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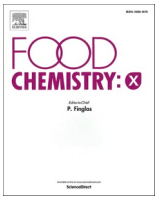

Phenolic compounds and multifunctional properties of thinned apples: revalorizing fruit by-products for industrial applications

Javier Cano-Lou [a,b], Gema Casado-Hidalgo [a], Francisco Les [a,b], Ana Pina [c], Laura Acquaticci [d], Giovanni Caprioli [d], Víctor López [a,b,*]

[a] Department of Pharmacy, Faculty of Health Sciences, Universidad San Jorge, 50830 Zaragoza, Spain
[b] Institute of agri-food research of Aragón, , IA2, Zaragoza University-CITA, 50013 Zaragoza, Spain
[c] Unidad de Hortofruticultura, Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA), Av Montañana 930, 50059 Zaragoza, Spain
[d] Chemistry Interdisciplinary Project (ChIP), School of Pharmacy, University of Camerino, Via Madonna delle Carceri, 62032 Camerino, Italy

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ABSTRACT

Thinned apples are a common by-product of apple orchards, produced in abundant quantities through thinning or natural drop, leading to the loss of substantial amounts of phenolic compounds with potential bioactive properties. Ten thinned apple samples, representing both commercial and autochthonous cultivars, were ultrasonicated using ethanol as green solvent, with the main goal of reusing their phenolic content for industrial and nutritional applications. For this reason, the phenolic content was characterized with high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS). The antioxidant, antidiabetic, and cardiovascular activities were evaluated and correlated with the targeted phenolic compounds. Under non-cytotoxic conditions in Caco-2, HepG2 and HFF-1 cells, Amarilla de Octubre and Pomera de Pomes Agrias showed the strongest antioxidant-antiglycation activity as well as ACE inhibition, while Reineta and Verde Doncella excelled in HMGR and ACE inhibition. Pomera de Pomes Agrias, Vadiello-02, Reineta and Verde Doncella led digestive enzymes under Caco-2 safety.

1. Introduction

Metabolic disorders are non-communicable diseases with a great impact nowadays, particularly in developed countries (Noubiap et al., 2022). Between them, metabolic syndrome (MetS) is a complex health disorder characterized by the synergy of dysglycemia, severe overweight, hypertension, hypertriglyceridemia and decreased high-density lipoprotein (HDL) cholesterol levels (Grima Serrano, 2010). Hyperglycaemia and obesity are major contributors to MetS by promoting oxidative stress, which drive to atherogenic dyslipidaemia, insulin resistance and hypertension (Masenga et al., 2023).

The rising prevalence of several of these risk factors have triggered a major scientific interest in functional food compounds. Particularly, regarding the role of plant-derived bioactive compounds, including phytochemicals, organic acids, dietary fiber, amino acids, monosaccharides and polysaccharides (Dou et al., 2025) in the modulation of metabolic pathways associated with the prevention and management of MetS (Capanoglu et al., 2022).

Among these, phenolic compounds, non-nutritive phytochemicals commonly found in plants classified as phenolic acids, flavonoids, tannins, stilbenes coumarins and lignans, are widely recognized for their antioxidant activity and their capacity to diminish oxidative stress and the risk of MetS (Tian et al., 2020). In particular, dietary flavonoid subclasses, including flavones, flavanones, flavanols, flavonols,

Abbreviations: ACE, Angiotensin-converting enzyme inhibitors; AGEs, Advanced glycation end products; Caco-2, Human colorectal adenocarcinoma cell line; CVD, Cardiovascular disease; DMEM, Dulbecco's modified eagle medium; DNS, Dinitrosalicylic acid; DPP-4, Dipeptidyl peptidase-4; ESI, Electrospray ionization; FBS, Foetal bovine serum; GAE, Gallic acid equivalent; HDL, High-density lipoprotein; HepG-2 Cells, human hepatocellular carcinoma cell line; HFF-1, Human foreskin fibroblast cell line; HMGR 3, hydroxy-3-methylglutaryl-CoA reductase; HPLC-MS/MS, High performance liquid chromatography coupled with mass spectrometry; IC_{50}, Half-maximal inhibitory concentration; MetS, Metabolic syndrome; MTT, Thiazolyl blue tetrazolium bromide; NPB p-4, Nitrophenyl butyrate; PCA, Principal component analysis; pNPG 4, nitrophenyl α-D-glucopyranoside; ROS, Reactive oxygen species; SEM, Standard error of mean; SGLTs, Sodium-dependent glucose transporters; SLR, Solid–liquid ratio; T2D, Type 2 diabetes; TPC, Total phenolic content; UAE, Ultrasound-assisted extraction.

* Corresponding author at: Department of Pharmacy, Faculty of Health Sciences, Universidad San Jorge, 50830 Zaragoza, Spain
E-mail address: ilopez@usj.es (V. López).

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isoflavones, anthocyanins or anthocyanidins are the most abundant group of polyphenols. These subclasses share a core of diphenylpropane (C_6–C_3–C_6) structure formed by a pair of aromatic rings linked by a three-carbon bridge that defines the central C ring (Jomova et al., 2025) and are closely associated with obesity reduction, improved lipid profiles, blood glucose control and blood pressure regulation (Khan et al., 2021). Similarly, phenolic acids, which represent about one-third of dietary phenolics (Yang et al., 2001) are associated with metabolic health benefits and are mainly classified into benzoic and cinnamic acids, both featuring a benzene ring and a carboxylic group, often with hydroxyl and/or methoxyl substitutions (Nunes et al., 2025).

Phenolic compounds are commonly contained in food of plant origin, but compared to other plant sources, phenolic content is particularly high in unripe fruits, which are often discarded as by-products during the thinning process. While thinning improves crop quality by reallocating resources to the remaining fruits, it tends to overlook the potential value of these phenolic-rich discarded fruits (Wei et al., 2021). Specifically, thinned apples are a considerable portion of apple production representing between 20 and 30% and tend to show higher concentrations of total phenolic compounds, flavonoids, and proanthocyanidins, as well as enhanced antioxidant activity, compared to mature apples (Zheng et al., 2012). Indeed, the valorization of this type of fruit by-products depends on the specific properties of the plant matrix and the targeted functional compounds (Ran et al., 2019).

The recovery of phenolic compounds can be achieved through novel approaches such as ultrasound-assisted extraction (UAE) which enhance the penetration of various solvents and provides higher yields along with several procedural advantages (Nirmal et al., 2023). In particular ethanol is a safe and efficient green solvent that typically yields phenolic enriched extracts with higher antioxidant activity, whereas adding water may increase total yield but dilute the phenolic fraction and weaken antioxidant related responses due to co extraction of abundant low activity matrix components that mask phenolic driven effects (Do et al., 2014).

In addition, the phenolic extracts obtained can be processed into dietary supplements as nutraceuticals for the treatment and prevention of MetS (Popiolek-Kalisz & Glibowski, 2023), and although still under investigation, they may offer an accessible, safe and novel therapy (Fahed et al., 2022).

This manuscript aims to evaluate whether thinned apple by-products from both autochthonous and commercial apple cultivars represent valuable natural sources of phenolic bioactive compounds, and to explore their biological properties and specific mechanisms of action in MetS along with evidence of safety in intestinal, hepatic and structural *in vitro* cell models, emphasizing their relevance for industrial applications through a sustainable and circular economy. We hypothesize that a metabolomics-driven profiling strategy, integrating LC–MS/HPLC chemical targeted analysis with chemometric modelling, will robustly link extract composition to pharmacodynamic profile (IC_{50}), enabling early identification of the metabolite features most predictive of activity. Consequently, this framework should support extract prioritization and guide targeted isolation of bioactive constituents thereby accelerating the discovery pipeline. For this purpose, their phenolic characterization was performed, along with the *in vitro* assessment of its antioxidant, antidiabetic, antiglycant, hypolipidemic, hypocholesterolemic, and hypotensive activities.

2. Materials and methods

2.1. Thinned apples samples preparation

Eight distinct cultivars of thinned apples (Reineta, Verde Doncella, Golden Smoothee, Fuji and Royal Gala as commercial cultivars; and Daroca-02, Camuesa de Castellano de Daroca and Vadiello-02 as autochthonous cultivars) were collected from Zaragoza (Aragón, Spain). Nevertheless, two specific varieties of autochthonous thinned apples (Amarilla de Octubre and Pomera de Pomes Agrias) were collected from the Garcipollera Valley, located in Huesca (Aragón, Spain). The thinning period took place in May 2024, and the samples of each variety were collected from two different trees and various parts of each tree. Subsequently, the thinned apples were frozen at −80 °C and lyophilized with LyoMicron −85 °C freeze-drying equipment (Dara-Lyo brand, Dara Pharma Group, Barcelona, Spain) for five days until completely dried. The dried samples were stored at −40 °C in hermetically sealed jars with silica gel.

2.2. Extraction of phenolic compounds

After a proper drying and storage, the samples were pulverized with a laboratory mill before the extraction method was applied. The procedure used was the ultrasound-assisted extraction (UAE) with the sonicator 505 (Fisherbrand™, Fisher Scientific, Madrid, Spain) equipped with a microtip of 13 mm diameter. The phenolic extraction process was achieved through the mixture of the powdered sample and absolute ethanol as green solvent, maintaining a solid liquid ratio (SLR) of 1:50 along with an exposure time of 20 min with an ultrasound amplitude of 40%. For each ratio, 5 g of powder were exactly weight and combined with 250 mL of absolute ethanol. To prevent overheating, the sample mixture was kept cool using ice bath throughout the extraction process. UAE conditions were preselected to ensure standardized extraction across apple cultivar by-products, and their plausibility defined within literature-reported operating ranges for phenolic extraction to balance mass transfer, rapid phenolic release, and moderate cavitation intensity, while limiting over-processing (Medina-Torres et al., 2017).

Every extracted solution mixture was centrifugated for 5 min, and the supernatant filtered by vacuum filtration with a 0.45 μm pore-sized polyamide 66 nylon membrane and a thickness of 90–140 μm. Additionally, the absolute ethanol was evaporated by rotary evaporator (Buchi UK Ltd., Newmarket, United Kingdom) until a semi-solid final mixture was obtained from each thinned apple sample. The extracts were stored in hermetic falcon tubes at −20 °C. The extraction yield, defined on a dry-weight basis as the mass of dried ethanolic extract recovered relative to the initial mass of lyophilized thinned-apple material, was approximately 30% (*w*/w) for most cultivars. In contrast, the autochthonous cultivars Amarilla de Octubre, Pomera de Pomes Agrias and Camuesa de Castellano de Daroca yielded approximately 20% (*w*/w). To contextualize yield in terms of the target phytochemical fraction, the summed targeted phenolic content quantified by HPLC–MS ranged from 9687.52 to 13,167.26 mg kg^{-1} in the 20% yield extracts, and from 8105.21 to 14,010.93 mg kg^{-1} in the 30% yield extracts. The lower yields observed in some autochthonous cultivars likely reflect differences in matrix properties rather than a reduced phenolic content.

2.3. Analysis of phenolic compounds by HPLC-MS/MS

2.3.1. Reagents and standards

A set of 38 reference compounds was utilized and obtained from PhytoLab (Vestenbergsgreuth, Germany) and Sigma-Aldrich (Milan, Italy). Stock solutions of pure compounds, formulated at 1000 mg L^{-1} in methanol of HPLC grade, served as the basis for subsequent analysis. These solutions were kept in glass containers with storage conditions at 4 °C for most solutions, while anthocyanins were preserved at −15 °C to prevent degradation. The daily preparation of standard working solutions at various concentrations was carried out by mixing stock solutions with HPLC-grade methanol. Formic acid (99%) was supplied by Merck (Darmstadt, Germany), and hydrochloric acid (37%, analytical grade) was sourced from Carlo Erba Reagents (Milan, Italy). HPLC-grade methanol was acquired from Sigma-Aldrich (Milan, Italy). Deionized water with a resistivity greater than 18 MΩ·cm was processed using a Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA). Solvents and prepared solutions were passed through 0.2 μm polyamide membrane filters acquired from Sartorius Stedim (Goettingen,

Germany). The filtration of the samples was carried out using Phenex™ RC syringeless filters (4 mm, 0.2 μm) provided by Phenomenex (Castel Maggiore, BO, Italy), prior to the analytical assay.

2.3.2. HPLC-ESI-MS/MS instrument and analysis

The analyse was carried out by an Agilent 1290 Infinity system coupled to a Triple Quadrupole 6420 mass spectrometer (Agilent Technologies, Santa Clara, CA), fitted with an electrospray ionization (ESI) source working in positive and negative ion mode. The method applied was previously developed and validated (Mustafa et al., 2022). Optimization of MS/MS parameters for target compounds was performed *via* flow injection analysis (FIA), using 1 μL injections of individual standard solutions (10 mg L^{-1}), with the aid of Agilent's Optimizer Software.

The separation of target analytes was carried out using a Synergi Polar–RP C18 analytical column (250 mm × 4.6 mm, 4 μm, Phenomenex, Cheshire, UK) and a Polar RP guard cartridge (4 mm × 3 mm ID) previously assembled. The mobile phase consisted of a solution of (A) water and (B) methanol, containing 0.1% formic acid, delivered at an elution rate of 0.8 mL min^{-1} under gradient conditions. The gradient program was structured as follows: an initial isocratic phase at 20% B from 0 to 1 min; a linear ramp from 20% to 85% B between 1 and 25 min; a second isocratic hold at 85% B from 25 to 26 min; followed by a return to 20% B from 26 to 32 min. Filtration of all solvents and solutions was performed using 0.2 μm polyamide membranes (Sartorius Stedim, Goettingen, Germany). An injection of 2 μL was used for the sample. Chromatographic separation was carried out with the column set to 30 °C. The electrospray ionization (ESI) source was configured under the following conditions: drying gas heated to 350 °C, flow rate of 12 L/min, nebulizer pressure of 55 psi, and a capillary voltage of 4000 V. Additionally, the detection was conducted in dynamic multiple reaction monitoring (dynamic-MRM) mode. The specified peak areas obtained from dynamic-MRM transitions were used for quantification purposes. The most intense product ion was used for quantitation, while additional fragment ions were used for qualification. A retention time window (Δt) of 2 min was applied for each target compound.

2.4. Cell viability assay in tumoral and non-tumoral cells

Three different cell lines were used to analyse cell viability. Human colorectal adenocarcinoma cell line (Caco-2) and human hepatocellular carcinoma cell line (HepG2) were obtained from American Type Culture Collection (ATCC) and cultured in Dulbecco's modified eagle medium (DMEM; Sigma-Aldrich, Barcelona, Spain) enriched with 10% fetal bovine serum (FBS), complemented by 1% penicillin-streptomycin (both from Sigma-Aldrich, Barcelona, Spain). As non-tumoral cell line, the human foreskin fibroblast cell line (HFF-1) from ATCC was selected and cultured with DEMEM, 1% penicillin-streptomycin, 1% pyruvate and 15% FBS. All cell cultures were incubated at 37 °C under a 5% CO_2 atmosphere. Cell viability was determined using thiazolyl blue tetrazolium bromide (MTT) (Fisher Scientific, Barcelona, Spain) by seeding cells in 96-well plates at a concentration of 2×10^4 cells per well and culturing them over a 24-h period. The accurate quantification of live cells was performed using an automatic cell counter (Logos Biosystems, South Korea) in combination with trypan blue (Sigma-Aldrich, Barcelona, Spain) exclusion staining. Following that, selected wells were exposed to thinned apple by-products within the range of 15.62 to 500 μg mL^{-1}, based on previous *in vitro* cytotoxicity studies with thinned apple extracts using similar ranges (Ferrario et al., 2022) (Zheng et al., 2020), prepared in DMEM containing 1% FBS. Control wells received only the medium with 1% FBS, without any extract treatment. After 24 h, 100 μL of MTT reagent, prepared at 0.4 mg mL^{-1} in a 10% FBS medium, was applied to each well. The plates were then maintained in the dark at 37 °C and 5% CO_2 environment for 3 h. After incubation, the MTT solution was removed, and 100 μL of DMSO was introduced into each well to solubilize the formazan precipitate. The resulting purple coloration was indicative of cell viability. The measurement of absorbance was carried out at 550 nm using a Synergy H1 Hybrid Multi-Mode Reader (Biotek, Bad Friedrichshall, Germany). The percentage of viable cells was determined according to the equation below:

$$\textit{Cell viability}\ (\%) = \left(Abs_{sample} / Abs_{control}\right) \times 100 \quad (1)$$

2.5. In vitro antidiabetic assays

2.5.1. α-Glucosidase (from Saccharomyces cerevisiae) inhibition

The inhibitory activity of α-glucosidase (Sigma-Aldrich, Barcelona, Spain) was evaluated with a 96-well microplate reader. The α-glucosidase derived from *Saccharomyces cerevisiae* was used as the enzyme source. Each well received 100 μL of α-glucosidase solution (1.0 U mL^{-1}) and 50 μL of the diluted samples at room temperature for a precise preincubation of 10 min. Following this, the enzymatic reaction was initiated through the addition of 50 μL of a 3 mM pNPG solution (4-nitrophenyl α-D-glucopyranoside) (Sigma-Aldrich, Barcelona, Spain) as a synthetic substrate. Following a 5-min incubation at 37 °C to enhance the reaction, the absorbance was recorded at 405 nm. All reagents and extracts were prepared using phosphate buffer. To ensure accurate absorbance readings, control and blank wells were included to eliminate background absorbance and to correct any signal interference caused by the extracts. Acarbose was used as positive control substance. The equation below was applied to quantify the percentage of inhibition:

$$\textit{Inhibition}\ (\%) = \left[\left(Abs_{control} - Abs_{sample}\right) / Abs_{control}\right] \times 100 \quad (2)$$

2.5.2. α-Amylase (from porcine pancreas) inhibition

The inhibition assay of porcine pancreatic α-amylase (Sigma-Aldrich, Barcelona, Spain) was conducted in 1.5 mL microtubes. A volume of 100 μL of the diluted extract was mixed with 100 μL of centrifuged α-amylase (2 mg mL^{-1}), followed by a 5-min incubation at 37 °C. Subsequently, 1% starch solution (Sigma-Aldrich, Barcelona, Spain) was incorporated, and the mixture was kept at 37 °C for an additional 10 min. After that period, 200 μL of a 1% solution of 3,5-dinitrosalicylic acid (DNS) colour reagent (Sigma-Aldrich, Barcelona, Spain) were mixed with 50 μL of 1 M NaOH, and the mixture was heated at 100 °C for five minutes. Once the reaction concluded, a colour change was observed. Phosphate buffer served as the solvent for all reagents and extract samples. Each reaction mixture (200 μL), including the control and blanks, was transferred to a 96-well microplate for spectrophotometric analysis at 540 nm. Acarbose served as the positive control substance in the assay. To evaluate inhibitory activity, the following formula was used (Andre et al., 2012).

2.5.3. Dipeptidyl peptidase-4 (human recombinant DPP-4) inhibition

The commercial DPP-4 inhibitor assay kit (Cayman) was employed to assess the antidiabetic activity of the extracts on the proteolytic activity of human recombinant DPP-4. The assay used the fluorogenic substrate Gly-Pro-Aminomethylcoumarin (AMC), and an incubation of 30 min at 37 °C was required to complete the reaction based on the procedure provided by the manufacturer. Fluorescence intensity was monitored employing wavelengths of 350–360 nm for excitation and 450–465 nm for emission. Sitagliptin served as the reference inhibitor. The degree of inhibition was quantified using the equation below.

$$\%\textit{Inhibition} = \left[\frac{(\textit{Initial Activity} - \textit{Inhibitor})}{\textit{Initial Activity}}\right] \times 100 \quad (3)$$

2.6. In vitro antiglycation and antioxidant assays

2.6.1. Advanced Glycation End Products (AGEs) inhibition

The accumulation of AGEs was assessed employing a 96-well black microplate format. Each well received 50 μL of sample extract, 100 μL of bovine serum albumin (10 mg mL^{-1}) and 100 μL of fructose (0.5 M),

both acquired from Sigma-Aldrich (Barcelona, Spain). All solutions prepared in phosphate buffer. The plate was kept at 37 °C for 24 h in the dark. Control and blanks were included for accurate measurement. Fluorescence intensity was monitored employing wavelengths of 355 nm for excitation and 460 nm for emission. Aminoguanidine was included as the reference inhibitor. To quantify the inhibitory effect, it was represented as a percentage and calculated using eq. (2).

2.6.2. Radical superoxide ($O_2\bullet^-$) scavenging from xanthine oxidase system, and xanthine oxidase enzyme inhibition

The neutralization of radical superoxide produced by xanthine oxidase from bovine milk (Sigma-Aldrich, Barcelona, Spain) was evaluated using a 96-well microplate reader. Firstly, a mix solution was prepared with xanthine (Sigma-Aldrich, Barcelona, Spain) (90 μM), nitro tetrazolium blue chloride (NBT) (Sigma-Aldrich, Barcelona, Spain) (22.8 μM) and Na_2CO_3 (Fisher Scientific, Barcelona, Spain) (16 mM) in phosphate buffer. Subsequently, 240 μL of the mixed solution was transferred into each well, followed by 30 μL of the extract in the same buffer and 30 μL of xanthine oxidase (0.168 U mL^{-1}). Control and blanks wells were also prepared for an accurate measurement. After incubation for 2 min at 37 °C, absorbance was recorded at 560 nm to determine the radical superoxide scavenging activity of the samples. Quercetin was used as the positive control substance. The percentage of inhibitory activity was calculated according to eq. (2).

The inhibition of xanthine oxidase from bovine milk was evaluated in 96-well microplate reader. In this enzymatic inhibition assay, a mix solution was prepared with xanthine (90 μM) and Na_2CO_3 (16 mM) in phosphate buffer. Subsequently, 240 μL of the mixed solution was added to each well, followed by 30 μL of the extract in the same buffer and 30 μL of xanthine oxidase (0.168 U mL^{-1}). Control and blanks wells were also included. After a 2-min incubation at 37 °C, absorbance was recorded at 295 nm to assess enzymatic inhibition capacity. Inhibition was quantified as a percentage according to eq. (2).

2.7. In vitro hypolipidemic and hypocholesterolemic assays

2.7.1. Lipase (from porcine pancreas) inhibition

The inhibition of pancreatic lipase (Sigma-Aldrich, Barcelona, Spain) was evaluated through a 96-well plate, with absorbance measured with a microplate reader. Each well contained a reaction mixture composed of 40 μL of diluted extract and 40 μL of centrifuged porcine pancreatic lipase (type II, 2 mg mL^{-1}). The reaction solution was preincubated at room temperature for 15 min. Following this, 20 μL of 10 mM *p*-nitrophenyl butyrate (NPB) (Sigma-Aldrich, Barcelona, Spain) as synthetic substrate was transferred to initiate the reaction, and subsequently the absorbance was recorded at 405 nm and 37 °C. Control and blanks wells were also included. All reagents and extracts were prepared using phosphate buffer. Orlistat was utilized as a reference substance for positive control. Lipase inhibition was expressed as a percentage based on the eq. (2)

2.7.2. HMG-CoA reductase (purified catalytic domain from HMGR) inhibition

The HMGR inhibitor assay commercial kit (Sigma-Aldrich) was employed to examine the inhibitory activity of the extracts on the HMGR from the purified catalytic domain of the enzyme. The assay relies on a decrease in absorbance at 340 nm caused by the tested extracts and monitors the oxidation of NADPH catalysed by the HMGR enzyme in the presence of its substrate, HMG-CoA. Control and blanks were also used. Based on the manufacturer's instructions, the reaction mixture was held at 37 °C for 10 min, and absorbance was recorded at 340 nm. Pravastatin served as the positive control substance. Inhibitory activity was calculated using eq. (2).

2.8. In vitro hypotensive assays

2.8.1. Angiotensin converting enzyme (ACE from rabbit lung) inhibition

The angiotensin-converting enzyme (ACE) inhibition was evaluated using the ACE commercial kit (Dojindo). This colorimetric bioassay is founded on the enzymatic conversion of the synthetic substrate 3-hydroxybutyryl-Gly-Gly-Gly (3HB-GGG) into 3-hydroxybutyrate (3HB) by ACE. Control and blanks were also performed for an accurate measurement. Following the manufacturer's guidelines, the resulting 3HB was subsequently detected *via* a coupled reaction that produces a colorimetric signal measurable at 450 nm. Captopril was employed as the positive control substance. Inhibitory activity was calculated using eq. (2).

2.9. Statistical analysis

Statistical procedures were carried out with GraphPad Prism version 10.0 (GraphPad Software, San Diego, CA, USA), including nonlinear regression, one-way ANOVA subsequently analysed using Dunnett's *post hoc* test, and Pearson correlation analysis. For multivariate data analyse MetaboAnalyst 5.0 (Pang et al., 2021) was carry out as exploratory tool, which included hierarchical clustering heatmaps, principal component analysis (PCA) to enhance comprehension of phenolic content variations. To complement the exploratory PCA visualization, we used PERMANOVA on the corresponding distance matrix to formally test whether overall phenolic profiles differed between cultivars. Statistical significance was assessed using a confidence level of 95% ($p < 0.05$).

Assays were performed in triplicate across separate days, with data reported as mean ± standard error of the mean (SEM).

2.10. Chemical structure generation

Structures of the main phenolic compounds detected in the extracts were drawn with ChemDraw v21.0.0 (PerkinElmer Informatics, Waltham, MA, USA).

3. Results

3.1. Phenolic content analyses by HPLC-MS/MS and chemometric analysis

3.1.1. HPLC-MS/MS analysis

In this research, HPLC-MS analysis was performed to target 38 relevant individual phenolic compounds in the 10 thinned apple by-products samples (Table 1). This analytical method identified and quantified 21 distinct phenolic compounds, which included 1 hydroxybenzoic acid, 5 cinnamic acids, 2 dihydrochalcones, 6 flavonols, 4 flavan-3-ols and 3 anthocyanidins. Among them, the greatest diversity of phenolic compounds was identified in the commercial samples of Verde Doncella and Fuji, with 19 compounds detected, while the lowest number, 13 compounds, was found in the autochthonous sample Vadiello-02. Nevertheless, none of the stilbenes nor flavanone compounds were detected in any of the thinned apples.

Furthermore, the highest TPC quantified was detected in the Fuji sample (14,010.93 mg kg^{-1} of extract) followed by Royal Gala, Verde Doncella and Amarilla de Octubre, whereas the lowest TPC was found in the Vadiello-02 sample, with a concentration of 8105.21 mg kg^{-1} of extract.

The most abundant phenolic compounds quantified in the thinned apple by-products were chlorogenic acid, quercitrin, rutin, hyperoside, isoquercitrin, catechin, procyanidin B2, epicatechin and delphinidin-3,5-diglucoside (Fig. 1). Among them, hyperoside, isoquercitrin and delphinidin-3,5-diglucoside were the predominant phenolic compounds in all the samples. In particular, the autochthonous sample Amarilla de Octubre showed the highest concentrations of several compounds, including chlorogenic acid (1945.66 mg kg^{-1}), quercitrin (1567.98 mg

Table 1
Quantitative determination of polyphenols in the thinned apple extracts (mg of phenolic compounds kg^{-1} of extract) by using HPLC-MS/MS triple quadrupole.

Compounds	Amarilla de Octubre	Pomera de Pomes Agrias	Camuesa de Castellano Daroca	Daroca-02	Vadiello-02	Verde Doncella	Reineta	Golden Smoothee	Fuji	Royal Gala
Hydroxybenzoic Acids										
Gallic acid	23.11	11.60	n.d.	6.78	n.d.	12.43	2.42	n.d.	n.d.	n.d.
3-Hydroxy benzoic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4-Hydroxy benzoic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Cinnamic Acids										
Chlorogenic acid	1945.66	1144.56	910.43	567.98	234.97	414.87	210.98	111.87	812.85	312.85
Neochlorogenic acid	423.56	34.80	5.98	n.d.	n.d.	n.d.	111.34	45.90	60.98	n.d.
Caffeic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Vanillic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Syringic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
P-Coumaric acid	13.44	4.05	n.d.	n.d.	n.d.	22.81	n.d.	n.d.	11.67	13.98
Ferulic acid	n.d.	n.d.	5,67	3,97	4,9	n.d.	1,98	n.d.	n.d.	n.d.
Ellagic acid	144.13	44.86	34.85	45.08	34.87	199.54	n.d.	109.98	48.80	106.86
Trans-cinnanic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Stilbenes										
Resveratrol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Dihydrochalcones										
Phlorizin	323.67	132.98	30.59	134.80	76.98	138.90	134.97	198.87	207.43	107.32
Phloretin	0.97	1.43	0.56	0.16	n.d.	2.22	1.14	0.34	1.14	1.09
Flavonols										
Quercetin	45.98	87.82	66.6	55.53	33.34	9.98	33.07	45.97	113.07	98.55
Kaempferol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Myricetin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Isorhamnetin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Kaempferol-3-glucoside	28.98	11.97	8.9	11.37	33.85	14.98	18.71	23.33	45.66	66.75
Quercitrin	1567.98	1447.40	442.78	456.86	954.97	45.89	342.78	517.20	824.56	723.97
Rutin	698.09	442.78	732.78	540.08	1142.78	923.98	122.78	322.78	1134.33	934.99
Hyperoside	1945.87	936.44	2345.28	1237.30	636.43	4389.09	1236.43	1123.89	2709.70	3809.05
Isoquercitrin	1031.45	2134.08	1678.34	2224.90	1228.09	3550.87	3755.56	4770.77	1704.55	1199.82
Flavan-3-ols										
Catechin	502.54	142.78	456.96	135.96	452.08	248.99	110.09	98.43	111.65	232.07
Procyanidin A2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.76	5.98
Procyanidin B2	1520.56	942.78	644.11	1441.87	742.19	1456.08	1232.78	992.42	986.9	776.09
Epicatechin	979.45	535.87	1077.12	1255.01	996.31	766.12	323.56	345.67	1412.45	1186.63
Flavanones										
Naringenin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Hesperidin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Anthocyanins										
Delphinidin-3,5-diglucoside	1960.71	1613.55	1777.93	1223.56	1533.45	1134.08	868.56	999.03	3567.45	4048.11
Delphinidin-3-galactoside	11.11	17.77	n.d.	n.d.	n.d.	7.06	18.8	11.08	249.87	153.43
Cyanidin-3-glucoside	n.d.	n.d.	n.d.	n.d.	n.d.	0.11	n.d.	n.d.	3.11	2.45
Petunidin-3-glucoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Pelargonidin-3-glucoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Pelargonidin-3-rutinoside	n.d.	n.d.	n.d.	n.d.	n.d.	1.34	n.d.	n.d.	n.d.	n.d.
Malvidin-3-galactoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total Flavonoid Content	10,617.36	8447.65	9261.95	8717.40	7830.47	12,689.69	8199.23	9449.78	13,076.63	13,346.30
Total Phenolic Content	13,167.26	9687.52	10,218.88	9341.21	8105.21	13,339.34	8525.95	9717.53	14,010.93	13,779.99

n.d: not detected.

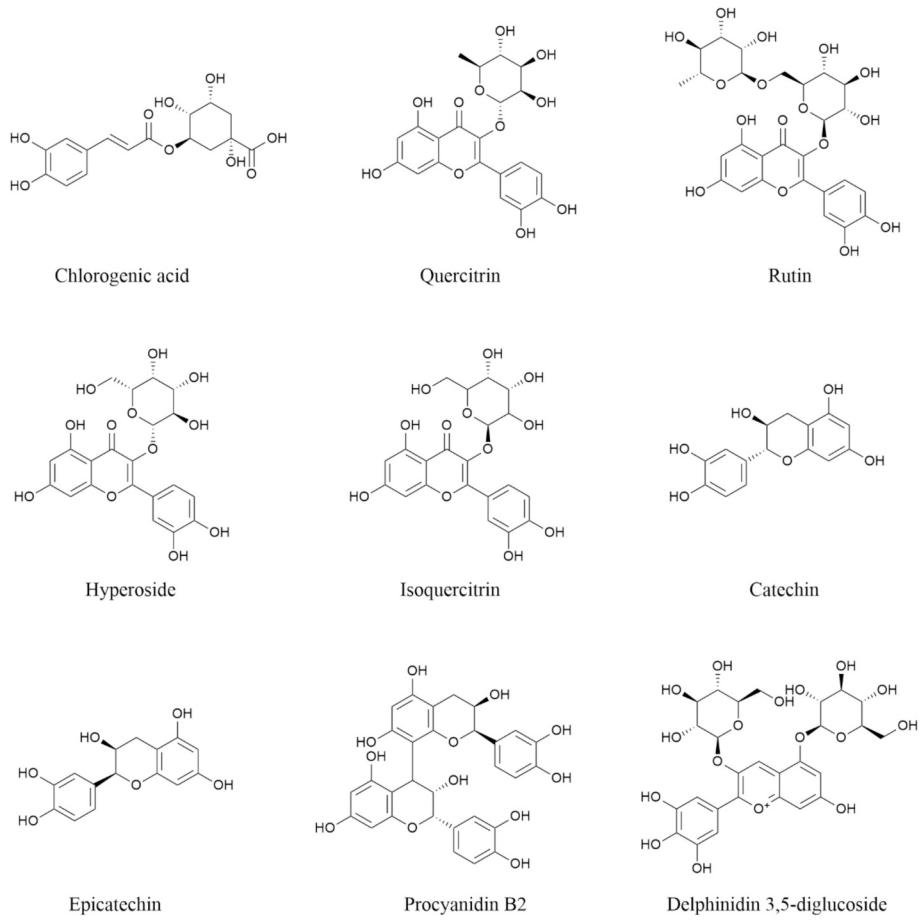

Fig. 1. Chemical structures of the predominant phenolic compounds in thinned apple by-products.

kg^{-1}), catechin (502.54 mg kg^{-1}), and procyanidin B2 (1520.56 mg kg^{-1}). Additionally, high levels were recorded for epicatechin in Fuji (1412.45 mg kg^{-1}), rutin in Vadiello-02 (1142.78 mg kg^{-1}), hyperoside in Verde Doncella (4389.09 mg kg^{-1}), isoquercitrin in Golden Smoothee (4770.77 mg kg^{-1}), and delphinidin 3,5-diglucoside in Royal Gala (4048.11 mg kg^{-1}).

3.1.2. Chemometric analysis

Hierarchical clustering heatmap was used to visualize relative enrichment or depletion of phenolic compounds, and PCA was performed to explore compositional trends between autochthonous and commercial cultivars.

Based on their phenolic profiles, the 10 thinned apple samples were grouped into four distinct clusters (Fig. 2). Cluster I grouped the autochthonous samples Daroca-02, Camuesa de Castellano de Daroca, and Vadiello-02, containing, in comparison with the rest of the samples, a higher proportion of ferulic acid, catechin and epicatechin. Cluster II included autochthonous and commercial samples such as Pomera de Pomes Agrias, Reineta, and Golden Smoothee, showing a higher enrichment of phlorizin, neochlorogenic acid, phloretin, and isoquercitrin. Cluster III was formed by the commercial samples Fuji and Royal Gala, which stood out for their higher proportion of quercitrin, hyperoside, p-coumaric acid, phloretin, delphinidin 3- galactoside, delphinidin 3,5-diglucoside, kaempherol-3- glucoside, quercetin, epicatechin, and rutin. Finally, cluster IV consisted of a mixed cultivar sample, such as Amarilla de Octubre and Verde Doncella with a higher proportion of phlorizin, procyanidin B2, gallic acid, catechin, hyperoside, p-coumaric acid, ellagic acid, phloretin, and rutin. Among them, clusters III and IV are notable for exhibiting the highest diversity of phenolic compounds.

The PCA of phenolic groups showed that PC1 and PC2 explained the 46.0% and a 28.9% of total variance, respectively, while the PCA of individual phenolic compounds revealed that PC1 and PC2 explained 28.9% and 25.8% of total variance, respectively. While full class separation is not expected in complex biological matrices the PCA biplot captures most of the phenolic-group variability (74.9% cumulative) and indicates moderate but statistically significant compositional trends in phenolic-group content across both cultivars ($R^2X = 0.22$; $p = 0.04$). as illustrated in Fig. 3. Samples from commercial cultivars are oriented in the direction of the anthocyanins, flavonols, total phenolic content, and total flavonoid content vectors, implying relatively higher concentrations. In contrast, the autochthonous samples are more closely associated with flavan-3-ols, cinnamic acids, dihydrochalcones, and hydroxybenzoic acids, indicating a different phenolic profile. Nevertheless, the PCA analysis of individual phenolic compounds did not reveal statistically significant differences between both cultivars ($R^2X = 0.01$, $p > 0.05$) consistent with the low variance explained by the model.

3.2. Cell viability in Caco-2, HepG2 and HFF-1 cell cultures

The cytotoxic potential of the thinned apple extracts was assessed by exposing two human tumoral cell lines (Caco-2, HepG2) added to a non-tumorigenic human cell line (HFF-1) to a wide concentration range of extracts, from physiologically relevant to non-physiological levels. Following an intestinal–systemic alignment strategy, we used Caco-2 viability to define non-toxic working concentrations and to support intestinal targets, while Caco-2 and HepG2 assays were used to contextualize safe conditions relevant to systemic action (Sanz-Buenhombre et al., 2016) as well as HFF-1 (Libero et al., 2024). Cell viability was quantified as a percentage, as shown in Fig. 4, with lower viability

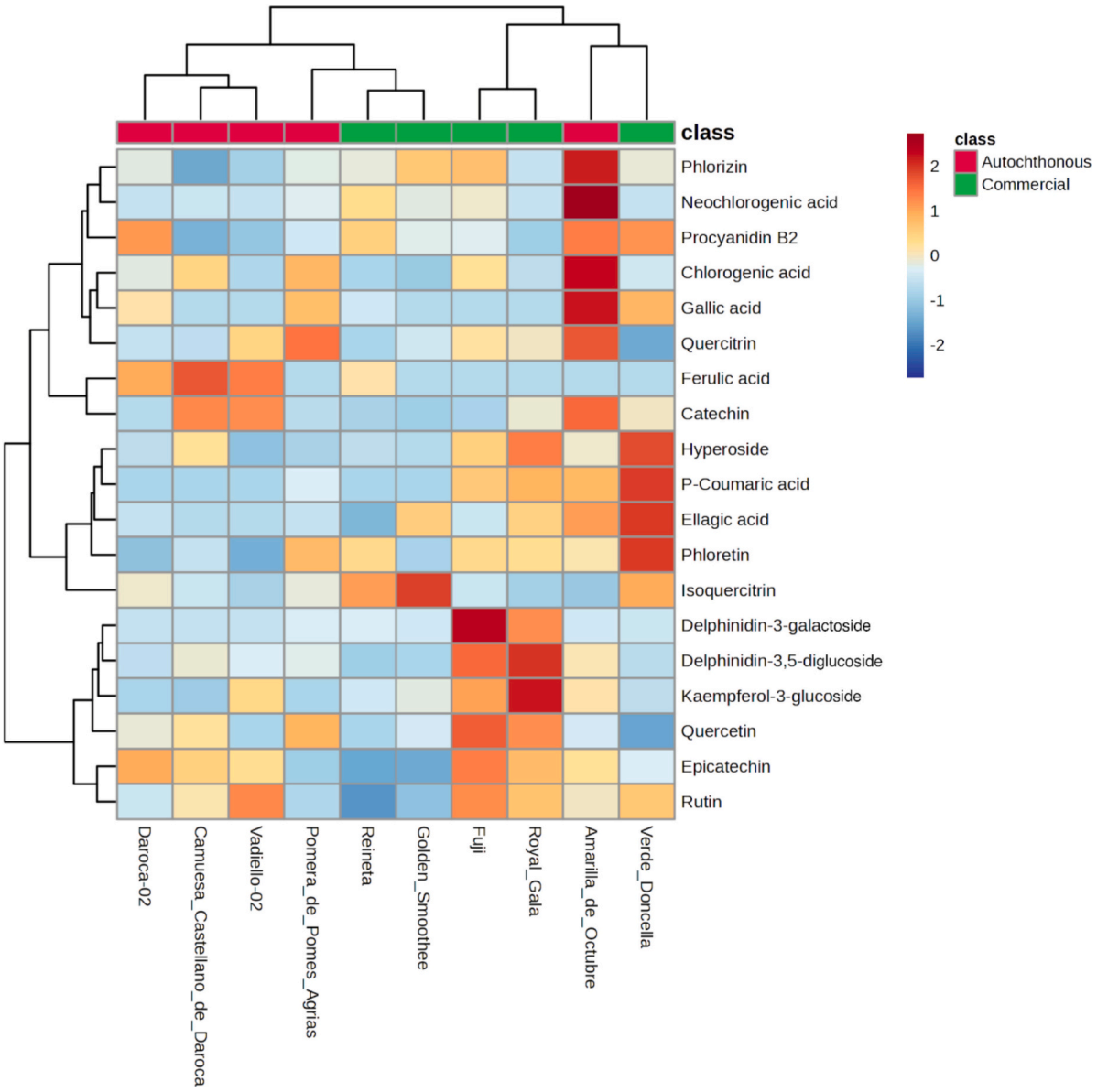

Fig. 2. Hierarchical clustering heatmap analysis. Heatmap values are *Z*-score normalized by compound. Red cells (Z > 0) indicate samples enriched in a given phenolic relative to its dataset mean, enabling row-wise comparison of enrichment patterns. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

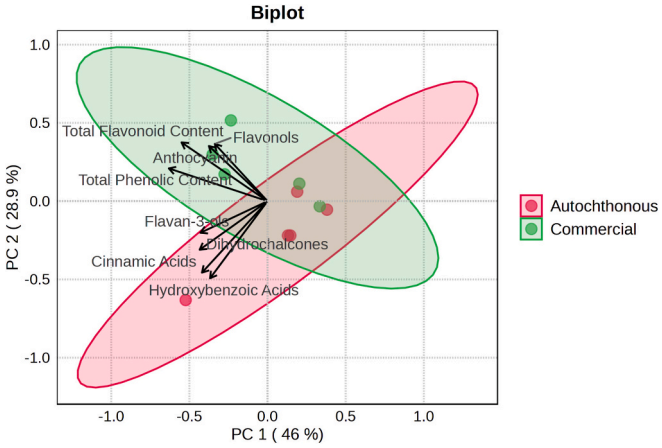

Fig. 3. PCA score plot of phenolic groups.

values indicating higher cytotoxicity. Therefore, to define non-cytotoxic *in vitro* working conditions, the commonly used MTT benchmark (viability ≥70%) was applied upon exposure of cells to the extracts (Casado-Hidalgo et al., 2025).

Overall, the three cell lines exhibited comparable cytotoxic profiles; however, the tumorigenic HepG2 and Caco-2 cells demonstrated greater sensitivity, particularly at higher concentrations in specific extracts, as evidenced by a more pronounced reduction in viability. The viability reduction induced by some thinned apple extracts followed a dose dependent pattern across most cell lines, with autochthonous cultivars (Amarilla de Octubre, Pomera de Pomes Agrias, Camuesa de Castellano de Daroca, and Daroca 02) and the commercial extracts (Verde Doncella and Reineta) showing the most pronounced effects at high concentrations.

In HepG2 cells, most of the extracts maintained cell viability above 70% at 500 μg mL^{-1}, except for Amarilla de Octubre, Pomera de Pomes Agrias, Reineta, and Verde Doncella. When the concentration was reduced to 250 μg mL^{-1}, only Pomera de Pomes Agrias and Amarilla de

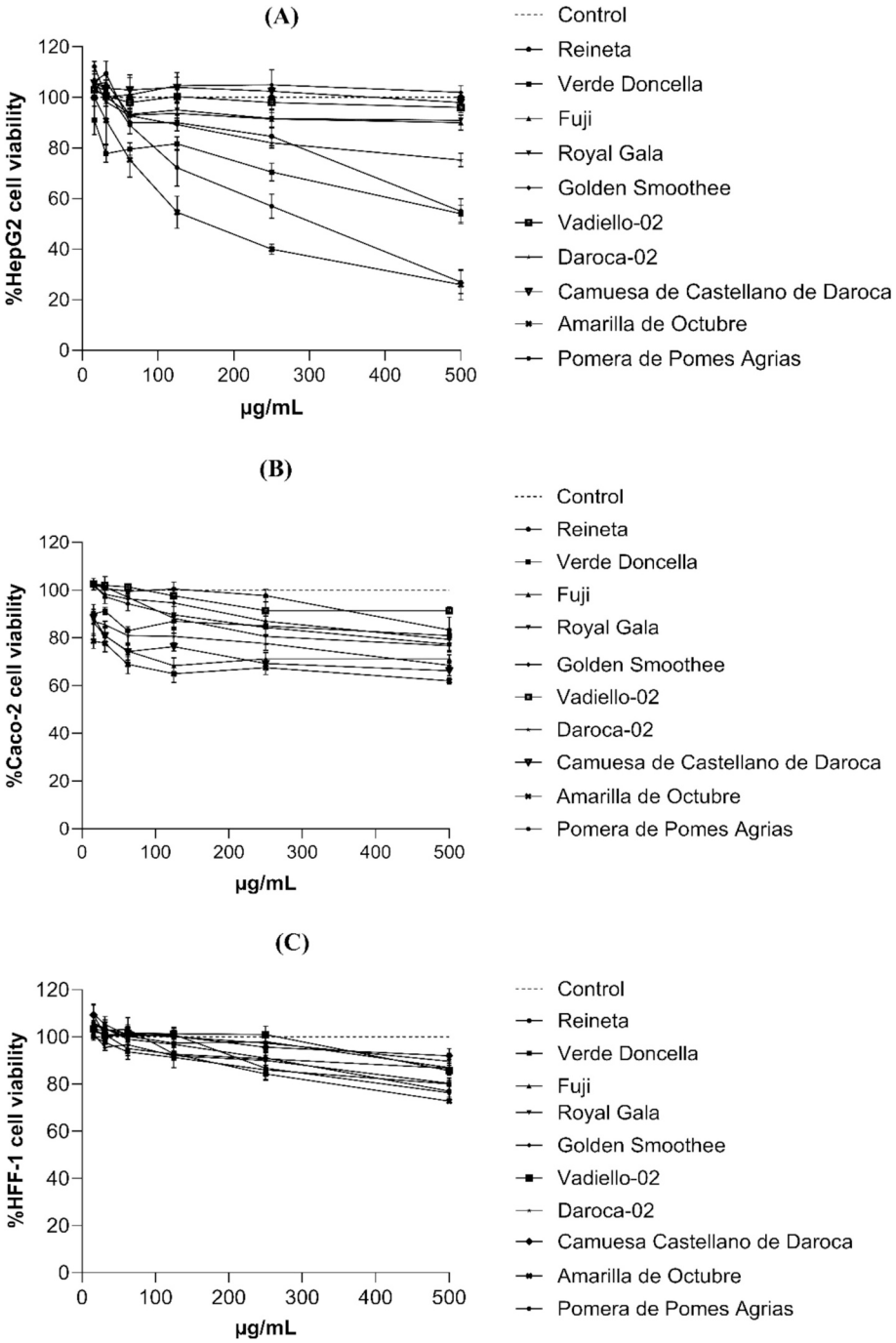

Fig. 4. Cell viability (%) determined in HepG2 (A), Caco-2 (B) and HFF-1 (C) cells treated with thinned apple extracts at concentrations ranging from 15.62 to 500 µg mL^{-1}. Results are presented as mean ± SEM from at least three independent experiments.

Octubre remained below this 70% threshold, while at 125 µg mL^{-1}, only Amarilla de Octubre showed significant growth inhibition.

A similar trend was observed in Caco-2 cells, where high concentrations of autochthonous (Amarilla de Octubre, Daroca-02, Camuesa Castellano de Daroca) extracts increased cytotoxicity. Specifically, at 500 µg mL^{-1}, viability dropped below 70% for the aforementioned cultivars, with Amarilla de Octubre failing to reach this threshold even within the 125–500 µg mL^{-1} range.

In summary, based on viability above 70%, the extracts of Golden Smothee, Fuji, Royal Gala, and Vadiello-02 are classified as non-cytotoxic up to 500 µg mL^{-1} in all cell types assayed. In contrast, Reineta and Verde Doncella are considered safe within physiological ranges (≤250 µg mL^{-1} in HepG2, ≤500 µg mL^{-1} in Caco-2), while Pomera de Pomes Agrias requires concentrations ≤125 µg mL^{-1} in HepG2 and ≤ 500 µg mL^{-1} in Caco-2. Nevertheless Daroca-02 and Camuesa de Castellano de Daroca are considered non-cytotoxic at ≤500 µg mL^{-1} in HepG2 and ≤ 250 µg mL^{-1} in Caco-2. At last, Amarilla de Octubre requires the lowest safe concentrations of all extracts (62.5 µg mL^{-1} in HepG2 and Caco-2) to ensure cytocompatibility.

Conversely, HFF-1 non-tumoral fibroblasts demonstrated higher resilience; even at 500 µg mL^{-1}, viability remained between 70 and 90% for all extracts. This suggests a selective response, where metabolic function is preserved in non-tumoral human cells. Since the MTT assay measures mitochondrial dehydrogenase activity rather than direct cell lysis, these results likely reflect a metabolic slowdown at supra-physiological concentrations rather than irreversible apoptosis in the

analysed cell types, which is supported by the maintenance of normal cell morphology and adherence observed under microscopic evaluation.

3.3. Evaluation of the in vitro antidiabetic activity

3.3.1. Inhibition of α-glucosidase enzyme

A dose-dependent inhibition of α-glucosidase was observed for all extracts. Notably, Amarilla de Octubre ($IC_{50} = 105.60 \pm 7.23$ μg mL^{-1}) exhibited the highest enzymatic inhibition followed by Pomera de Pomes Agrias ($IC_{50} = 123.71 \pm 11.70$ μg mL^{-1}), which showed a lower half-maximal inhibitory concentration (IC_{50}) than the therapeutic agent acarbose ($IC_{50} = 220.00 \pm 64.10$ μg mL^{-1}), indicating greater antidiabetic activity, although without statistically significant difference compared to the control with p values of 0.58 and 0.76, respectively. Furthermore, Vadiello-02 ($p = 0.99$), and Verde Doncella ($p = 0.22$), produced results comparable to the reference drug as well, with IC_{50} values of 244.14 ± 15.08 μg mL^{-1} and 361.56 ± 6.04 μg mL^{-1}, respectively, showing no statistically significant differences compared to acarbose ($p > 0.05$). The Reineta extract ($IC_{50} = 486.47 \pm 8.85$ μg mL^{-1}) also stood out above the rest of the samples, showing less significant differences respect to the control ($p = 0.005$), in contrast to the other samples ($p < 0.0001$).

3.3.2. Inhibition of α-amylase enzyme

Only five extracts were capable of inhibiting α-amylase enzyme in a dose-dependent manner. Among them, Vadiello-02 extract exhibited the highest inhibitory activity, followed by Pomera de Pomes Agrias, Amarilla de Octubre, Verde Doncella and Reineta extracts. Notably, Vadiello-02 ($p = 0.07$) showed an IC_{50} value of 194.79 ± 22.88 μg mL^{-1}, which was comparable to the reference antidiabetic drug acarbose (IC_{50} = 7.72 ± 1.89 μg mL^{-1}), with no statistically significant difference observed ($p > 0.05$) compared to control. In addition, Pomera de Pomes Agrias ($IC_{50} = 337.12 \pm 59.73$ μg mL^{-1}) showed great enzymatic inhibition as well, with a less significant difference ($p = 0.002$) compared to control, in contrast to the other samples ($p < 0.0001$).

3.3.3. Inhibition of DPP-4 enzyme

All extracts demonstrated DPP-4 inhibition in a dose-dependent manner, contributing to the overall synergistic antidiabetic potential of the samples, although with generally low inhibitory potency against DPP-4. Among these, Pomera de Pomes Agrias ($IC_{50} = 480.63 \pm 3.39$ μg mL^{-1}), followed by Amarilla de Octubre (IC_{50} = 628.03 ± 21.09 μg mL^{-1}), demonstrated the highest inhibitory activity. Both showed less pronounced statistically significant differences compared to the reference antidiabetic drug sitagliptin ($IC_{50} = 0.04 \pm 0.01$ μg mL^{-1}), with p-values of 0.003 and 0.0001, respectively, in contrast to the other samples ($p < 0.0001$). These results emphasize the antidiabetic potential of Pomera de Pomes Agrias and Amarilla de Octubre extracts.

3.4. Evaluation of the in vitro assay of the antiglycant and antioxidant activity

3.4.1. Inhibition of AGEs formation

In this non-enzymatic assay, all extracts demonstrated the ability to inhibit albumin protein glycation. Specifically, Amarilla de Octubre and Pomera de Pomes Agrias extracts, with IC_{50} values of 49.77 ± 3.52 μg mL^{-1} and 60.08 ± 3.01 μg mL^{-1}, respectively, exhibited the most pronounced antiglycation effects. The Reineta sample (IC_{50} = 189.36 ± 13.15 μg mL^{-1}), followed by Verde Doncella (IC_{50} = 203.44 ± 5.61 μg mL^{-1}) also stood out in terms of antiglycation bioactivity. It is noteworthy that Amarilla de Octubre ($p = 0.99$), exhibited stronger activity, as indicated by a lower IC_{50} value, whereas Pomera de Pomes Agrias (p = 0.99) showed a higher IC_{50} than the investigational drug aminoguanidine ($IC_{50} = 55.01 \pm 5.63$ μg mL^{-1}). Both extracts showed notable antiglycation effects without statistically significant differences compared to the control ($p > 0.05$), in contrast to the remaining samples ($p < 0.0001$).

3.4.2. Antioxidant activity against radical superoxide ($O_2\bullet^-$) and xanthine oxidase

This assay allowed us to differentiate the antioxidant mechanisms of action between superoxide radical scavenging and xanthine oxidase enzyme inhibition. A clear antioxidant mechanism was identified, as all extracts demonstrated superoxide radical scavenging activity, while none of the extracts were able to inhibit the enzyme. The antioxidant reference substance was the pure phenolic compound quercetin (IC_{50} = 4.16 ± 0.24 μg mL^{-1}), which stood out due to its strong radical scavenging ability. Nevertheless, the extracts of Amarilla de Octubre (IC_{50} = 1.18 ± 0.21 μg mL^{-1}) followed by Pomera de Pomes Agrias ($IC_{50} = 1.98$ ± 0.02 μg mL^{-1}), Fuji ($IC_{50} = 2.13 \pm 0.29$ μg mL^{-1}), Reineta ($IC_{50} = 2.47$ ± 0.50 μg mL^{-1}) and Verde Doncella (IC_{50} = 2.75 ± 0.26 μg mL^{-1}) achieved even higher scavenging activity than the control substance. All extracts showed comparable antioxidant activity to quercetin, with no statistically significant differences observed ($p > 0.05$), except for the Vadiello-02 extract ($p < 0.001$), which exhibited the lowest antioxidant capacity.

3.5. Evaluation of the in vitro hypolipidemic and hypocholesterolemic activity

3.5.1. Inhibition of lipase enzyme

All extracts showed dose-dependent inhibition of lipase activity. Notably, the Reineta extract ($IC_{50} = 225.43 \pm 6.31$ μg mL^{-1}), followed by the Verde Doncella extract (IC_{50} = 363.90 ± 25.91 μg mL^{-1}), Fuji ($IC_{50} = 397.1 \pm 44.77$ μg mL^{-1}), Amarilla de Octubre (IC_{50} = 419.31 ± 17.76 μg mL^{-1}) and Pomera de Pomes Agrias ($IC_{50} = 447.02 \pm 25.45$ μg mL^{-1}) showed the most pronounced hypolipidemic effects. These extracts exhibited statistically significant differences compared to the reference hypolipidemic agent orlistat ($IC_{50} = 27.12 \pm 11.49$ μg mL^{-1}). However, the Reineta extract ($p = 0.03$) and the Verde Doncella extract ($p = 0.0001$) showed less pronounced statistical differences than the other samples ($p < 0.0001$), indicating a closer similarity to the control treatment.

3.5.2. Inhibition of HMGR enzyme

In this enzymatic assay, only four extracts were able to inhibit HMGR activity, acting through a more specific and complex mechanism of action in HMGR enzyme in a dose-dependent manner. Among them, Verde Doncella (IC_{50} = 100.88 ± 3.93 μg mL^{-1}) exhibited the highest hypocholesterolemic activity, followed by Reineta ($IC_{50} = 149.39 \pm 9.39$ μg mL^{-1}), Vadiello-02 (IC_{50} = 233.04 ± 17.78 μg mL^{-1}) and Golden Smoothee ($IC_{50} = 469.49 \pm 8.04$ μg mL^{-1}) extracts. In particular, Verde Doncella extract exhibited less pronounced statistically significant differences ($p = 0.0001$) compared to the reference hypocholesterolemic agent pravastatin ($IC_{50} = 0.012 \pm 0.0001$ μg mL^{-1}), in contrast to the other thinned apple extracts ($p < 0.0001$). These findings emphasize the potential of Verde Doncella and Reineta extracts as promising hypolipidemic and hypocholesterolemic agents, acting through a synergistic mechanism of action.

3.6. Evaluation of the in vitro activity against ACE enzyme

All extracts were able to inhibit the ACE enzyme, showing an additional hypotensive activity. Among them, Verde Doncella ($IC_{50} = 42.17$ ± 3.11 μg mL^{-1}) followed by Amarilla de Octubre ($IC_{50} = 55.94 \pm 1.18$ μg mL^{-1}), Pomera de Pomes Agrias (IC_{50} = 81.32 ± 10.33 μg mL^{-1}), Reineta ($IC_{50} = 84.34 \pm 11.58$ μg mL^{-1}) and Daroca-02 (IC_{50} = 90.91 ± 12.09 μg mL^{-1}) showed the highest ACE inhibition. Furthermore, Verde Doncella ($p = 0.26$) and Amarilla de Octubre ($p = 0.07$) showed no statistically significant differences ($p > 0.05$) compared to the hypotensive agent captopril (IC_{50} = 0.003 ± 0.001 μg mL^{-1}), revealing a similarly interesting hypotensive activity. In addition, the samples of

Pomera de Pomes Agrias ($p = 0.004$), Reineta ($p = 0.003$), and Daroca-02 ($p = 0.001$), exhibited a notable capacity to inhibit the ACE enzyme, showing less statistically significant differences ($p < 0.05$) compared to captopril, in contrast to the remaining extracts which displayed more pronounced differences ($p < 0.0001$).

3.7. Determination of correlation between all in vitro bioassays and bioassays with individual phenolic content

The analysis of the IC_{50} of the enzymes (Table 2) revealed several statistically significant positive correlations, suggesting potential functional relationships among metabolic and regulatory pathways. The strongest positive correlation was observed between AGEs and radical superoxide scavenging activity ($R = 0.92$, $p = 0.0001$), indicating a close link between oxidative stress and glycation processes. Other notable positive correlations include ACE with AGEs ($R = 0.82$, $p = 0.004$), as well as ACE with radical superoxide scavenging ($R = 0.71$, $p = 0.02$. These findings highlight possible interactions between oxidative stress, glycation, and blood pressure regulation mechanisms. Similarly, α-glucosidase and α-amylase exhibited a strong positive association ($R = 0.88$, $p = 0.0007$), consistent with their complementary roles in carbohydrate digestion. A moderate correlation was also found between α-glucosidase and DPP-4 ($R = 0.65$, $p = 0.04$), reinforcing the interplay in glucose metabolism.

In addition, several phenolic compounds demonstrated strong and statistically significant negative correlations with the IC_{50} of the enzymes, as shown in Fig. 5, suggesting potential inhibitory effects and relevant bioactivity. Significant negative correlations were identified for AGEs with procyanidin B2 ($R = -0.72$, $p = 0.02$), gallic acid ($R = -0.70$, $p = 0.02$), and phlorizin ($R = -0.65$, $p = 0.04$), indicating potential roles in reducing advanced glycation end-products. Furthermore, radical superoxide scavenging exhibited negative associations with phlorizin ($R = -0.69$, $p = 0.02$), procyanidin B2 ($R = -0.64$, $p = 0.04$), and phloretin ($R = -0.64$, $p = 0.04$), while gallic acid ($R = -0.78$, $p = 0.008$) together with procyanidin B2 ($R = -0.86$, $p = 0.001$) with ACE, reinforcing their cardiovascular relevance, with procyanidin B2 being the most pronounced inverse association. These mechanisms exhibited multiple significant negative correlations with different phenolics, suggesting synergistic inhibitory effects and emphasizing the importance of polyphenol synergy and features in enhancing enzyme inhibition and improving cardiometabolic health.

Additionally, quercitrin showed a strong negative correlation with DPP-4 ($R = -0.83$, $p = 0.003$), highlighting its possible contribution to glycemic control. Other notable findings include phloretin with lipase ($R = -0.79$, $p = 0.006$), suggesting an effect on lipid digestion, also HMGR correlated negatively with isoquercitrin ($R = -0.65$, $p = 0.043$), suggesting possible implications for cholesterol metabolism. Nevertheless, α-glucosidase (R = -0.59) and α-amylase ($R = -0.55$) enzymes showed moderate negative correlations with gallic acid, but none reached statistical significance ($p > 0.05$) indicating a potential mechanistic trend towards inhibition of carbohydrate digestion without strong evidence under current conditions.

Furthermore, there was no significant correlation ($p > 0.05$), between any of the enzymes and TPC nor TFC of the targeted phenolic compounds. However, TPC appears to be biologically relevant for the antioxidant activity of the extracts in the scavenging of superoxide radicals, as indicated by the observed correlation ($R = -0.51$). However, given that only a partial phenolic analysis was performed, the absence of significant differences is understandable.

Collectively, these findings support the hypothesis that specific polyphenols, particularly procyanidin B2, quercitrin, gallic acid, phloretin, phlorizin, and isoquercitrin may exert beneficial effects by inhibiting enzymes involved in hypertension, diabetes, oxidative stress and lipid metabolism. Nevertheless, other phenolic compounds showed some degree of correlation with enzymatic inhibition, although without statistical significance, as this could bias enzymes associated with a

Table 2
Results of antidiabetic, antiglycation, antioxidant activity hypolidemic, hypocholesterolemic and hypotensive activity against α-glucosidase, α-amylase, DPP-4, AGEs formation, radical superoxid, lipase, HMG-COA reductase and ACE. Data are expressed as mean ± SEM from at least three independent experiments. IC_{50} values were calculated using nonlinear regression. Statistical significance: ns = not significant; *$p < 0.05$; **$p < 0.001$; ***$p < 0.0005$; ****$p < 0.0001$ *vs.* control.

IC_{50} values (μg mL^{-1}) of the *in vitro* assays								
Extracts	α-Glucosidase	α-Amylase	DPP-4	AGEs	$O_2\bullet^-$ scavenging	Lipase	HMGR	ACE
Verde Doncella	361.56 ± 6.04 ns	875.84 ± 72.132****	1766.89 ± 29.57****	203.44 ± 5.61****	2.75 ± 0.26 ns	363.90 ± 25.91***	100.88 ± 3.93***	42.17 ± 3.11 ns
Golden Smoothee	1342.37 ± 58.26****	n.a	1114.76 ± 49.74****	381.16 ± 24.83****	4.03 ± 0.48 ns	875.02 ± 13.57****	469.49 ± 8.04****	209.28 ± 17.26****
Reineta	486.47 ± 8.85*	1474.71 ± 69.02****	1193.10 ± 48.02****	189.36 ± 13.15****	2.47 ± 0.50 ns	225.43 ± 6.31*	149.39 ± 9.39****	84.34 ± 11.58*
Fuji	826.19 ± 51.25****	n.a	1281.03 ± 133.44****	434.48 ± 15.64****	2.13 ± 0.29 ns	397.1 ± 44.77****	n.a	206.28 ± 12.12****
Royal Gala	1644.86 ± 43.83****	n.a	1763 ± 89.71****	419.44 ± 20.90****	4.18 ± 0.46 ns	635.65 ± 13.48****	n.a	290.02 ± 22.81****
Amarilla de Octubre	105.60 ± 7.23 ns	606.94 ± 17.23****	628.03 ± 21.09***	49.77 ± 3.52 ns	1.18 ± 0.21 ns	419.31 ± 17.76****	n.a	55.94 ± 1.18 ns
Pomera de Pomes Agrias	123.71 ± 11.70 ns	337.12 ± 59.73*	480.63 ± 3.39*	60.08 ± 3.01 ns	1.98 ± 0.02 ns	447.02 ± 25.45****	n.a	81.32 ± 10.33*
Vadiello-02	244.14 ± 15.08 ns	194.79 ± 22.88 ns	1110.34 ± 66.72****	871.90 ± 9.20****	9.74 ± 2.78*	948.38 ± 5.26****	233.04 ± 17.78****	287.24 ± 21.32****
Daroca-02	1484.60 ± 66.79****	n.a	1496.99 ± 131.97****	343.74 ± 14.95****	4.10 ± 0.92 ns	1332.2 ± 83.88****	n.a	90.91 ± 12.09*
C. de Castellano de Daroca	1733.29 ± 112.18****	n.a	1617.74 ± 116.73****	835.12 ± 34.16****	7.12 ± 0.45 ns	901.74 ± 89.56****	n.a	242.44 ± 20.55****
Acarbose	220 ± 64.10	7.72 ± 1.89	–	–	–	–	–	–
Sitagliptin	–	–	0.04 ± 0.01	–	–	–	–	–
Aminoguanidine	–	–	–	55.01 ± 5.63	–	–	–	–
Quercetin	–	–	–	–	4.16 ± 0.24	–	–	–
Orlistat	–	–	–	–	–	32.20 ± 4.28	–	–
Pravastatin	–	–	–	–	–	–	0.012 ± 0.0001	–
Captopril	–	–	–	–	–	–	–	0.003 ± 0.001

n.a: no activity.

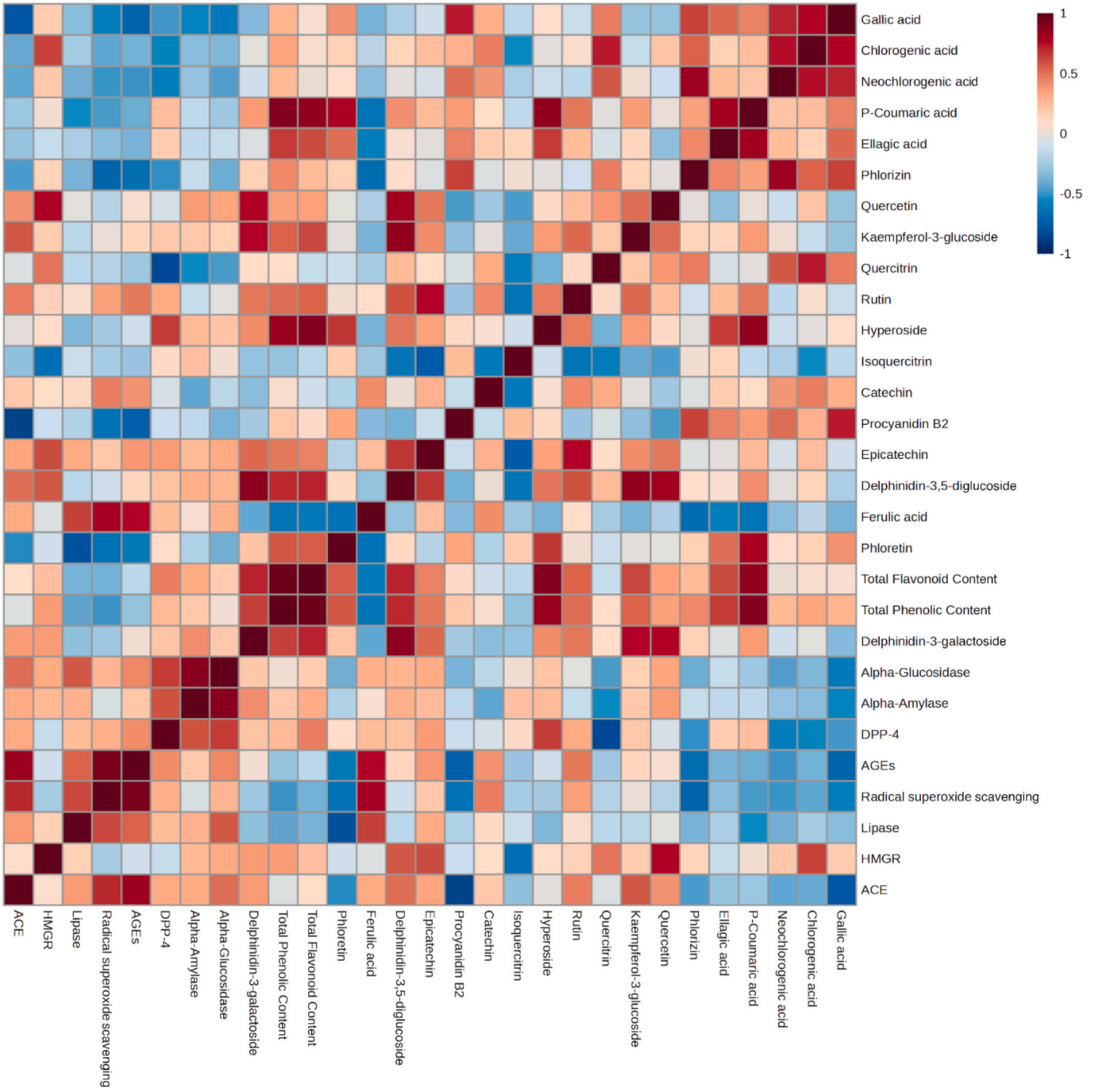

Fig. 5. Pearson correlation heatmap showing the relationships between IC_{50} values of the enzymes obtained from all *in vitro* bioassays and the individual phenolic content. Each cell displays the Pearson correlation coefficient, where a value of 1.0 denotes a positive linear correlation (red) and − 1.0 indicates negative correlation (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

single phenolic compound. However, their potential contribution to the overall inhibitory biological effect cannot be ruled out, since enzyme inhibition by complex plant matrices often results from synergistic or additive interactions among multiple bioactive compounds. Due to the limited sample size along with the limited bioactive compounds analysed, it is not possible to determine the actual impact of these secondary correlations.

4. Discussion

By delving deeper into the targeted analysis of the phenolic profile using HPLC/MS along with the chemometric analysis, the highest TPC was found in the commercial cluster of Fuji and Royal Gala, followed by the cluster of Verde Doncella and Amarilla de Octubre. This result contrasts with other previous studies focused on the phenolic content of pulp (Millán-Laleona et al., 2025) and peel (Cano-Lou et al., 2025) from different apple cultivars, where autochthonous samples were richer in the TPC than the commercial samples. This could be explained by the rise of sugar content, added to a reduction in phenolic compounds and the antioxidant activity during the ripening process, especially in commercial samples (Zheng et al., 2012).

As shown, all thinned apple by-products are rich sources of phenolic compounds such as hyperoside, isoquercitrin, and delphinidin-3,5-diglucoside, followed by chlorogenic acid, quercitrin, rutin, catechin,

procyanidin B2, and epicatechin with yields ranging between 20 and 30% (*w*/w) of dry ethanolic extracts relative to the weight of the lyophilized samples and between 8105.21 and 14,010.93 mg kg^{-1} of TPC. Indicating that bulk extract recovery does not necessarily translate into proportional differences in phenolic loading. Additionally, the great yield as well as the rich phenolic matrix obtained suggests that this type of residue is a great source of phenolic compounds, and that the extraction method along with the ethanol solvent system is efficient (Do et al., 2014).

The PCA biplot analysis suggests compositional trends in phenolic group content, with a higher ratio of anthocyanins and flavonols in commercial samples, and a greater presence of flavan-3-ols, cinnamic acids, dihydrochalcones, and hydroxybenzoic acids in autochthonous samples. These results align with previous research comparing commercial and autochthonous apple peel by-products (Cano-Lou et al., 2025).

Regarding the PCA of individual phenolic compounds and the hierarchical clustering heatmap, both indicate that autochthonous and commercial samples display high variability in phenolic distribution, with no clear separation and mixed clusters containing both commercial and autochthonous cultivars. Differences in phenolic composition among cultivar clusters clearly contributed to the wide range of bioactivities observed across the thinned apple extracts against multiple MetS enzymatic targets. Within all samples, cluster II of the hierarchical clustering heatmap, especially Reineta and Pomera de Pomes Agrias, as well as cluster IV of Verde Doncella-Amarilla de Octubre exhibited the highest results in all the bioactivities performed, pointing out the relationship between their individual phenolic content and the great bioactive properties. However, the autochthonous samples of both clusters (Pomera de Pomes Agrias, Amarilla de Octubre) exhibited higher antioxidant, antiglycant and antidiabetic activity, in contrast to the commercial samples of both clusters (Reineta, Verde Doncella) which exhibited greater hypolipidemic and hypocholesterolemic activity. In addition, cluster IV exhibited the greatest hypotensive activity. Apart of these samples, Vadiello-02 from all autochthonous cluster I also showed great antidiabetic and hypocholesterolemic activity, despite being the sample with the lowest TPC.

These findings suggest that the inhibitory activity seems to be more strongly associated with the specific molecular structures of the phytochemicals and the conformational properties of the target proteins, which govern the formation of protein–phenolic complexes essential for inhibition (Dalar & Konczak, 2013). The evidence presented indicates that the targeted TPC is not a reliable predictor of enzyme inhibitory capacity of thinned apple extracts. Depending on their specific chemical structures such as the quantity and positional distribution of hydroxyl groups, the glycosylation and the structural complexity of phenolic compounds may influence the interaction with the enzymatic catalytic site of digestive enzymes (Martinez-Gonzalez et al., 2017), DPP-4 (Huang et al., 2019), ACE (Chen et al., 2021) and HMGR through hydrophobic interactions, metal chelation, or hydrogen bonding. However, in HMGR, steric limitations often redirect binding to the NADPH cofactor site (Ojha et al., 2015) making it a unique exception among these enzymes, which may explain why only 4 out of 10 tested extracts exhibited inhibitory activity against this enzyme.

Consequently, the proportion of individual phenolic compounds and their interactions may significantly influence the antioxidant activity, as well as the antidiabetic, hypotensive, hypolipidemic, and hypocholesterolemic effects (Yeh et al., 2017) of thinned apple by-products on MetS.

For instance, procyanidin B2 and phlorizin were present at higher levels in clusters II and IV than the other cultivars, and their significant contribution to radical-scavenging capacity and AGEs inhibition, together with elevated gallic acid concentrations as well, is consistent with enhanced antiglycation potential. This compositional interplay may explain why these autochthonous and commercial clusters outperform the remaining samples in these specific activities. Specifically, Amarilla de Octubre showed the strongest antioxidant and antiglycation activities, which coincided with the highest concentrations of procyanidin B2 (1520.56 mg kg^{-1}), gallic acid (23.11 mg kg^{-1}), and phlorizin (323.67 mg kg^{-1}). The co-enrichment of phlorizin, procyanidin B2, and gallic acid in the most active extracts is consistent with prior studies that report antioxidant and antiglycation activities of these individual phenolic compounds (Bai et al., 2021; Chen et al., 2023; Maisto et al., 2022).

Furthermore, quercitrin was the only phenolic compound significantly associated with an antidiabetic *in vitro* bioactivity, showing a strong significant correlation with DPP-4 inhibition and reaching its highest levels in Amarilla de Octubre and Pomera de Pomes Agrias (1567.98 and 1447.40 mg kg^{-1}). Notably, these two extracts also exhibited the highest DPP-4 inhibitory activity, supporting quercitrin as a key contributor to glycaemic regulation. Likewise, the enrichment in quercitrin can reasonably support DPP-4 inhibition, since isolated quercitrin (quercetin-3-O-rhamnoside) inhibited purified DPP-4 (from Cayman) in a biochemical assay with an IC_{50} of 71.79 ± 1.05 μg mL^{-1} (Liu et al., 2021).

In addition, isoquercitrin and phloretin emerged as the phenolic features most aligned with lipid related *in vitro* activities. Isoquercitrin was particularly abundant in Verde Doncella and Reineta (3550.87 and 3755.56 mg kg^{-1}) and showed a significant correlation with HMGR activity, supporting a potential contribution to hypocholesterolemic effects *via* modulation of cholesterol biosynthesis. In addition, phloretin, detected in clusters II and IV, correlated strongly with the lipase inhibition, and despite its very low abundance it may still contribute to the activity pattern, particularly in Reineta and Verde Doncella. The enrichment of isoquercitrin in the extracts showing stronger HMGR effects are biologically plausible because isolated isoquercitrin (quercetin-3-O-glucoside) has been shown to inhibit purified HMGR (from Sigma Aldrich) in a spectrophotometric assay with an IC_{50} of 80.6 μM (Kwon et al., 2010) corresponding to 37.4 μg mL^{-1}. In addition, the enrichment of the extracts in phloretin and their bioactivity in inhibiting pancreatic lipase are confirmed by the inhibition of the enzyme by pure phloretin, which exhibits an IC_{50} of 164.7 ± 2.4 μM (Jeong et al., 2021) corresponding to 45.2 μg mL^{-1}.

Moreover, procyanidin B2 emerged as the key phenolic driver of ACE inhibition, showing the strongest significant correlation and reaching high levels in Amarilla de Octubre (1520.56 mg kg^{-1}) and Verde Doncella (1456.08 mg kg^{-1}), among the highest across all samples. Consistently, these two varieties displayed the most pronounced ACE inhibitory activity, supporting procyanidin B2 as a major contributor to renin–angiotensin system modulation. Procyanidin B2 is known as the most effective ACE inhibitor among plant flavonoids, as epicatechin dimers have been shown to inhibit ACE (from rabbit lung) with an IC_{50} of 97 μM (Nileeka Balasuriya & Vasantha Rupasinghe, 2011), corresponding to 56.1 μg mL^{-1}.

The IC_{50} value of the most bioactive extracts was within the same order of magnitude as that IC_{50} reported for the previous isolated phenolic compounds. Considering that the previous pure compounds are present in the thinned apple extracts at a lower concentration, this comparable activity suggest that weaker active constituents may contribute to the observed bioactivity (Atanasov et al., 2015). Whether these interactions are additive or synergistic cannot be determined from the present data and warrants further investigation through combination studies (Caesar & Cech, 2019). Furthermore, the lack of significant correlation between antidiabetic digestive enzymes and the targeted phenolics indicates that α-glucosidase and α-amylase inhibition may depend on specific phenolic mixtures or non-targeted metabolites. For instance, lipophilic triterpenes (ursolic and oleanolic acids), reported in apple matrices and linked to carbohydrate enzyme inhibition, may contribute alongside phenolic mixtures in the most active extracts (Amarilla de Octubre, Pomera de Pomes Agrias, Vadiello-02) (Andre et al., 2012; Hussain et al., 2017).

Accordingly, to ensure that the observed bioactivity is interpreted

within a cytocompatible window, we evaluated extract-induced cytotoxicity across representative structural, hepatic, and intestinal models as phenolic compounds can induce some disrupting mitochondrial functions (Procházková et al., 2011).

Most of the thinned apple extracts exhibited a robust safety profile at physiological exposure levels (≥ 70% viability) in non-tumorigenic cells. HFF-1 non-tumorigenic cells were the most resilient model, maintaining the defined cytocompatibility across the full tested range for all extracts (15.62–500 μg mL^{-1}). By comparison, HepG2 and Caco-2 cells, which are tumorigenic models, showed moderate tolerance for most cultivars, but a reduction in viability was detected particularly in Amarilla de Octubre and Pomera de Pomes Agrias, which are enriched in specific antiproliferative phenolic compounds such as quercitrin (Cincin et al., 2014) and chlorogenic acid (Jiang et al., 2021). This fact could explain the cytotoxic/antiproliferative activity of certain extracts in these hepatic and colon cancer-related cell lines.

Collectively, these cell-type and cultivar-dependent thresholds suggest different upper tolerance boundaries under *in vitro* exposure conditions, with fibroblasts exhibiting the widest safety window and intestinal-hepatic models revealing cultivar-specific sensitivity. These findings agree with previous studies on ethanolic thinned-apple polyphenol extracts, which reported significant antioxidant and anti-inflammatory *in vitro* activities with minimal cytotoxicity at comparable doses, maintaining viability ≥70% up to 250–300 μg mL^{-1} in HEK293 and R3/1-NF-κB cells (Ferrario et al., 2022), as well as in murine macrophage-like cells (Zheng et al., 2020). This indicates that *in vitro* bioactivity can be achieved within defined non-cytotoxic windows.

The increased sensitivity observed in HepG2 and Caco-2 tumoral cell lines at high concentrations such as 500 μg mL^{-1} for specific cultivars (Amarilla de Octubre, Pomera de Pomes Agrias, Camuesa de Castellano de Daroca, Daroca-02, Reineta and Verde Doncella) is consistent with dose-dependent cytotoxicity reported of thinned apple extracts, where tumor-derived murine macrophage cells showed a viability decline to 50% at 600 μg mL^{-1} (Zheng et al., 2020). This dose-responsive loss of viability supports the notion that, although apple-derived complexes are generally well tolerated, certain cultivar-specific compositions may approach cellular stress thresholds at certain concentrations. For instance, the samples that induced the strongest reduction in HepG2 cell viability at lower concentrations (Amarilla de Octubre and Pomera de Pomes Agrias) were those most enriched in chlorogenic acid, a phenolic compound highly associated with enhanced antiproliferative activity in hepatic tumor models (Jiang et al., 2021).

In addition, HepG2 hypersensitivity should be interpreted cautiously because *in vivo* hepatocyte exposure occurs only after intestinal absorption and first-pass metabolism, resulting in substantially lower exposition concentrations than those applied in *in vitro* assays. In contrast, the generally higher tolerance observed in Caco-2 supports the feasibility of local intestinal applications, where luminal concentrations higher than systemic levels may be required for efficacy without compromising epithelial viability within defined cytocompatible ranges (Sahu et al., 2014).

Moreover, *in vitro* IC_{50} values of thinned apple extracts should be interpreted in the context of achievable exposure, ideally by benchmarking them against unbound plasma concentrations and target tissue levels (Abdel-Tawab, 2021). Considering that phenolic compounds typically circulate at relatively low concentrations (10–500 ng mL^{-1}), direct systemic inhibition may be modest, whereas higher local tissue levels (up to 1 μg mL^{-1}) can make target modulation more plausible (Yoshida et al., 2017). In this setting, efficacy may be further enhanced by the combined action of metabolites, tissue accumulation, and site-specific exposure such as in hepatic parenchyma for HMGR, vascular endothelium for ACE, and multiple systemic tissues for DPP-4 (Abdel-Tawab, 2021). Crucially, these concentrations remain within the safety boundaries of Caco-2, HepG2 and HFF-1 cells enhancing metabolic modulation.

The multitarget effect towards different enzymes (ACE, HMGR), combined with antiglycan activity and a potent antioxidant protection towards $O_2\bullet -$ scavenging, may yield meaningful benefits by acting on several pathways simultaneously. Specifically, Verde Doncella and Reineta demonstrated a superior therapeutic window for cardiovascular and lipid management, inhibiting ACE and HMGR below 250 μg mL^{-1} under non-cytotoxic conditions. Additionally, Amarilla de Octubre and Pomera de Pomes Agrias also contributed to ACE, AGEs inhibition and radical scavenging at concentrations that can be considered cytocompatible, particularly in the non-tumoral HFF-1 cells. The most biological relevance of these thinned apple extracts lies in the correlation of antioxidant, anti-glycation, and ACE-inhibitory bioactivities together with an enriched profile of bioactive compounds such as procyanidin B2, gallic acid and phlorizin. This convergence suggests a coherent chemical feature underlying the observed functional effects.

Notably, several extracts demonstrated *in vitro* bioactivities comparable to reference substances with no statistically significant differences ($p > 0.5$) in AGEs inhibition (Pomera de Pomes Agrias, Amarilla de Octubre), ACE inhibition (Verde Doncella, Amarilla de Octubre), and antioxidant activity (all extracts except Vadiello-02); such bioactivities reinforce the potential translational value of these extracts. In contrast, HMGR inhibition was considered of lower biological relevance because it was only detected in four extracts (Reineta, Verde Doncella, Golden Smoothee and Vadiello-02), associated with a single phenolic marker (isoquercitrin), and markedly less potent than pravastatin. Meanwhile, the DPP-4 inhibition was not considered interesting as IC_{50} values were very high. Within this framework, Clusters II and IV, and particularly Amarilla de Octubre, emerge as key candidates for biological relevance in MetS. This claim is supported by the highest contain in procyanidin B2, gallic acid, and phlorizin, which, despite representing only 0.19% of the total extract mass in Amarilla de Octubre, potentially points towards a high antiglycation and antioxidant activity along with inhibitory interaction with ACE.

In addition, the cultivar Pomera de Pomes Agrias also stand out as a prominent multitarget inhibitor in key intestinal enzymes involved in carbohydrate metabolims (α-glucosidase, α-amylase, DPP-4) while maintaining IC_{50} values under non-cytotoxic conditions in Caco-2. This activity profile supports their biological relevance as candidate for the development of nutraceuticals with antidiabetic properties.

Furthermore, the obtained data encourage deeper investigation for potential industrial applications and incorporation of isolated compounds or complex mixtures into nutraceuticals and dietary supplements in the health and well-being sector.

5. Conclusion

Targeted HPLC–MS profiling of UAE ethanolic extracts from thinned apples identified hyperoside, isoquercitrin, and delphinidin-3,5-diglucoside as the predominant phenolics across samples. The marked cultivar-driven diversity supported the efficiency of the extraction approach, and PCA clearly separated commercial cultivars from autochthonous cultivars phenolic groups. However, hierarchical clustering at the level of individual phenolics produced mixed clusters containing both origins, indicating clusters enriched in specific phenolic compounds. Integrating these chemical targeted fingerprints with chemometric modelling linked composition to biological potency (IC_{50}), enabling early prioritization of predictive metabolite features and supporting a data-guided selection of candidate extracts or enriched mixtures for health applications. Accordingly, phenolic compounds such as procyanidin B2, quercitrin, gallic acid, phloretin, phlorizin, and isoquercitrin emerge as plausible contributors to multi-pathway metabolic modulation supported by significant correlation.

In addition, the cultivars Amarilla de Octubre and Pomera de Pomes Agrias showed the best antioxidant-antiglycation activity, with interesting antidiabetic activity (due to α-glucosidase, α-amylase and DPP-4 inhibition) while Reineta and Verde Doncella showed the most pronounced hypolipidemic/cardiovascular activities (due to lipase, HMGR

and ACE inhibition). Such findings highlight a possibility to use thinned apples as food matrices for the extraction of phenolic compounds and development of nutraceuticals, emphasizing the value of reusing by-products generated by the agronomy sector for new industrial applications.

CRediT authorship contribution statement

Javier Cano-Lou: Writing – original draft, Methodology, Investigation, Formal analysis. **Gema Casado-Hidalgo:** Methodology, Formal analysis. **Francisco Les:** Writing – review & editing, Supervision, Methodology. **Ana Pina:** Methodology, Investigation. **Laura Acquaticci:** Methodology, Investigation. **Giovanni Caprioli:** Methodology, Investigation. **Víctor López:** Writing – review & editing, Supervision, Resources, Investigation, Funding acquisition, Conceptualization.

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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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2026.103640.

Data availability

The authors confirm that all relevant data supporting the findings of this research are included in the manuscript. Additional raw and processed datasets can be obtained from the corresponding author upon reasonable request.

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