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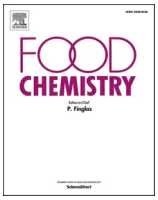

Extraction of phenolic compounds from grape pomace using ohmic heating: Chemical composition, bioactivity and bioaccessibility

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ABSTRACT

This study addresses the effectiveness of ohmic heating technology (OH) for the sustainable recovery of phenolic compounds from Grape Pomace (GP) by hydroethanolic extraction. GP extracts biological potential was evaluated in terms of antioxidant activity, cytotoxicity and preventive effect against reactive oxygen species (ROS). To understand if GP extracts can be used as a functional ingredient, simulated gastrointestinal digestion was performed to evaluate the bioaccessibility. OH-assisted hydroethanolic extraction proved to be an effective process for the recovery of GP phenolic compounds with high antioxidant capacity. The digestion process increased the concentration of total phenolics and the biotransformation of high-molecular phenolics (anthocyanins, flavonoids and resveratrol) in simpler phenolic acids, improving bioaccessibility. GP extract displayed a selective action against cancer cells (Caco-2 and HeLa) and promoted ROS prevention. The results highlighted the ability of OH to extract bioactives from GP and its potential application as a nutraceutical or for functional food formulations.

1. Introduction

Grape (*Vitis* sp.) is an extensively produced crop. In 2022 the world production was 73.5 million tons (FAOStat, 2022). The grape production in Europe represents 36% of the total production, and the main European producers are Italy, Spain, France, Germany, and Portugal. According to the International Organisation of Vine and Wine, in 2022, the estimated world wine production was 258 $\times$ 10^8 L (*World* Wine Production Outlook OIV First Estimates., 2022).

The wine industry generates a significant amount of waste, being the most abundant the grape pomace (GP). GP signifies about 20% (w/w) of the total grapes used for winemaking. GP is produced upon pressing the grapes and comprises pressed skins, seeds and grape stems. GP has traditionally been employed for the production of spirits or as fertilizer. It is also used to obtain valuable products including enzymes, grape seed oil, dyes, and tartaric acid (Errichiello et al., 2023). As this is a by-product widely produced in the winemaking process, it is important to find an alternative use for it.

During regular metabolic processes or in response to environmental factors, such as temperature, UV radiation, illness, and other, plants can produce a wide range of chemical molecules known as secondary metabolites (Alara et al., 2021). These metabolites are grouped according to the number of phenol rings, and bonds formed to hold the ring in place. So, phenolic compounds can be divided into flavonoids (including anthocyanins), phenolic acids, stilbenes and lignans (Ferreira-Santos, Zanuso, et al., 2020). Several studies have shown that agri-food wastes, vegetables, fruits, micro and macro-algae, among others, are extraordinary sources of phenolic compounds (Ferreira-Santos, Genisheva, et al., 2020; Ferreira-Santos, Miranda, et al., 2021; Geada et al., 2018; Mármol et al., 2021; Pereira et al., 2016).

GP is rich in phenolic compounds (anthocyanins, flavanols, resveratrol and phenolic acids), which have interesting biological properties including antioxidant, cytotoxic, anti-inflammatory and antimicrobial activity (Caponio et al., 2022; Milinčić et al., 2021; Oliveira et al., 2013; Peixoto et al., 2018).

Usually, the conventional extraction systems of valuable compounds from agri-food residues and other bioresources involves the use of organic solvents and heating for prolonged periods of time, promoting

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the degradation of compounds of interest (Ferreira-Santos, Zanuso, et al., 2020; Milinčić et al., 2021), making it an expensive and highly polluting process, often not suitable for the expected purpose. Taking in consideration the exposed facts, it is important to develop and optimize a sustainable and effective extraction methodology according to Green Chemistry principles (Ferreira-Santos, Zanuso, et al., 2020).

Faster and automated methods have been applied to extract phenolics and other bioactive compounds, such as ultrasounds, microwaves, supercritical and subcritical extraction, and distinct electrotechnologies (Ferreira-Santos, Zanuso, et al., 2020). These methods aim to save energy, reduce the use of organic solvents, considerably decrease the environmental impact and lowering the overall costs.

Ohmic (OH) heating is an electrotechnology that focuses on the use of an electric current passing directly through the process product, resulting in a rapid and homogeneous heating (Joule effect). Furthermore, the electric field induces electroporation and electropermeabilization of the cell membrane increasing the intracellular compounds diffusion into the solvent (Hashemi Gahruie et al., 2020). However, the extent of permeabilization depends on several variables, such as electric current intensity, processing time and others (Rocha et al., 2018). OH has been used in various industrial applications, like blanching, evaporation, dehydration, and pasteurization (Sarkis et al., 2013). Recently, this technology has been applied for bio-functional compounds extraction (Rocha et al., 2018). OH in contrast with other electrotechnologies has an advantage, the ability to precisely control the heating rate and thermal load. Ferreira-Santos et al. (2019) demonstrated that OH applied in the extraction of phenolic compounds, leads to an increase of the extraction yield, promoting selective compound extraction, decreasing energy consumption. The combination of this characteristics leads to a process with a lower cost and higher efficiency.

If food or therapeutic applications, like functional foods and dietary supplements, are considered for the GP extract (GPE), it is required to assess its effect in terms of toxicity and overall bioactive potential (Milinčić et al., 2021; Salehi et al., 2019). If the oral administration route is considered, it is mandatory to evaluate the changes induced by the conditions of the gastrointestinal tract, as well as its bioaccessibility upon digestion.

Taking in consideration the previously exposed facts, this study was designed to assess the suitability of water and ethanol thermal extraction assisted by electric fields (*i.e.* OH) to extract phenolic compounds from GP. Results of OH were also compared with conventional thermal extraction method (CH) performed under the same conditions. Analytical studies were performed to assess the extracts chemical characteristics, and GP cells morphological changes were observed by scanning electronic microscopy (SEM). In order to evaluate GPE potential as a functional ingredient for oral administration, several studies were performed, particularly the changes induced by simulated gastrointestinal digestion (GID). Additionally, the bioactive potential of GPE was assessed in terms of chemical antioxidant capacity, cytotoxicity and the ability to prevent an exacerbated formation of intracellular reactive oxygen species (ROS).

2. Material and methods

2.1. Chemicals and raw material

The grape pomace of the Croatina variety was obtained in the production of wine from the 2019 vintage of the Mossi winery (Ziano Piacentino, Piacenza, Italy). The pomace was dried at 35 °C for 5 days (moisture content < 10%). The material was milled in a cutting mill (Retsch Mill MM 2000, Retsch, Haan, Germany) to a granulometry of 1–1.6 mm. The ground GP was sealed in bags and stored in a dry dark location, for further use.

All chemical reagents and standards were obtained from Sigma-Aldrich (St. Louis, MO, USA): Folin-Ciocalteu reagent, 2,2-Di(4-*tert*-octylphenyl)-1-picrylhydrazyl (DPPH), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), aluminium chloride ($AlCl_3$), porcine pepsin, pancreatin, bile extract, $(NH_4)_2{\cdot}CO_3$, $NaHCO_3$, $MgCl_2{\cdot}6H_2O$, HCl (37 % (w/v)), NaCl, KCl, NaOH, KH_2PO_4, $CaCl_2{\cdot}2H_2O$, dimethyl sulfoxide (DMSO, ≥99.9%), fetal bovine serum (FBS), penicillin–streptomycin, Dulbecco's Modified Eagle Medium (DMEM), resazurin sodium salt, and all standards for HPLC (3,4-hydroxybenzoic acid (DHB acid), 2,5-DHB acid, ellagic acid, caffeic acid, ferulic acid, *o*-cumaric acid, gallic acid, rosmarinic acid, apigenin, naringenin, quercetin, rutin, taxifolin, catechin and resveratrol). Water Milli-Q was used during the experiments.

2.2. Conventional and ohmic heating extraction conditions

The extractions were carried out in a double-walled water-jacketed cylindrical glass reactor (overall volume of the reactor 250 mL) with two stainless-steel electrodes coated with polytetrafluoroethylene. The electrode distance was kept constant (5 cm). To avoid direct light incidence, the reactor was adequately covered with aluminum foil.

For OH experiments (7–14 V/cm), the power source worked with a sinusoidal wave at 25 kHz, originated from a function generator (Agilent 33220A, 1 Hz − 25 MHz and 1–10 V; Penang, Malaysia) amplified by a power amplifier (Peavey CS3000, Meridian, MS, USA). The temperature was monitored with a stainless-steel type-K thermocouple (Omega Engineering, Inc., Stamford, CT, USA), placed in the center of the reactor. The thermocouple was coupled to a data logger (USB-9161, National Instruments Corporation, Austin, TX, USA), and the data was extracted using Lab View 7 Express software (National Instruments, NI Data logger). During OH treatments, an oscilloscope (ScopeMeter R 125/S, Fluke, WA, USA) was utilized to detect electrical frequency, voltage, and current intensity.

To achieve the same heating rates in all treatments, a thermostatic circulator water system (F25-ED, Julabo, Seelbach, Germany) was utilized for conventional thermal extractions (CH, 0 V/cm).

For the extractions, 15 g of dried GP was mixed with 150 mL of solvent (water or ethanol 50% (v:v)). The extractions were performed in a glass reactor vessel (previously described) at 50 or 80 °C for 30 or 60 min with a magnetic stirrer (size of 0.5 cm) at 150 rpm to promote homogeneous heat transfer during the heating cycle. Before each experiment, electrical conductivity was adjusted to 3.0 ± 0.1 mS/cm using NaCl and controlled by a conductivity meter (HANNA Instruments Inc., edge, HI2003, USA).

The obtained extracts were centrifuged at 7000 *g* for 10 min (Fiberlite™ F146x250LE Fixed Angle Rotor, Thermo Scientific™, USA) and filtered through 10 μm filter paper. The extracts were stored at 4 °C, and some were dried by freeze-drying. Extractions were performed by quadruplicates.

2.3. Extraction yield

The extraction yield can be used to evaluate the extraction methodology suitability for the recover the target compounds from a dry GP biomass. The yield results (presented in %) were considered taking into account the cumulative mass of the grape pomace extract (GPE) recovered relatively to the initial GP biomass.

2.4. Morphological characterization of biomass

Morphological analyses of GP biomass were performed in an Ultra-high resolution Field Emission Gun Scanning Electron Microscope (FEG-SEM; NOVA 200 Nano SEM, FEI Company, Hillsboro, Oregon, USA) applying an acceleration voltage of 10 kV. All SEM images were acquired using the secondary electron (SE) detector.

Previous to the analysis, freeze-dried GP samples were coated with a thin film (40 nm) of Au-Pd (80–20 wt%), using a high-resolution sputter coater (208HR Cressington Company, Watford, UK) coupled to an MTM-20 Cressington High-Resolution Thickness Controller.

2.5. Phytochemical characterization of extracts

2.5.1. Total phenolic content

The total phenolic content (TPC) was measured in liquid extracts by the Folin–Ciocalteau method, following the procedure descrived by Ferreira-Santos et al. (2019). Gallic acid was used as a standard compound (100–2,000 mg/L, $R^2 = 0.995$), and the data were expressed as milligrams of gallic acid equivalents (GAE) per gram of dry GP (mg GAE/g GP).

2.5.2. Total flavonoid content

The total flavonoid content (TFC) was determined in liquid extracts by a colorimetric assay described by Ferreira-Santos et al. (2020). The standard curve was developed using (+)-catechin (5–500 mg/L, $R^2 = 0.996$) and the results were reported as mg of catechin equivalents (CE) per g of dry GP (mg CE/g GP).

2.5.3. Total anthocyanin content

The content of total anthocyanins (TA) was quantified in liquid extracts by pH difference method (AOAC, 2005). The anthocyanin content was represented as cyanidin-3-glucoside (cyd-3-glu) equivalents per gram of dry GP (mg cyd-3-gluE/ g GP), using Equation (1):

$$TA\ (\text{cyd}-3-\text{gluE, mg/L}) = \frac{A \times MW \times DF \times 10^3}{\varepsilon \times 1} \quad (1)$$

Where A = ($A_{520nm} - A_{700nm}$) pH 1.0 – ($A_{520nm} - A_{700nm}$) pH 4.5; MW (molecular weight) = 449.2 g/mol for cyd-3-glu; DF = dilution factor; 1 = pathlength in cm; ε = 26 900 M extinction coefficients (L × mol^{-1} × cm^{-1}) for cyd-3-glu; and 10^3 = g to mg conversion factor.

2.5.4. Determination of phenolic compounds by HPLC

The identification and quantification of individual phenolic compounds were carried out as previously described by Ferreira-Santos, Genisheva, et al. (2020) using a Shimadzu Nexpera X2 UPLC chromatograph equipped with Diode Array Detector (DAD) (Shimadzu, SPD-M20A, Columbia, MA, USA). A Aquity BEH C18 column (2.1 mm × 100 mm, 1.7 μm particle size) (Waters, Milford, MA, USA) and mobile phase composed by water-formic acid (0.1%) and acetonitrile at flow rate of 0.4 mL/min was used for separation. The oven temperature was 40 °C, and injection volume of 5 μL. Phenolics were identified and quantified by comparation of UV spectra and retention times with standards. Calibration curves for each compound were prepared at concentrations ranging from 2.5 to 250 mg/mL, and the coefficient of linear correlation (R^2) was higher than 0.99 in all curves. Compounds were quantified and identified at different wavelengths (209–370 nm), as previously validated (Ferreira-Santos, Ibarz, et al., 2021).

2.6. Antioxidant activity

The antioxidant capacity of extracts was accessed in liquid extracts by two different assays: Ferric reducing antioxidant power (FRAP) and free radical scavenging (DPPH).

FRAP method was performed according to Ferreira-Santos, Duca, et al. (2021) using a solution of ferrous sulfate as a standard (800–100 μM, $R^2 = 0.993$). FRAP results are reported as micromoles of ferrous equivalent per g of dry GP (μmol Fe^{2+}/g GP).

The DPPH assay was performed following the method reported by Ferreira-Santos et al. (2019). Calibration curves were obtained with Trolox as a standard compound (15 to 250 μM, $R^2 = 0.999$). DPPH values were reported as micromoles of Trolox equivalent (TE) per gram of dry GP (μmol TE/g GP).

2.7. Cell viability

The toxicity of the freeze-dried GPE [obtained by OH using ethanol 50% (v/v), 80 °C for 30 min] was assessed *in vitro* using two normal cell lines - mouse fibroblast (L929, ATCC® CCL-1, passage 7) and human embryonic kidney (HEK293T, ATCC® CRL-11268, passage 14), and two cancer cell lines - human colorectal cell lines (Caco-2, ATCC® HTB-37™, passage 19) and human cervix adenocarcinoma (HeLa, ATCC® CCL-2™, passage 22). The cells were cultivated under the same conditions already used by us in the same laboratory (Ferreira-Santos, Genisheva, et al., 2020). After, the cells were seeded in a 96-well plate at a density of 1 × 10^5 cells per well for 24 h and then incubated with GPE diluted in supplemented DMEM in concentration between 25 and 4,000 μg/mL for 36 h (supplemented DMEM as used as control). After incubation, cell viability was determined using the resazurin method (Ferreira-Santos, Genisheva, et al., 2020), and the pink fluorescent resulting product (resorufin) was detected using a microplate reader (Cytation 3, BioTek Instruments, Inc., Winooski, VT, USA) at 560 nm (λex) and 590 nm (λem). The cell viability (%) was obtained by subtracting blank data (cell-free medium) and comparing to untreated controls.

2.8. Reactive oxygen species

To determine intracellular ROS levels in L929 and HeLa cells, a commercial kit (ab 113851, DCFDA/H2DCFDA – Cellular ROS Assay Kit by Abcam plc®, Cambridge, UK) was used. Cells were seeded in 96-well plates at a density of 2.5 × 10^4 cells/mL and were incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 12 h. For treatment, catechin was employed as a reference standard (100 μM) and a dose of 100 μg/mL of freeze-dried GPE [obtained by OH using ethanol 50% (v/v), 80 °C for 30 min] dissolved in supplemented DMEM, and placed in contact with different cell lines for 8 h. Afterward, cells were washed with PBS and incubated with 25 μM 2′,7′-dichlorofluorescein diacetate (DCFDA) for 45 min at 37 °C. *Tert*-butyl hydrogen peroxide (TBHP, 100 μM) dissolved in PBS was used to induce ROS for 1 h. Control cells were incubated with PBS. The resulting fluorescence intensity was measured at wavelengths of 485 (λex) and 535 nm (λem) and considered a reflection of the total intracellular ROS level. Each experiment was performed in triplicate.

2.9. In vitro digestion of phenolic compounds

In this work, a static protocol made available by the international consensus INFOGEST group (Brodkorb et al., 2019) was used to simulate human gastrointestinal digestion (GID).

A simulated salivary fluid for the oral (SSF), gastric (SGF), and intestinal (SIF) digestion was prepared at a 1.25x concentration, for further addition through digestion. The SSF stock solution was composed as follows (mmol/L): 15.1 KCl, 3.7 KH_2PO_4, 13.6 $NaHCO_3$, 0.15 $MgCl_2{\cdot}6H_2O$, 0.06 $(NH_4)_2.CO_3$, and 1.1 HCl; while SGF was composed of (mmol/L): 6.9 KCl, 0.9 KH_2PO_4, 25 $NaHCO_3$, 47.2 NaCl, 0.12 $MgCl_2{\cdot}6H_2O$, 0.5 $(NH_4)_2.CO_3$ and 15.6 HCl; and SIF of (mmol/L): 6.8 KCl, 0.8 KH_2PO_4, 85 $NaHCO_3$, 38.4 NaCl, 0.33 $MgCl_2{\cdot}6H_2O$, and 8.4 HCl. Solutions were then adjusted to a 1x concentration with Milli-Q water during the digestion. To avoid precipitation, a 0.3 M $CaCl_2{\cdot}2H_2O$ solution was prepared separately for further addition during the digestion steps.

For the oral phase digestion, 250 mg of freeze-dried GPE was suspended in 5 mL of Milli-Q water and mixed with 4 mL of SSF, 25 μL of $CaCl_2$, 0.5 mL of salivary α-amylase solution (activity of 75 U/mL), and 0.475 mL of Milli-Q water. To simulate the gastric phase, it was then added 6.4 mL of SGF stock solution, 0.4 mL of pepsin solution (2000 U/mL), and 4 μL of 0.3 M $CaCl_2$.The pH was adjusted to 3.0 (with HCl, 1 M) and the required volume of water was added to complete 16 mL of total gastric volume. The intestinal phase was reproduced by adding 5.95 mL of SIF, 3.5 mL of a pancreatin solution (trypsin activity 100 U/mL), 1.75 mL of bile solution (10 mmol/L), 28 μL of $CaCl_2$. The pH was adjusted to 7.0 (with NaOH, 1 M) and the required volume of water was added to complete 28 mL of total intestinal volume. The assay was conducted at 37 °C under agitation, using a water bath. The oral, gastric and intestinal

steps were simulated for 2 min, 2 h, and 2 h, respectively.

Samples (2.0 mL) were collected at the end of the oral and gastric phases. The collected samples and final intestinal phase were filtered (0.22 μm) and frozen (−20 °C) and used for subsequent phenolic (TPC, TA and HPLC) and antioxidant analyses. The *in vitro* digestions were performed at least in triplicate.

2.10. Bioaccessibility index

The bioaccessibility index of phenolic compounds was determined according to Equation (2):

$$\text{Bioaccessibility}\ (\%) = \frac{PCd}{PCa} \times 100 \quad (2)$$

where PCd, phenolic content after digestion; PCa, phenolic content before digestion.

2.11. Statistical analysis

The results were examined using GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA, USA). Data values were expressed as mean ± standard deviation (SD) and the analysis of variance (ANOVA) followed by Tukey's multiple comparisons test were employed for statistical analyses performed at 95% confidence interval ($p < 0.05$).

3. Results and discussion

3.1. Extraction conditions and morphological changes in biomass

The results obtained for the different extraction conditions, *i.e.* extraction solvent (water and ethanol 50% (v:v)), temperature (50 and 80 °C) and time (30 and 60 min), tested on the ohmic heating extraction process, are represented in Fig. 1 and Table S1 (Supplementary material).

The factors represented in the Principal Component Analysis (PCA) clearly indicate that component 1 (PC1) is responsible for 95% of the data distribution. As it can be seen, the extraction with water:ethanol (50:50) results are represented on the positive PC1 axis, showing higher values for all studied parameters (yield, TPC, TFC, anthocyanin's, and antioxidant activity - DPPH and FRAP). Hydroethanolic extraction at 80 °C for 60 min presented higher extraction yield and lower content of antioxidant phenolic compounds. Greater selectivity or less degradation

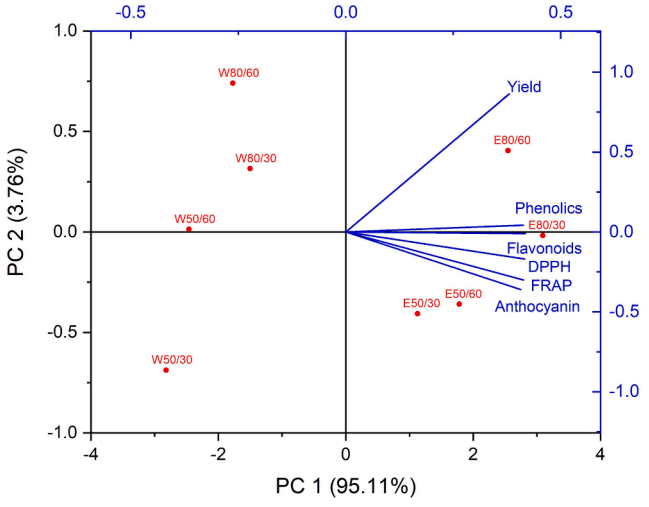

Fig. 1. Principal component analysis (PCA) of the studied conditions on the ohmic heating extraction process. W - aqueous extraction at 50 or 80 °C/30 or 60 min. E - hydroethanolic extraction at 50 or 80 °C/30 or 60 min.

was observed for hydroethanolic extraction at 80 °C for 30 min, with a higher content in phenolic compounds (TPC, TFC and TA), and as expected with greater chemical antioxidant capacity measured by DPPH and FRAP methods. Overall, the hydroethanolic solution was more efficient than water to recover the target compounds, *i.e.* phenolic compounds.

Chemical molecules solubility depends on the type of solvents (and its related polarity) used in the extraction method. It is known that most phenolic compounds have greater affinity to organic solvents (*e.g.* ethanol) mixed with water in comparison to extractions with only water (Ferreira-Santos et al., 2019; Mir-Cerdà et al., 2023). The original structure and structural changes of phenolic molecules can alter their solubility and separation ability. It is known that the structure of a molecule has a substantial impact on its polarity, conjugation, and interaction with biomass. Due to the structural composition of biomolecules, high molecular weight phenolics are often insoluble (Alara et al., 2021). When choosing an extraction method, it is necessary to take into account the several parameters described above, in order to increase extraction efficiency of the target compounds and avoid structural changes of the extracted molecules.

The extraction condition, water:ethanol (50:50 (v:v)) at 80 °C for 30 min, was selected to further understand the influence of electric fields (OH) on the extraction of phenolic compounds. For this, a control at selected conditions was performed without the application of electric fields - CH (0 V/cm). When comparing the extraction yield obtained using the two distinct extract methods, OH with CH, it is clear that the OH-assisted thermal extraction resulted in a higher extraction yield (yield: 21.0 ± 0.5 % for CH and 26.7 ± 0.5 % for OH). This increase may be due to compromised cell wall integrity by the simultaneous effect of increased internal temperature (Joule effect) and permeation, due to electric fields (electropermeabilization effect). The combination of these two mechanisms, resulted in an increased solvent-sample contact, enhancing recovery of intracellular compounds, like phenolic compounds (Coelho et al., 2021; Ferreira-Santos et al., 2019; Pereira et al., 2016).

In Fig. 2, it is possible to identify several changes in the structure of GP biomass treated with CH and OH. Structural changes are clear upon the application of OH, particularly cell wall disintegration and porous structures (electroporation phenomenon) (Fig. 2C). The observed structural change facilitates the migration of intracellular phenolic compounds, leading to an increased extraction yield, has demonstrated by the presented results. Electroporation and permeabilization caused by electric fields (moderate electric fields (MEF), OH, pulsed electric field (PEF), etc.) has also been reported while using different raw materials, like pine bark, microalgae, stevia leaves, date palm fruit, chicory, *etc* (Barba et al., 2015; Ferreira-Santos et al., 2019; Geada et al., 2018; Hai et al., 2023).

The use of OH for the recovery of high-value compounds from plants and agro-food by-products is described in the literature (Hashemi Gahruie et al., 2020; Jesus et al., 2020; Pereira et al., 2020). All the studies describe a positive effect on the recovery of nutritional and bioactive compounds, namely phenolics, with increased recovery yield (Hashemi Gahruie et al., 2020; Jesus et al., 2020; Pereira et al., 2020). None of the studies reported chemical or structural changes (or loss of bioactivity) of the recovered phenolic compounds.

An additional advantage of this method, is the selectivity towards the extraction of phenolic compounds. Ferreira-Santos et al. (2019) demonstrated that electric fields (5 – 15 V/cm) combined with heat and hydroethanolic solvents, induced changes in the structure of pine bark cells, and consequently increasing a selective extraction of phenolic compounds. Another study found that MEF extraction (10 V/cm) resulted in a higher sugar extraction yield from palm fruits due to electroporation and permeabilization effects which allowed the diffusion of intracellular molecules. The study demonstrated that this technique is more efficient for juice extraction when compared to a conventional extraction method (Hai et al., 2023).

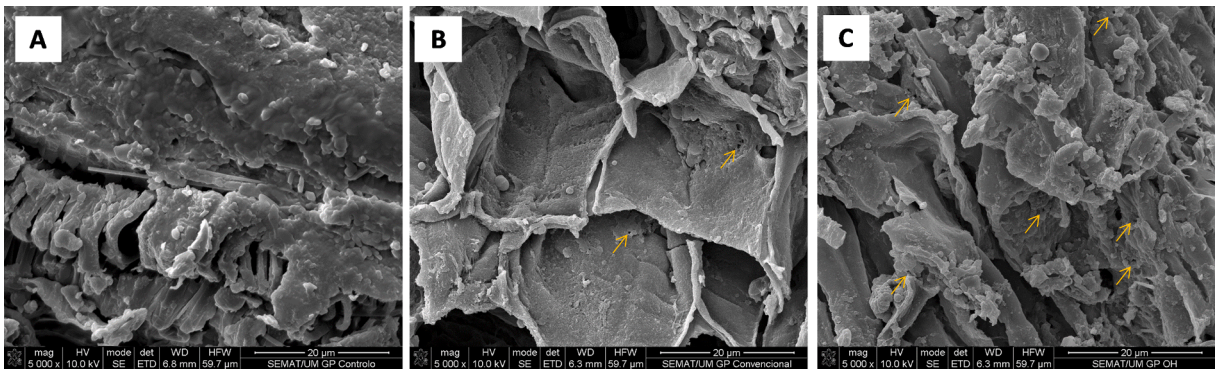

Fig. 2. Micrographs by scanning electron microscopy of untreated (**A**), conventional heating (**B**), and ohmic heating (**C**) treatments of grape pomace. Extraction conditions of water:ethanol (50:50),80 °C, 30 min (electric field: 0 V/cm and 14 V/cm for CH and OH, respectively).

3.2. Phytochemical characterization of extracts

In this study, OH influence on the extraction process is clear, particularly on biomass fragmentation (Fig. 2), as well as, in the composition and bioactivities of the obtained extracts (Fig. 3).

Fig. 3 shows the effect of both thermal treatments - OH and CH - on the phytochemical content and antioxidant capacity of the obtained extracts from GP residues [extraction condition: 15 g of dried GP was mixed with 150 mL of solvent water:ethanol (50:50 (v:v)) at 80 °C for 30 min]. Significant differences between extraction methods were observed for all the parameters studied (TPC, TFC, TA and antioxidant activity). OH-assisted extraction provided a significantly higher concentration of phenolic compounds than CH. In the case of TPC, the application of continuous electric fields at 14 V/cm allowed a 40% increase on the total phenolic compound's extraction compared to the conventional heat treatment (without the application of electric fields). This increment was accompanied by a 44% increase in flavonoid content upon the application of an electric field (37.98 ± 2.3 *vs* 26.32 ± 1.4 mg CE/g dry GP, for OH and CH, respectively). Furthermore, the anthocyanin content (TA) quantified by the standard pH differential method demonstrated that the application of this innovative technology, *i.e.* OH, allowed a 60% increase in the recovery of bioactive pigments from wine residues. Authors like Peixoto et al. (2018), reported the presence of different anthocyanins in the hydroethanolic GPE, being malvidin derivatives the main group of anthocyanins (malvidin-hexoside, malvidin-acetylhexoside, malvidin-dihexoside and malvidin-rutinoside).

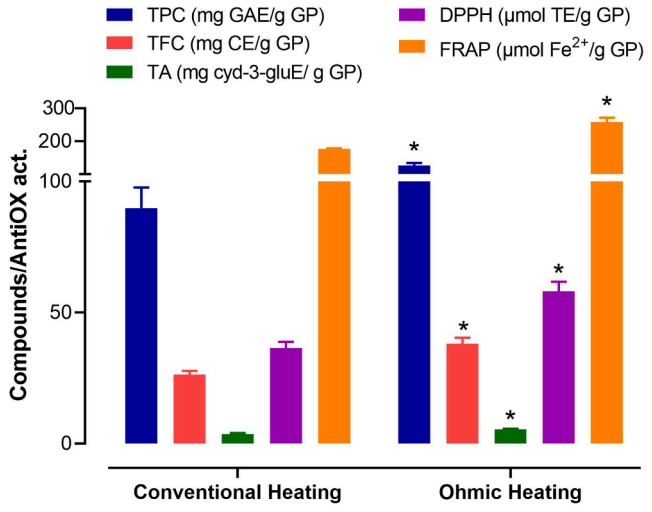

Fig. 3. Phenolic content and antioxidant capacity of obtained GP extracts from different treatments (CH and OH) at selected conditions (water:ethanol (50:50 (v:v)) at 80 °C for 30 min). TPC: total phenolic content; TFC: total flavonoid content; TA: total anthocyanins; DPPH (radical scavenging) and FRAP (antioxidant reduction power) were antioxidant assays. * Significantly different versus conventional heating for the same assay.

The increase in phenolic compounds concentration could be explained by the electro-thermal effects of OH treatment on plant cell wall permeabilization. Electrical disturbances in cell membranes (electroporation phenomenon); or thermal effects can cause the breakdown of complexes between phenolics and other compounds such as proteins, polysaccharides and fibers, leading to an easiest release of phenolics (Barrón-García et al., 2022; Ferreira-Santos et al., 2019; Pereira et al., 2020; Rocha et al., 2018).

The antioxidant capacity of foods and food by-products is mainly due to the existence of different molecules, particularly phenolic compounds. These antioxidant molecules are known to be beneficial for human health by decreasing the production of cellular ROS (oxidative stress). The incorporation of these molecule in functionalized foods, cosmetic and pharmaceutical industries increases their values.

The antioxidant activity of the obtained GPE measured by DPPH and FRAP (Fig. 3), indicate the presence of antioxidant molecules with high radical scavenging activity and antioxidant-reducing power action. The antioxidant capacity values varied according to the assay used, which can be attributed to the chemical structures of the radicals, molecules mechanism of action, as well as, the type of phenolic compounds present in GPE (Ferreira-Santos, Genisheva, et al., 2021). In both antioxidant assays (DPPH and FRAP), GPE obtained from biomass processed with OH showed higher antioxidant capacity (60% for DPPH and 46% for FRAP, respectively) than samples obtained by CH. These results are in line with another work performed by Coelho et al. (2021), who showed that OH treatment (≈30 V/cm) using citric acid increased anthocyanin extraction of red GP from Portugal. El Darra and coworkers (2013) reported the effect of pulsed electric fields (100–800 V/cm) and ethanol percentage on the phenolic compounds extraction. Their results (high electric field intensity, 400 V/cm, and temperature of 50 °C) suggest an effect on cell membrane denaturation and increased diffusion of phenolic compounds from GP using 30% ethanol solution. Our results, using continuous OH with lower electric field strength (lower energy spending), allowed us to obtain similar results, both in cell membrane permeabilization and diffusion of intracellular compounds using 50% ethanol. Our results regarding the antioxidant activity of the hydroethanolic GPE are in agreement with other studies reporting values of DPPH between 60 and 135 µmol TE/g GP (Caponio et al., 2022). Another study by Barrón-García et al. (2022), demonstrate that OH treatments (15–20 V/cm) can preserve the phenolics structure from mango pulp, preserving its potential health benefits when compared to CH.

3.3. Cell viability and oxidative stress prevention

The GPE obtained by OH using ethanol 50% (v/v), 80 °C for 30 min

has a high antioxidant activity, indicating that it may have a beneficial effect for human health. In order to validate this hypothesis, several studies were performed in this work using different cell lines (cancerous and non-cancerous cell lines).

One of the first tests required prior to considered the use of GPE for human consumption is GPE cytotoxicity assessment.

As it can be seen in Fig. 4 (A and C), the different cell lines appear to metabolize GPE compounds differently. Human cervix adenocarcinoma cells (HeLa) were the most susceptible to GPE presence, showing the lowest IC_{50} (122.7 mg/mL), followed by human colorectal cell (Caco-2) with IC_{50} of 234.1 mg/mL. Interestingly, both cell lines are derived from cancerous tissue. On the other hand, the non-cancerous cell lines, human embryonic kidney cells (HEK293T) and mouse fibroblasts (L929) had the highest IC_{50}, 431.4 mg/mL and 397.9 mg/mL, respectively. The IC_{50} of HEK293T (the highest) was 352 % higher than HeLa (the lowest). This is a very important result, as it shows that for the same GPE concentration, the compounds present elicit a toxic effect on the cancerous cell lines, but not on non-cancerous cell lines (visible in microscopy images - Fig. 4 C), opening the door to natural anti-cancer therapies. Even though, further analyses are required. Peixoto et al. (2018), reported similar results, where GPE were only effective against human breast adenocarcinoma - MCF7 cell line (IC_{50} = 332 μg/mL) and HeLa cell line (IC_{50} = 253 μg/mL), not showing toxicity for liver non-cancerous cells (PLP2).

When analyzing the composition of GPE it is possible to detect the presence of several phenolic compounds like taxifolin (see Table 1), which is known for its anti-proliferative activity on tumor cells (Hossain & Ray, 2014). Hossain & Ray (2014) demonstrated that the combination of shRNAS-binding protein EWS (EWS – Ewinǵs Sarcoma) with taxifolin decreased in 80% cell viability of human Ewing's sarcoma cells (SK-*N*-MC) and epithelial cells from humerus bone (RD-ES), with a decrease in EWS expression at mRNA and protein levels. Reports in the literature

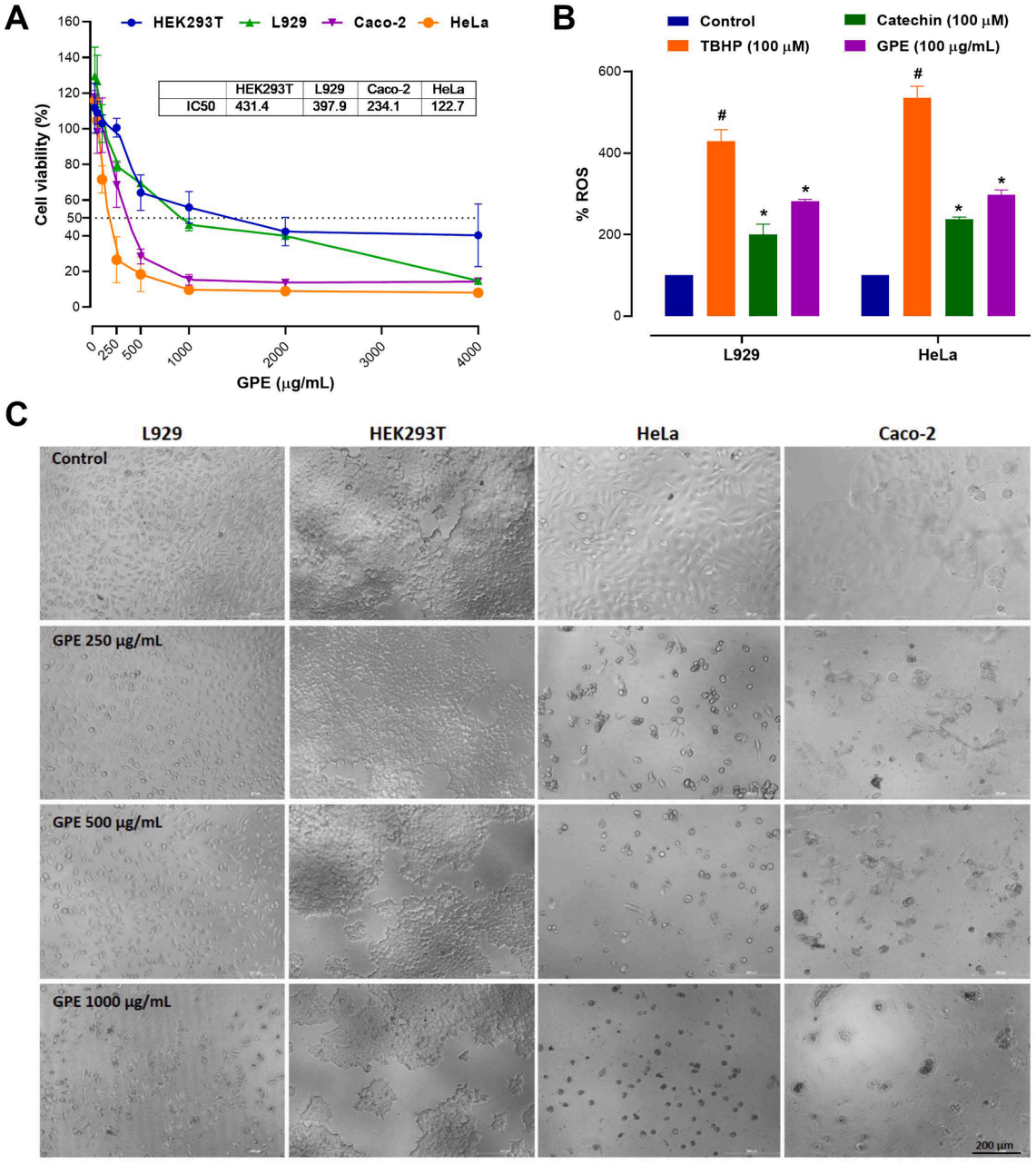

Fig. 4. Cellular viability (**A**), cellular antioxidant capacity (**B**) and microscopy images of cells (**C**) treated with different concentrations of OH grape pomace extract. # Significantly different versus Control, and * significantly different versus *Tert*-butyl hydrogen peroxide - TBHP (for the same cell line).

Table 1
Influence of *in vitro* gastrointestinal digestion on composition, antioxidant activity and bioaccessibility of GP phenolic extract.

Components	Non-digested	Oral	Gastric	Intestinal	Bioaccessibility index (%)
TPC (mg GAE/g GPE)	511.11 ± 6.6	528.99 ± 8.8	508.71 ± 4.0	535.26 ± 5.9*	122
TA (mg cyd-3-gluE/g GPE)	48.29 ± 1.2	45.22 ± 2.4	37.41 ± 4.1*	8.27 ± 0.5*	17
Individual phenolic compounds (mg/g GPE)					
Phenolic acids					
Ellagic acid	6.58 ± 0.3	7.94 ± 1.0	10.70 ± 2.1*	13.79 ± 1.4*	210
Gallic acid	2.61 ± 0.0	3.96 ± 0.4	4.08 ± 0.2*	n.d.	0
3,4-DHB acid	1.03 ± 0.1	0.95 ± 0.02	0.87 ± 0.01	2.92 ± 0.2*	283
2,5-DHB acid	1.50 ± 0.0	2.31 ± 0.01*	4.21 ± 0.2*	n.d.	0
o-coumaric acid	4.35 ± 0.1	5.06 ± 0.7	5.93 ± 0.2*	6.06 ± 0.3*	139
Rosmarinic acid	2.57 ± 0.2	2.96 ± 0.3	5.6 ± 0.5*	10.37 ± 3.1*	404
Caffeic acid	0.13 ± 0.00	0.23 ± 0.01	0.60 ± 0.01*	0.92 ± 0.02*	707
Ferulic acid	0.89 ± 0.04	1.04 ± 0.02	1.05 ± 0.01	1.42 ± 0.02*	156
Flavonoids					
Catechin	± 0.02	4.50 ± 0.12	5.26 ± 0.3*	n.d.	0
Rutin	5.19 ± 0.1	1.89 ± 0.04*	2.79 ± 0.02*	6.01 ± 0.2*	116
Quercetin	3.58 ± 0.2	3.22 ± 0.1	2.12 ± 0.3*	1.20 ± 0.01*	28
Taxifolin	5.68 ± 0.3	5.79 ± 0.2	4.23 ± 0.1	0.57 ± 0.01*	10
Kaempferol	5.32 ± 0.06	5.58 ± 0.3	3.89 ± 0.1*	1.44 ± 0.2*	27
Naringenin	28.26 ± 0.5	32.43 ± 2.7	30.30 ± 3.0	21.98 ± 1.1*	78
Apigenin	25.35 ± 0.7	28.59 ± 4.84	24.75 ± 0.6	32.12 ± 0.9*	127
Stilbene					
Resveratrol	2.38 ± 0.2	1.99 ± 0.05	1.91 ± 0.04	1.58 ± 0.3*	67
Antioxidant activity					
DPPH (μmol TE/g GPE)	95.55 ± 1.4	104.18 ± 5.6	79.63 ± 7.5	124.44 ± 5.1*	–
FRAP (μmol Fe^{2+}/g GPE)	247.83 ± 0.2	290.99 ± 20.8*	304.12 ± 11.1*	321.77 ± 9.9*	–

Values of phenolic compounds are expressed as concentration (mg/L) mean ± SD of 3 experiments. n.d.: not detected; DHB: Dihydroxybenzoic acid. * Significantly different versus non-digested GPE.

described several mechanisms by which flavonoids exert anti-cancer activity, specifically, metabolizing enzymes are suppressed, cell cycle regulators are inhibited, phase II metabolizing enzymes are activated to inhibit reactive oxygen species formation, apoptosis is induced, and angiogenesis is prevented by inhibiting vascular endothelial and fibroblast growth factors, among other things (Sunil & Xu, 2019).

The chemical characterization of the recovered compounds demonstrated that the GPE is rich in antioxidants molecules, particularly phenolics compounds, like catechin, apigenin, naringenin, taxifolin, rutin, phenolic acids, among others (Table 1). Naringenin is present in GPE in large concentration. It has been shown that naringenin potently suppresses endothelial cells migration, tube formation and formation of microvessels, which are essential for tumor development. It is also involved in tumor cell death mechanism (Choi et al., 2020).

Apigenin is also present in large concentrations in GPE, similar to the previously described phenolics compounds, apigenin has cytostatic and cytotoxic properties against several cancer cells types. Moreover, when compared to other flavonoids, apigenin has been reported as having minimal intrinsic toxicity on normal versus malignant cells (Ali et al., 2016), similarly to the results demonstrated in this study.

It is known that upon ingestion, some phenolic compounds are metabolized, increasing the concentration of phenolic acids that can be absorbed by the organism exerting their therapeutic effect, namely antioxidant and anti-cancer (Heleno et al., 2015). Furthermore, we cannot forget the possible synergistic effect of the compounds present in the GPE, which can increase its therapeutic efficacy and improve nutritional value (Ferreira-Santos, Duca, et al., 2021; Honaiser et al., 2022; Sarabandi et al., 2023).

Oxidative stress can be defined as an imbalance between the pro-oxidant and anti-oxidant mechanisms, due to an exacerbated production of ROS and the inability of the body to degrade them (Enogieru et al., 2018). This imbalance can lead to the development of several pathologies, like cancer and premature aging (Hajam et al., 2022).

The need to develop more efficient therapies with fewer side effects is of upmost importance. The potential of phytochemicals has recognized for many years, although the mechanism behind is still poorly understood (Kumar et al., 2023). Therefore, cellular antioxidant capacity of GPE was evaluated in two cell lines, one normal cell line (L929) and one cancerous cell line (HeLa). The obtained results are presented in Fig. 4B. Cells were placed in contact with 29 mg/mL (100 μM) of catechin and 100 mg/mL of GPE for 8 h. Upon this period the cells were challenged with TBHP, the oxidant reagent. Catechin was chosen as a positive control, due to its recognized antioxidant properties (Bernatoniene & Kopustinskiene, 2018).

Similarly, to catechin, GPE was able to protect cells against the oxidative insult. When observing Fig. 4B, it is clear that ROS production profile was similar for both cell lines.

Even though the mechanism behind the therapeutic effect of phytochemicals is unclear, there are some hypothesis raised and described in the literature. Heleno and colleagues report regarding catechins (Heleno et al., 2015); It seems that catechins exert their antioxidant activity by two distinct methods: scavenging ROS and chelating metal ions, and by the induction of antioxidant enzymes, inhibiting pro-oxidant enzymes (Heleno et al., 2015). The results observed in this study strengthen the proposed mechanism, regarding the ability of catechins to scavenge for ROS. Catechins are present in GPE, which may contribute for the observed result, protection of cells from an oxidant insult. Although, it is not possible to single out only one component as responsible for the antioxidant effect of GPE. Rutin, also present in GPE is known for its antioxidant capacity (Enogieru et al., 2018). Enogieru et al. (2018) described the ability of rutin to improve antioxidant enzymes activity, activation of the mitogen-activated protein kinase cascade, restoration of the mitochondrial complex enzymes activity, among others.

Milinčić et al. (2021) presented similar results, regarding the cellular antioxidant activity of GP skins, which they related to the presence of flavanols and anthocyanins. Similarly, to Milinčić, in this study it is not possible to attribute the observed results to the presence of a single compound, but to a synergetic effect of the bioactive molecules present in the GPE composition.

These results clearly demonstrate the benefits of dietary GPE for prevention of different pathologies, encouraging its use in the preparation of functional foods or formulation of nutraceuticals.

3.4. *In vitro* digestion and bioaccessibility of phenolic compounds

Several chemical reactions (by pH changes, enzymes, salts, etc.) are known to play a role in the metabolism/biotransformation of phenolic compounds in the human body, resulting in enhanced solubility of bioactive molecules and so changing their distribution, absorption, and excretion (Eker et al., 2020).

Therefore, foods and dietary metabolites, *e.g.* phenolics, are subject to their bioaccessibility and bioavailability after consumption.

Bioaccessibility values superior than 100% indicate that compounds were liberated from the food matrix and/or metabolized from more complex phenolic compounds. The enzymes, pH and the action of bile salts in the stomach and intestinal environments can cause variations in the chemical structures of phenolic compounds, resulting in new molecules with different bioavailability and biological activities (Heleno et al., 2015). During GID, complex phenolic compounds can be biotransformed into low molecular weight compounds, which are easier absorb than large molecules (Ferreira-Santos et al., 2022). Due to the limited bioavailability of phenolics, only a small portion of the ingested phenolics (5–10%) are absorbed in the small intestine, with the remainder reaching the colon. They are biotransformed in the small intestine by conjugation reactions such as glucuronidation, sulfation, and/or methylation and absorbed by enterocytes. Moreover, phenolics which are not absorbed in the small intestine reach the colon, where can be transformed by the gut microbiota (via deglycosylation, dehydroxylation and demethylation reactions) and further absorbed by the colonocytes (Singh et al., 2020). Finally, the absorbed phenolic molecules are delivered to various tissues and organs via systemic circulation (Ávila-Román et al., 2021).

Recently, it has been reported that phenolics have similar effects to prebiotics, modulating the growth of beneficial bacteria, like *Bifidobacterium*, *Lactobacillus* and *Akkermansia muciniphila* (Rodríguez-Daza et al., 2021). This characteristic confers health benefits like immune system stimulation, allergy and cancer prevention, lipids deposition reduction, and metabolic function regulation, promoting eubiosis and homeostasis of the body (Rodríguez-Daza et al., 2021).

In this work, the phenolic constituents (TPC, TA and phenolic profile by HPLC-DAD) and antioxidant activity were evaluated to understand the alterations observed in dry GPE promoted by the digestive process, and the results are displayed in Table 1. GID considerably increased the concentration of phenolic compounds (TPC measured by the Folin-Ciocalteu method) (511 to 535 mg GAE/g GPE, $p < 0.05$) representing a bioaccessibility of 122%. These results suggest the biotransformation of high-molecular phenolics towards their depolymerization to produce simpler low-molecular compounds (Honaiser et al., 2022).

Anthocyanin (TA) had the opposite behaviour. In this case, the digested extracts showed a significantly lower concentration of TA than the non-digested extract, with a decrease of 83% (bioaccessibility of 22%). These results are in line with those reported by Victoria-Campos et al. (2022) who describe that anthocyanins were highly stable in the gastric phase of GID and their content decreased drastically ($p < 0.05$) during the intestinal phase of digestion. Moreover, the stability depends on the anthocyanidin structure and type of glycation. Also, after oral consumption, a significant low proportion of the ingested anthocyanins are detected in the circulation (plasma and urine) (Victoria-Campos et al., 2022). Anthocyanins are highly reactive molecules, being considered one of the most unstable with pH changes. They are hydrolysed by several enzymes in the small intestine, which results in the destruction of the anthocyanin's chromophore and transformed into simple phenolics (Ferreira-Santos et al., 2022).

As it can be seen in Table 1, sixteen phenolic compounds were quantified in GPE, belonging to the groups of phenolic acids, flavonoids and stilbenes. In the initial extract (non-digested), flavonoids like catechin, rutin, quercetin, taxifolin, kaempferol, naringenin and apigenin are the compounds with the highest content, with concentrations ranging from 3.43 to 28.26 mg/g in dry GPE.

During the simulated *in vitro* GID, including the mouth (oral phase), stomach (gastric phase), and small intestine (intestinal phase), significant changes in GPE composition regarding the major phenolic compounds were observed. Our results suggest that large amounts of flavonoids were metabolized, biotransformed and/or degraded during digestion, except for rutin and apigenin, which increase their concentration at the end of digestion by 16% and 27%, respectively. Catechin was not detected at the end of digestion. At the same time, the content of quantified phenolic acids like ellagic acid, 3,4-dihydroxybenzoic acid, *o*-coumaric acid, rosmarinic acid, caffeic acid and ferulic acid (with exception of gallic acid and 2,5- dihydroxybenzoic acid) increased dramatically during the digestion process, suggesting that larger molecules (flavonoids and stilbenes) were biotransformed into simpler phenolic acids, favouring their bioaccessibility and consequent absorption. Recently, Taladrid et al. (2021) reported the presence of some of compounds in GPE from a Tempranillo variety from Spain like phenolic compounds including gallic acid (1772 μg/g), catechin (205 μg/g), epicatechin (71 μg/g), kaempferol (12 μg/g), and quercetin glucoside (60 μg/g), in addition to carbohydrates (810 mg/g), protein (77 mg/g), fibre (19 mg/g) and ash.

As far as it is known, plant flavonoids are available in aglycone and glycoside forms. However, the number of glycosides significantly surpasses the number of glycoside-free forms. Studies have demonstrated that when flavanols are present in the diet as aglycones, they could be partly absorbed in the intestine, but their glycosidic forms cannot (Santangelo et al., 2019). For example, quercetin is an abundant bioactive flavonoid found in vegetables and fruits, and this compound is mainly present in the glycoside form (quercitrin). After ingestion, quercetin is naturally biotransformed in DHB acids (Santangelo et al., 2019). Das et al. (2021) performed a comprehensive review about flavonoid taxifolin (dihydroquercetin), reporting interesting bioactive properties against inflammation, microbial infection, oxidative stress, and other associated diseases like cancers, cardiovascular and liver pathologies. Furthermore, the authors reported that taxifolin is rapidly metabolized by the gastrointestinal (GI) tract and converted to hydroxyphenylacetic acid, an identical metabolite excreted after ingestion of quercetin.

The most studied property of GPE is its antioxidant capability, which is related to the presence of phenolic high content. However, comparisons with other works are difficult due to the variety of methodologies applied for the extraction and quantification of phytochemicals, antioxidant standards used, and raw material (grape varieties). GPE (non-digested) presents antioxidant values of 95.55 ± 1.4 μmol TE/g of GPE for DPPH and 247.83 ± 0.2 μmol Fe^{2+}/g GPE for FRAP results. After GID, the scavenging capacity of the GPE increased to 124.44 ± 5.1 μmol TE/g GPE (DPPH), and their reducing antioxidant power was improved to 321.77 ± 9.9 μmol Fe^{2+}/g GPE, which is in accordance with the increase TPC and higher bioaccessibility of phenolic acids after the digestion process. Our results, in accordance with the literature (Caponio et al., 2022; Taladrid et al., 2021), provide information regarding the behaviour of GPE upon the digestion process. It is shown that anthocyanins and flavonoids can be degraded or biotransformed into low molecular weight compounds (phenolic acids), and antioxidant activity of extracts can be changed positively or negatively, upon GID.

4. Conclusion

The search for new natural molecules and compounds that can be employed to improve food characteristics for health benefits or molecules for medicinal purposes has accelerated research on Green Chemistry.

Regarding the extraction process of target compounds from grape pomace residues, the application of electric fields related to OH technology causes cell wall permeabilization and electroporation. Concomitantly, innovative OH-assisted extraction in combination with hydroethanolic solvent contributes to an enhanced extraction of bioactive phenolic compounds from grape pomace residues.

Depending on the application intended for the recovered bioactive compounds it is necessary to further characterize the extract and understand its modifications along biological and environmental processes. If the bioactive molecules are to be ingested as food constituents (functional food, preservatives, colorant, etc) or dietary supplements, it is necessary to assess the changes generated by the gastrointestinal system conditions, as well as their bioaccessibility.

This study showed that the phenolic composition of grape pomace

extracts can vary during digestion. Some phenolic compounds (flavonoids and anthocyanins) were biotransformed into low molecular weight molecules (phenolic acids) during gastrointestinal digestion, increasing their bioaccessibility (and possible absorption) and bioactivity. The cellular assays clearly demonstrated significant cytotoxicity of grape extracts against cancerous cell lines (antiproliferative effect) and low toxicity towards normal cell lines. In addition, the grape extract confers antioxidant prevention against an oxidative insult.

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CRediT authorship contribution statement

P. Ferreira-Santos: Project administration, Funding acquisition, Writing – review & editing, Writing – original draft, Investigation, Methodology, Conceptualization. **C. Nobre:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **R.M. Rodrigues:** Writing – review & editing, Investigation. **Z. Genisheva:** Writing – review & editing, Writing – original draft, Investigation. **C. Botelho:** Writing – review & editing, Writing – original draft, Investigation, Methodology. **J.A. Teixeira:** Project administration, Funding acquisition, Writing – review & editing, Conceptualization.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.137780.

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