ionization source was 350 °C. The gas flow was 12 L/min, the nebulizer pressure was 55 psi, and the capillary voltage was 4000 V. Detection was performed in the dynamic-multiple reaction monitoring (dynamic-MRM) mode. The selected ion transitions and the mass spectrometer parameters for the analyzed compounds are reported in Supplementary 2.

2.3. Cell cultures and cell viability assay

HepG2 (liver cancer cell line) and HeLa (cervical cancer cell line) were purchased from ATCC. They were cultured in DMEM medium (Sigma Aldrich), 10 % Fetal bovine serum (FBS) (Sigma Aldrich) and 1 % penicillin-streptomycin (Sigma Aldrich) in the presence of 5 % CO2 at 37 °C.

Cytotoxicity was evaluated using the MTT-based colorimetric method (Mosmann, 1983). HepG2 and Hela cells were seeded in 96-well plates at 4×10^4 and 1.5×10^4 cells/well respectively. After 24 h, cells were treated with various concentrations of the extract for 24 h (from 32.5 to 500 μg/mL in HepG2 and Hela). The total cell culture medium was removed, and then 100 μL of MTT solution (0.4 mg/mL in DMEM) was added to each well. The plate was incubated at 37 °C 5 % CO_2 for 2.5 h. Finally, MTT solution was removed and replaced with 100 μL of DMSO. Agitation of the plates was performed to dissolve the formazan crystals in DMSO for 15 min at 70 rpm. The absorbance was read at 550 nm in a Synergy H1 Hybrid Multi-Mode Reader (Biotek, Bad Friedrichshall, Germany). This assay was performed over three days and different weeks and passages. The cell viability was calculated according to the following formula (Eq. (1)):

$$\textit{Cell viability}\ (\%) = \frac{\textit{Abs sample}}{\textit{Abs control}} \times 100 \quad (1)$$

2.4 Nitric oxide-scavenging activity

Different concentrations of the apple pulp extracts were dissolved in phosphate buffer 0,1 M pH 7.4 and mixed with the substrate sodium nitroprusside dihydrate (Sigma Aldrich). Extracts (at the range 0–1000 μg/ml) with sodium nitroprusside mixtures were incubated 60 min at

room temperature under a source of light. Griess reagent (Sigma Aldrich) was added to the mixture after the incubation. The absorbance was measured at 560 nm after 10 min of dark incubation. Quercetin (Fluorochem, Barcelona, Spain) was performed as positive control from 62,5 to 250 μg/ml (Ebrahimzadeh, Pourmorad, & Hafezi, 2008). The procedure is based on the assay by Sreejayan & Rao (Sreejayan & Rao, 1997). Each measurement was repeated three times, in triplicate and results are expressed in μg/ml.

2.5 5-Lipoxigenase (5-LOX) inhibition assay

The potential interference with the enzymatic activity of 5-LOX was determined by the linoleic acid oxidation, according to a previously described procedure (Macedo et al., 2020). Inhibitory reactions were performed at a final volume of 240 μL, containing 200 μL of phosphate buffer (pH = 9), 20 μL of soybean 5-LOX (EC 1.13.11.12; Sigma-Aldrich, St. Louis, MO, USA) and 20 μL of extract. Preincubation at 25 °C was followed by the addition of linoleic acid (20 μL, 4.18 mM), formation of its oxidation product being followed for 3 min. The absorbance was measured spectrophotometrically at 234 nm. Each measurement was repeated three times, in triplicate and results are expressed in μg/ml (from 0 to 1000 μg/ml of extract).

2.4. Statistical analysis

Each experiment was performed at least three times on different days and results were expressed as the mean ± standard error of the mean (SEM) of different assays. GraphPad Prism v.6 (GraphPad Software, San Diego, CA, USA) was required to perform data analyses, nonlinear regressions, and statistics. Bioactivity studies assays were statistically analyzed by using ANOVA following Tukey post-test.

3. Results

3.1. Characterization of phenolic compounds by HPLC MS/MS

We monitored and quantified 38 individual phenolic compounds of interest in apple pulp extracts from different accesions (Table 1). Three of the autochthonous samples, Amarilla de Octubre, Pomera de Pomes agrias and Ziordia have major TPC than the rest of apple varieties (Table 1). Particularly, Amarilla de Octubre contains more than 10 times total polyphenolic content (108,446.71 μg/g) than commercial apples such as Royal Gala, Verde Doncella and Pinova (10,700.20, 7511.54 and 7378.12 μg/g respectively).

Twenty among the 38 monitored phenolic compounds were found and quantified in the fifteen apple samples analyzed Table 1). The highest number of the monitored phenolics (13 different analytes) was found in “Doncella de San Martin”, “Verde Doncella” and “Pinova” meanwhile twelve different polyphenolic compounds were detected in “Amarilla de Octubre” sample. The lowest number of analytes (6) was found in “Pomera del pais” apple sample. Considering the amount of the targeted analytes, the total ranged from 7378.12 μg/g (Pinova) to 108,446.71 μg/g (Amarilla de Octubre) being Procyanidin B2 was the most abundant targeted analyte in the different samples with concentrations ranging from 362.20 μg/g (Gordoncha) to 70,236.55 μg/g (Amarilla de Octubre). The two dihydrochalcons, phloridzin and phloretin, were quite abundant, with levels ranging from 67.89 μg/g (Verde Doncella) to 1160.08 μg/g (Pompera del Pais) for phloridzin and from 429.09 μg/g (M. Tomate) to 1009.28 μg/g (Ziorda) for phloretin. According to the hydroxycinnamic acids family, the main compound was 3-caffeoylquinic acid whose levels varied from 714.47 μg/g (Verde Doncella) to 15,653.70 μg/g (Amarilla de Octubre). The last sample also showed the highest concentration of neochlorogenic acid (84.82 μg/g) and 3,5-dicaffeoylquinic acid (150.14 μg/g) among all. Regarding the flavan-3-ols subclass, epicatechin and catechin were found at varying levels in the different apple samples with concentrations ranging from 49.48 μg/g (Pomera del pais) to 18,029.00 μg/g (Amarilla de Octubre) for epicatechin, and from 90.33 μg/g (Pinova) to 3489.20 μg/g (Amarilla de Octubre) for catechin, respectively. Other phenolic compounds were quite common in the different apples such as 4- and 3- hydroxy benzoic acid, ellagic acid and others. Phenolic acid such as ferulic, vanillic, syringic, and coumaric acid were found in any samples meanwhile gallic acid was found only in three samples, i.e. “Doncella de San Martin”, “Verde Doncella” and “Pinova” in low amount.

The flavanols dominated in most autochthonous apples and all commercial varieties, particularly epicatechin and procyanidin B2 (PB2) reaching percentages of 16.6 and 64.8 respectively in the sample Amarilla de Octubre. 3-caffeoylquinic acid was also abundant (for instance 14.4 % in Amarilla de Octubre) except for the group of Ziordia, Gordoncha, Arraiza, De Mine and M. Tomate. Ziordia showed the major TPC with some compositional differences in comparison to some autochthonous and the commercial varieties (Figs. 1A). Other compounds like 4-hydroxy benzoic acid (4HA) and 3-hydroxy benzoic acid have an important presence in contrast to the absence of epicatechins on this group of five autochthonous apple varieties.

Ellagic acid just appears as an important compound for Pomera del pais, achieving 59.89 % of its TPC. Other phenolic compounds such as phloretin or its glycoside form, phloridzin, are also part of most samples but regarding their TPC, their presence remain minoritarian.

These phenolic compounds were also analyzed but they are not present at none of the apple pulp extracts in Table 1: 3-hydroxy benzoic acid, caffeic acid, vanillic acid, resveratrol, syringic acid, procyanidin A2, p-coumaric acid, ferulic acid, naringin, rutin, myricetin, delphindin-3-galactoside, cyanidin-3-glucoside, pentunidin-3-glucoside, pelargonidin-3-glucoside, malvidin3–galactoside, hesperidin, trans-cinnanic acid and kaempferol.

3.2. Cell viability

The widely used MTT assay was performed to determine mitochondrial activity to elucidate antiproliferative effects in two different tumour cell lines. HepG2 did not show significant differences in viability at tested concentrations with any phenolic extract obtained from the region of Aragón (Fig. 2 A) or the region of Navarra (Fig. 2 B). Despite Hela cells had more susceptibility to apple extracts, they do not affect to their viability at tested concentrations (Fig. 2 C and D), which corroborates the absence of toxicity for these samples…

3.3. Nitric oxide-scavenging and 5-lipoxigenase (5-LOX) inhibition assays

Nitric oxide scavenging was tested with quercetin, a potent antioxidant flavonoid, as positive control, achieving an IC_{50} of 60.73 ± 4.73 μg/ml. Apple pulp extracts were tested from 62.5 to 1000 μg/ml. Only the sample known as Pomera de Pomes Agrias, Amarilla de Octubre, Doncella de San Martin and Arraiza showed some remarkable NO^- scavenging activity. Comparing them at 1000 μg/ml significant differences were found between Pomera de Pomes Agrias, Amarilla de Octubre and Doncella de San Martin against Arraiza (Fig. 3). Commercial samples, known as Royal Gala, Pinova and Verde Doncella, were not able to scavenge nitric oxide radicals.

Inhibition of the 5-lipoxigenase enzyme was performed for a deeper understanding of enzymatic anti-inflammatory potential mechanisms and as complementary assay of NO^- scavenging. Quercetin was also used as positive control tested from 0.625 to 20 μg/ml achieving an IC_{50} of 3.52 ± 9.15 μg/ml.

According to apple pulp samples, we tested concentrations from 62.5 to 1000 μg extract/ml. Despite previous results in NO^- scavenging assay, Doncella de San Martín did not achieve an $IC_{50} < 1000$ μg/ml. However, Pomera de Pomes Agrias (IC_{50} 578.76 ± 5.73 μg/ml), Ziordia (IC_{50} 660.24 ± 6.29 μg/g), Arraiza (IC_{50} 709.84 ± 6.00 μg/g) and Amarilla de Octubre (IC_{50} 891.12 ± 6.71 μg/g) were the extracts with inhibitory potential. Significant differences were observed when comparing those

Table 1
Quantitative determination of the analyzed phenolic compounds in the 15 samples (μg phenolic compounds/g extract) ordered by their TPC by HPLC MS/MS.

Compounds	Amarilla de Octubre	Pomera de Pomes agrias	Ziordia	Manzana helada	Esperiega de Ademuz	Doncella de San Martin	Gordoncha	Arraiza	Borau 01	De Mine	M. Tomate	Royal Gala	Pomera del pais	Verde Doncella	Pinova
Gallic acid	n.d.	n.d.	n.d.	n.d.	n.d.	18.86	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	14.12	26.36
5-caffeoylquinic acid	84.82	32.44	n.d.	n.d.	n.d.	17.03	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Catechin	3489.20	2077.58	n.d.	640.45	1385.76	934.82	n.d.	n.d.	144.88	n.d.	n.d.	507.79	n.d.	514.35	90.33
Procyanidin B2	70,236.55	25,344.97	1743.71	11,706.32	3799.72	9918.01	362.20	n.d.	3897.39	542.33	342.01	4379.36	n.d.	4296.40	2871.02
3-caffeoylquinic acid	15,653.70	8933.89	10,948.54	7292.10	11,256.09	5963.69	2447.87	4558.89	9786.04	4196.51	3509.76	4266.80	1761.72	714.47	2848.37
4-hydroxy benzoic acid	n.d.	n.d.	18,115.15	n.d.	n.d.	n.d.	11,991.45	9280.61	n.d.	7401.07	8225.38	n.d.	n.d.	n.d.	n.d.
Epicatechin	18,029.00	8069.33	n.d.	2776.88	1750.12	1469.36	n.d.	n.d.	854.74	n.d.	n.d.	1313.13	49.48	864.72	761.91
3,5-dicaffeoylquinic acid	150.14	45.23	n.d.	12.06	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Isoquercitrin	23.63	24.97	n.d.	38.54	27.22	24.42	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	39.40	13.15
Delphindin 3,5 diglucoside	20.51	15.58	n.d.	37.05	24.98	20.85	n.d.	n.d.	n.d.	n.d.	n.d.	9.01	n.d.	34.03	9.40
Phloridzin	269.51	913.02	n.d.	643.48	1024.95	299.94	n.d.	n.d.	436.19	n.d.	n.d.	156.84	1160.08	67.89	124.65
Quercitrin	63.50	12,01	39.40	82.55	13.56	68.15	6.45	n.d.	73.96	n.d.	n.d.	35.43	n.d.	44.16	40.69
Kaempferol-3-glucoside	n.d.	n.d.	41.11	n.d.	n.d.	n.d.	41.21	94.79	n.d.	28.44	57.36	n.d.	n.d.	31,62	n.d.
Ellagic acid	n.d.	n.d.	n.d.	n.d.	n.d.	420.51	21.80	41.83	n.d.	16.01	23.93	n.d.	4505.83	778.72	543.71
Quercetin	n.d.	n.d.	19.59	n.d.	n.d.	n.d.	19.10	34.60	n.d.	12.30	23.07	n.d.	n.d.	n.d.	n.d.
Phloretin	n.d.	n.d.	1009.28	n.d.	n.d.	n.d.	354.86	638.25	n.d.	832.72	429.09	n.d.	n.d.	n.d.	n.d.
Isorhamnetin	n.d.	n.d.	42.05	n.d.	n.d.	n.d.	14.50	44.68	n.d.	20.18	36.61	n.d.	6,08	n.d.	n.d.
Pelargonidin-3-rutinoside	395.49	12.09	n.d.	55,71	19.05	68.80	n.d.	n.d.	19.29	n.d.	n.d.	22.66	n.d.	35.42	23.15
Hyperoside	30.66	24.54	n.d.	50.69	36.42	43.75	n.d.	n.d.	10.45	n.d.	n.d.	9.16	39.33	76.26	25.38
TPC (μg/g)	**108,446.71**	**45,505.66**	**34,970.54**	**23,335.84**	**19,337.87**	**19,268.19**	**15,911.58**	**15,588.61**	**15,222.95**	**13,977.26**	**13,325.86**	**10,700.20**	**7522.52**	**7511.54**	**7378.12**

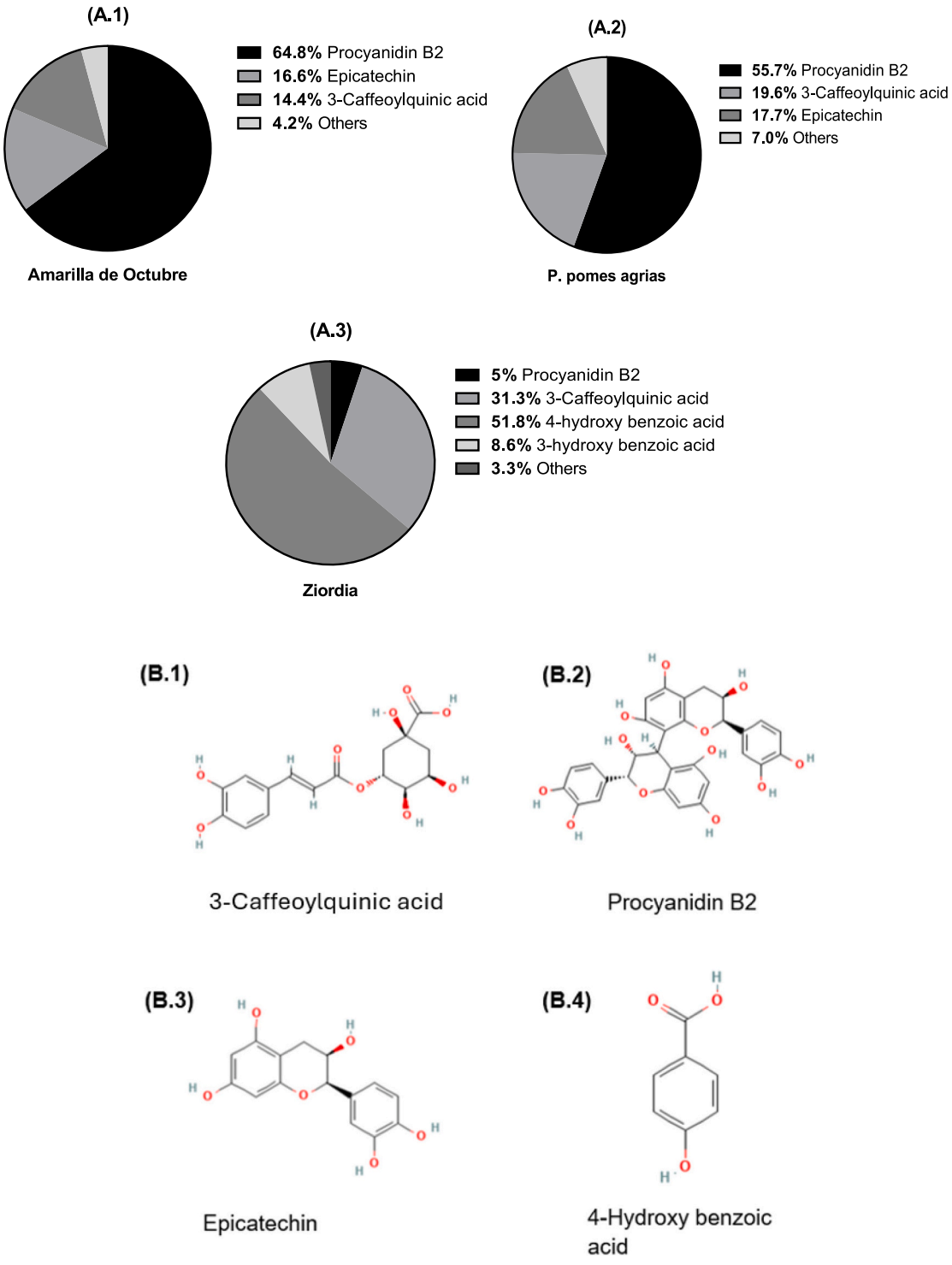

Fig. 1. (A) Representation of the individual phenolic compounds of the three apple pulp extracts with major TPC, Amarilla de Octubre, Pomera de pomes agrias and Ziordia, (A.1, A.2 and A.3 respectively). (B) Chemical structure of the most abundant phytochemicals found in the varieties with major TPC: procyanidin B2, 3-caffeoylquinic acid, epicatechin and 4-hydroxy benzoic acid (B.1, B.2, B.3, B.4 respectively) (PubChem, 2024).

apple pulp extract varieties to their negative controls (Fig. 4). The rest of apple pulp samples did not show activity differences in comparison to the negative control at the tested concentrations. Like the NO^- scavenging assay, none of the commercial samples (Royal Gala, Pinova or Verde Doncella) were able to exhibit bioactivity modulation through 5-LOX inhibition.

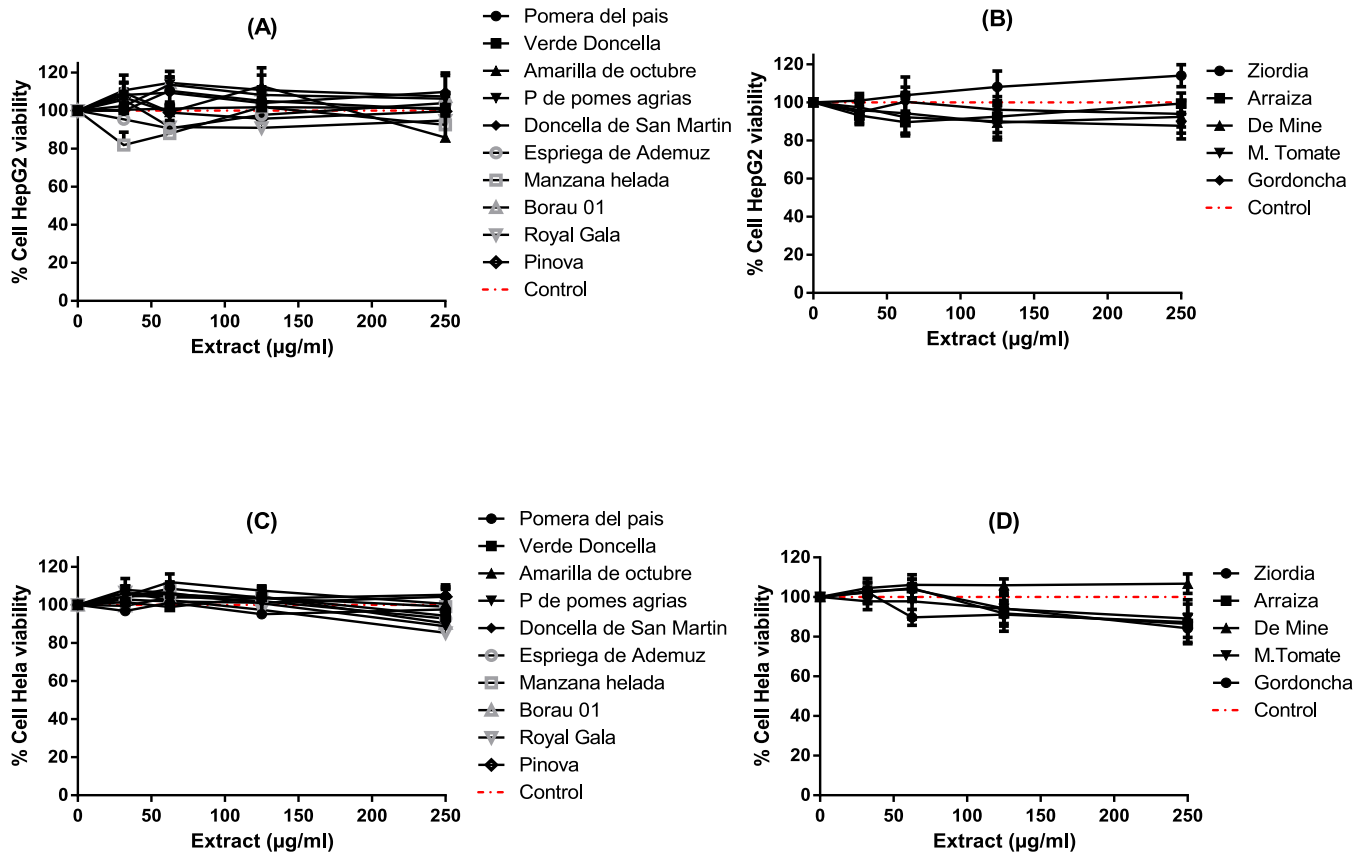

Fig. 2. Cell viability percentage expressed as the mean of different concentrations in micrograms per millilitre (from 32.5 to 250 µg extract/ml) of the sample pulp extracts in HepG2 (A and B) and Hela cell cultures (C y D). No significant differences were found versus control. *Note: Results are expressed as average SEM of at least three independent experiments. ANOVA with post-hoc Tukey test was performed for the statistical analysis.*

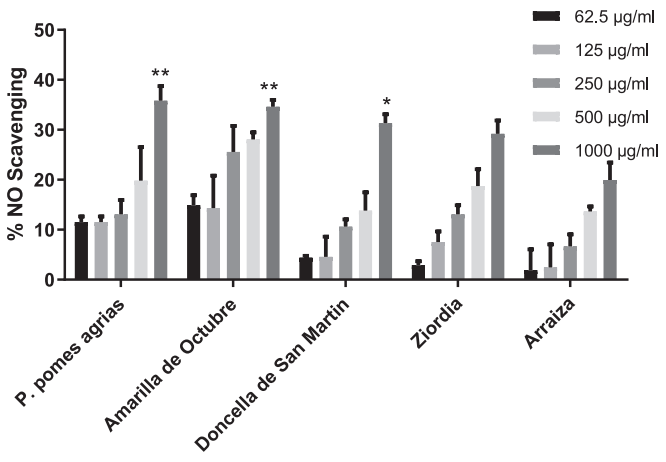

Fig. 3. NO^{-} scavenging percentage expressed as the means of different concentrations in micrograms per millilitre (from 62.5 to 1000 µg extract/ml) of apple pulp extracts. Significant differences ($p < 0{,}005$) are pointed between Arraiza and the rest of apple pulp samples at 1000 µg/ml. *Note: Results are expressed as average SEM of at least three independent experiments. ANOVA with post-hoc Tukey test was performed for the statistical analysis.*

4. Discussion

Regarding the phenolic pulp composition, autochthonous local varieties contain a higher total phenolic content. Interestingly, Amarilla de Octubre is an outstanding sample with 10 times more TPC (108,446.71 µg/g) than commercial samples such as Pinova, Royal Gala or Verde Doncella (7378.12, 7511.54 and 10,700.20 µg/g respectively). Commercial varieties show in general lower values of total phenolic compounds quantified by HPLC MS/MS but these results confirm other previous studies performed with other spectrophotometric methodologies like the Folin-Ciocalteu assay (Millán-Laleona et al., 2023).

It is important to consider individually its composition, appearing PB2 as the main compound for most apple varieties except for Ziordia, Gordoncha, Arraiza, De Mine and M. Tomate varieties. Regarding the sample with the highest TPC content, Amarilla de Octubre, the percentage of PB2 achieve a 64.76 % of the TPC. Previously, PB2 has been related to high antioxidant activity (Bai, Zhang, & Ren, 2013), neuronal protection due to scavenging potential against reactive species (Sutcliffe, Winter, Punessen, & Linseman, 2017) and also hair growth stimulant factor because its protective action against cellular apoptosis (Tenore et al., 2018).

Additional studies have also linked the PB2 consumption with the improvement metabolic syndromes on induced fat mice due to its anti-inflammatory potential in terms of triglyceride accumulation in the liver and insulin resistance (Sutcliffe et al., 2017). Several inflammatory processes seem to be modulated by procyanidins, such as mitogen-activated protein kinase (MAPK) or the arachidonic acid pathways (Martinez-Micaelo, González-Abuín, Ardèvol, Pinent, & Blay, 2012).

Another abundant compound are epicatechins, molecules related to mitochondrial functions modulation (Bernatoniene & Kopustinskiene, 2018). Variations in epicatechin content is related its dimer degradation, PB2. This fact explains the major presence of those two compounds in most varieties like in Amarilla de Octubre, Pomera de Pomes agrias or Manzana helada.

In addition, 3-caffeoylquinic acid, a compound with an antioxidant capability similar to that of ascorbic acid (Veberic et al., 2005), is also a

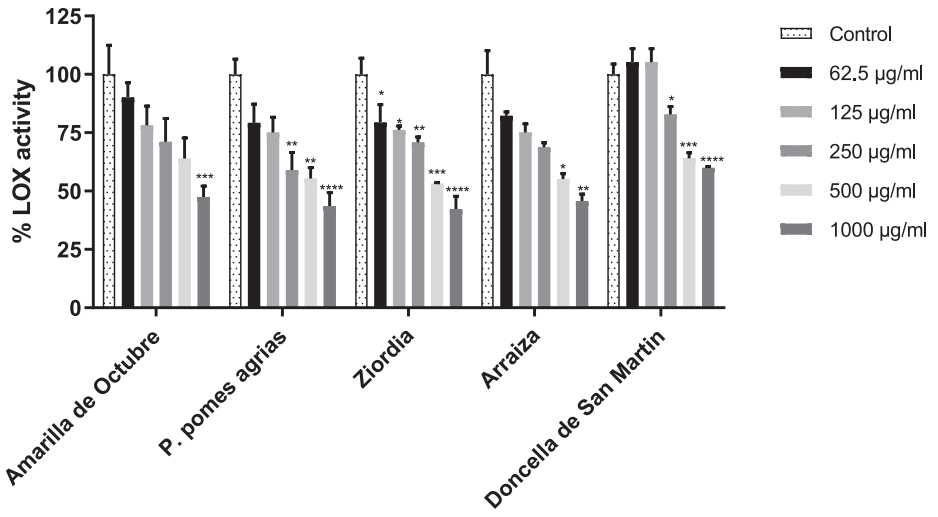

Fig. 4. LOX activity percentage results expressed as the means of different concentrations in micrograms per millilitre (from 62.5 to 1000 µg extract/ml) of apple pulp extracts. Significant differences were marked versus their controls. *Note: Results are expressed as average SEM of at least three independent experiments. ANOVA with post-hoc Tukey test was performed for the statistical analysis.*

main phenolic acid for most extracts, including an important fraction of Amarilla de Octubre and Pomera de Pomes agrias composition. Commercial varieties also contain significant fractions of 3-caffeoylquinic acid. However, upon comparing HPLC MS/MS results and considering the TPC, Verde Doncella, for instance, exhibits an almost 22-fold lower total content of this compound compared to Amarilla de Octubre.

3-Caffeoylquinic acid has also been related to antioxidant and anti-inflammatory properties. Modulation of the levels of serum TNF-α, nitric oxide synthase activity and cyclooxygenase-2 inhibition on liver show the relationship between this molecule and health benefits (Tajik, Tajik, Mack, & Enck, 2017). Moreover, decreasing the secretion of pro-inflammatory cytokines IL-8 and IL-6 (Melillo de Magalhães et al., 2012) or regulation of nitric oxide levels on rats reinforce this evidence (Bagdas et al., 2015).

Interestingly, the group of samples composed by Ziordia, Gordoncha, Arraiza, De Mine and M. Tomate, cultivated all of them in the region of Navarra, showed different phenolic composition, appearing 4-hydroxibenzoic acid (4HA), and not the PB2, as the most abundant analyte. 4HA is a well-known molecule used as maintaining agent in food and cosmetic industry due to its low toxicity and antioxidant capacity (Das, Kumar, Wankhade, & Mandavgane, 2022). It is also related to neuro and cardio protective effects (Hurtado-Barroso et al., 2019). Nonetheless, none of this autochthonous samples showed epicatechin content by HPLC MS/MS analysis. This remarkable variation in the apple pulp profiles could be attributed to the lower PB2 content observed for this group of samples because epicatechins are oligomers of these molecules (Fraga, Oteiza, & Galleano, 2018). Other phenolics traditionally related to apple flesh such as quercetin derivates (Jakobek, García-Villalba, & Tomás-Barberán, 2013) does not appear on this pulp extracts.

According to these results, they suggest the environment as one important factor involved for individual accumulation and the presence of these phytochemicals changes widely among apple varieties, influenced by factors such as location, season, light exposure, and altitude (Shafi et al., 2019). Additionally, phenolic compounds accumulation is an important strategy to confront biotic and abiotic stress particularly in wild varieties (Varela, Arslan, Reginato, Cenzano, & Luna, 2016). They act as part of non-enzymatic mechanisms to maintain physiological levels of reactive oxygen species (ROS) to avoid potential cellular damage (Singh, Kaur, & Kariyat, 2021).

Cytotoxic and antiproliferative activities mediated by phenolic compounds can be regulated via several cellular mechanisms such as caspase enzymes, tumour vascularization or even induction of mitochondrial damage (Davatgaran-Taghipour et al., 2017). Despite this potential effect, we did not observe toxicity in tested cell cultures by MTT assay (Kumar, Nagarajan, & Uchil, 2018). We decided to employ the HepG2 cell line to investigate the potential toxicity resulting from the hypothetical interaction of these compounds with hepatic human cells, given their detoxifying properties due to previous studies related to hepatotoxicity linked to an excessive phenolic concentration (Duda-Chodak & Tarko, 2023). Despite the high phenolic content of Amarilla de Octubre or Pomera pomes agrias, we did not observe a significant decrease in HepG2 cell viability. In addition, to accomplish a deeper toxicity test, we decided to probe them against Hela cells with similar results. Apple pulp extracts did not show important antiproliferative data at tested extract concentrations. Due to the basis of MTT assay, we can also conclude they do not affect negatively to mitochondrial activity. However, antioxidants can exhibit dual roles in cancer cells, they act as inhibitors of tumorigenesis while simultaneously maintaining tumour surveillance by preventing oxidative damage to DNA (Saeidnia & Abdollahi, 2013) (Davatgaran-Taghipour et al., 2017). This condition could explain the fact that, for instance, Ziordia shows an increment of viability when we added any of the extract concentration tested to the Hela cell cultures.

As we already know, NO^- has an important presence in pro-inflammatory process. Those autochthonous pulp extracts showed scavenging properties against nitrogen reactive species, underlining the protective action of bioactive dietary compounds in the non-enzymatic regulation of RNs (Ji, Gong, Li, Wang, & Li, 2020). Considering the three extracts with the lowest IC_{50} for NO- scavenging assay (Pomera de pomes agrias, Amarilla de Octubre and Doncella de San Martín), we cannot relate linearly the TPC with the effect observed. Those results suggest that individual phenolic compounds combination could provide better results than just a higher TPC.

For these varieties, PB2, 3-caffeoylquinic acid and epicatechin are the predominant compounds, suggesting a relationship between the scavenging activity and the presence of these phytochemicals. On the other hand, Ziordia and Arraiza apples may justify their promising result due to their high 4HA content (51,80 % and 59,53 % of their TPC respectively), another close related antioxidant compound. Moreover, the impact on 5-LOX activity enhances the potential effect observed in the previous NO^- scavenging assay in most samples. This enzymatic assay showed that Pomera de pomes agrias, Ziordia, Arraiza and Amarilla de Octubre were the extracts with inhibitory potential with the lowest IC_{50} (578.76 ± 5.73, 660.24 ± 6.29, 709.84 ± 6.00 and 891.12 ± 6.71 µg/g respectively). However, no correlations were found between de % of each phenolic compound of the samples with the

inhibitory 5-LOX activity.

Despite regulation of the inflammatory process is very complex and involves multiple enzymes, these results highlight the influence of phenolics as potential modulators. They could represent a complementary approach in which natural compounds from food sources may alleviate proinflammatory processes (Charlier & Michaux, 2003).

Phytochemical variations in apple pulp extract composition may influence the NO^- scavenging assay and 5-LOX inhibition results. None of the commercial varieties (Royal Gala, Pinova or Verde Doncella) seems to achieve the potential of the autochthonous apples. Nevertheless, Amarilla de Octubre, Pomera de pomes agrias and Ziordia are, regarding both assays, the most promising samples with anti-inflammatory potential at tested conditions. This is, to the best of our knowledge, the first approach between Spanish autochthonous and commercial apples in terms of phenolic composition analysing individual phenolics and health benefits related to inflammatory mediators and bioassays.

5. Conclusions

Autochthonous apple cultivars present, in general, a higher total phenolic content than commercial apples, being in some cases up to ten times higher. The main phenolic compounds detected in the samples by HPLC MS/MS analysis were phenolic acids like 3-caffeoylquinic acid or 4-hydroxybenzoic acid and flavonoids such as procyanidin B2 and epicatechins. Amarilla de Octubre, Pomera de pomes agrias and Ziordia are the apple pulps with the most promising polyphenolic content. Despite the elevated differences in composition, apple pulp extracts were not toxic at physiological or high concentrations for the cell lines tested. NO^- scavenging and 5-LOX inhibition significant effects were observed also for the autochthonous local Spanish accessions known as Amarilla de Octubre, Pomera de pomes agrias and Ziordia.

In conclusion, traditional local varieties are a better source of natural antioxidants and phenolic compounds than commercial apples without any toxic effects on cell cultures. Although further analysis is required, this study reveals an initial association between apple pulp phenolics and the potential inhibition of some inflammatory pathways, which could be linked to the maintenance of a healthy lifestyle.

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Ethical statement

The authors declare that this study was conducted without animal nor human samples.

CRediT authorship contribution statement

Adrián Millán-Laleona: Writing – original draft, Methodology, Investigation, Formal analysis. **Pilar Cebollada:** Methodology, Investigation. **Giovanni Caprioli:** Investigation, Formal analysis. **Diletta Piatti:** Investigation, Formal analysis. **Filippo Maggi:** Investigation. **Ana Pina:** Resources. **Carlota Gómez-Rincón:** Conceptualization. **Víctor López:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The funders had no role in study design, data collection, analysis, decision to publish, or preparation of the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2024.106631.

Data availability

Data will be made available on request.

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