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Ohmic heating polyphenolic extracts from vine pruning residue with enhanced biological activity

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ABSTRACT

Vine Pruning residue was submitted to conventional heating and ohmic heating (OH) for the extraction of bioactive compounds and analyzed for total phenolic content (TPC), polyphenolic profile, antioxidant activity, antimicrobial activity and anticancer activity. The OH extracts were obtained using Low electric field (496.0 V/cm) or Intermediate electric field – IEF (840.0 V/cm). The tests were performed using 45% (v/v) ethanol–water extraction solution at 80 °C at different extraction times (20–90 min). The extract that stood out among the others concerning anticancer potential was the one obtained by OH when used, IEF, where the TPC was significantly higher than in the other extracts which correlated with higher antioxidant, antimicrobial and antiproliferative activity on different tumor cell lines (HepG2, MDA-MB-231, MCF-7 and Caco2). Vine pruning OH extracts obtained using green solvents by an eco-friendly procedure were revealed as a source of compounds with relevant antioxidant and anticancer activity.

1. Introduction

Vine Pruning residue (VPR) is a lignocellulosic material widely generated in the Mediterranean region by the wine industries, as a consequence of the great wine market. Conversely, this waste represents a pollution hazard if discharged into the environment. On the

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Nomenclature

Abbreviations

ABTS	2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid
ATCC	American Type Culture Collection
CH	Conventional Heating
CFU	colony forming unit
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picryl-hydrazyl-hydrate
ECCA	European Collection of Authenticated Cell Cultures
FBS	Fetal Serum Bovine
FE (II)	ferrous equivalents
GAE	Gallic Acid Equivalent
Hz	hertz
kHz	kilohertz
IEF	Intermediate electric field
LEF	Low Electric Field
MEM	Minimum Essential Medium
mha	Million Hectares
mhl	Million hectoliters
MHz	Megahertz
MTT	Methylthiazolyldiphenyl-tetrazolium bromide
OH	Ohmic heating
RPMI-1640	Medium Roswell Park Memorial Institute-1640
RT	Room temperature
UHPLC	Ultra-high-performance liquid chromatography
V	Volts
VPR	Vine Pruning Residue

Parameters and constants

A_0	Absorbance of control (DPPH or ABTS)
A_1	Absorbance of extracts
Ia	Inhibition activity (%)
IC_{50}	Concentration of sample or standard that can inhibit 50% of DPPH, ABTS (mg/mL) or 50% of cell viability (μg/mL)
CV	cell viability (%)
V_1	cell viability of control sample
V_2	cell viability of extracts

other hand, VPR is economically underutilized, however, it has attracted enormous attention due to its interesting properties and its possible use as a raw material to obtain added-value compounds. In fact, this by-product can be considered a rich source of energy and bioactive compounds, and the recovery of these compounds from VPR could avoid the possible economic and environmental concerns. In this context, some authors have proposed different alternative applications for the valorization of this residue. Thus, VPR can be used to produce biochar (Azuara, Sáiz, Manso, García-Ramos, & Manyà, 2017), enological additives (Cebrián-Tarancón et al., 2019), bioethanol (Jesus, Romaní, Genisheva, Teixeira, & Domingues, 2017), bioactive compounds (Rajha et al., 2018), or foliar fertilizers (Sánchez-Gómez, Garde-Cerdán, et al., 2016), reducing sugars, and soluble lignin (Rajha et al., 2018) as well as others. In spite of these applications, VPR is still underutilized as a valuable material for industrial processes.

Nowadays, studies for reusing wine production residues as potential sources of phenolic compounds have gained great interest since these compounds present multiple biological effects, including antioxidant (Jesus et al., 2019), anti-mutagenic, anti-inflammatory, antimicrobial and anti-carcinogenic properties (Tartaglione et al., 2018), which provide enormous benefits for human health. According to some authors, extracts of different residues from the wine industry, such as leaves, seeds and, vine pruning may be toxic to different human cancer cells. As a result, leaf extracts were tested for human colon cancer cells and A549 lung cancer cells (Abed, Harb, Khasib, & Saad, 2015; Ramadan, Abou-Taleb, Galal, & Abdel-Hamid, 2017). The extracts from the VPR was subjected to the extraction, purification and isolation of oligostilbenoids in order to be tested against normal MRC-5 lung fibroblasts, AGS gastric adenocarcinoma cells, SK-MES-1 lung stem cells and bladder carcinoma cells J82 (Sáez et al., 2018).

Factors such as the origin and composition of the raw material, the type of solvent used and its concentration, the solid/solvent ratio, time of contact, temperature, and extraction methodology, significantly influence the efficiency of the process extraction of bioactive compounds (Jesus et al., 2019). Previous works regarding the extraction of phenolic compounds using VPR as a raw material have tested different solvents including methanol (Ju et al., 2016), butanone and acetone (Alexandru et al., 2014). However, these solvents are mostly toxic to humans. Taking into account the potential application of these extracts in human health sector, researchers are looking for technologies able to use green solvents such as water (Gullón et al., 2017; Jesus et al., 2017; Moreira et al., 2018; Sánchez-Gómez, Zalacain, Alonso, & Salinas, 2016), ethanol (Alexandru et al., 2014) or ethanol–water mixtures (Alexandru et al., 2014; Jesus et al., 2019; Moreira et al., 2018) in order to ensure the attainment of phenolic compounds suitable for the food, pharmaceutical and cosmetic industries.

Thus, alternative eco-friendly methodologies including alkaline hydrolysis treatments (Max, Salgado, Cortés, & Domínguez, 2010; Rajha, Boussetta, Louka, Maroun, & Vorobiev, 2015b), ultrasound-assisted extraction (Alexandru et al., 2014; Delgado-Torre, Ferreiro-Vera, Priego-Capote, Pérez-Juan, & Luque De Castro, 2012; Farhadi, Esmaeilzadeh, Hatami, Forough, & Molaie, 2016), hydrothermal treatment at high temperatures (Gullón et al., 2017; Jesus et al., 2017), solid-liquid dynamic extraction (Delgado-Torre et al., 2012; Luque-Rodríguez, Pérez-Juan, & de Castro, 2006; Sánchez-Gómez, Zalacain, Alonso, & Salinas, 2014), subcritical water extraction (Gabaston et al., 2018; Moreira et al., 2018), as well as high-voltage electrical discharge (Rajha, Boussetta, Louka, Maroun, & Vorobiev, 2015a), have been applied. However, the current methods used for the extraction of bioactive compounds present limitations due to the long extraction times and high process temperatures, which can cause oxidation and denaturation of the phenolic compounds since they are relatively unstable, leading to low extraction percentages. Therefore, it is necessary to study novel extraction methodologies capable of extracting high concentrations of bioactive compounds using less aggressive conditions and environmentally friendly and sustainable solvents. One of the eco-friendly trends in the techniques of extracting polyphenolic compounds is the use of emerging technologies such as ohmic heating (OH) to reduce the damaging effects of conventional thermal processes.

OH is one of the emerging high potential technologies where the passage of alternating electric current is used to generate heat internally (Pereira et al., 2018). This process was used in different studies for the production, pasteurization and shelf-life enhancement of beverages, flours and sausages (Cappato et al., 2018; Ferreira et al., 2019; Inmanee, Kamonpatana, & Pirak, 2019; Makroo, Prabhakar, Rastogi, & Srivastava, 2019; Ramírez-Jiménez et al., 2019). Additionally, this technique involves less aggressive heat treatments than others usually used because of its ability to heat materials evenly and rapidly, preventing thus, the denaturization of thermosensitive substances including the phenolic compounds (Khan et al., 2018; Ramírez-Jiménez et al., 2019). Some authors have reported that the use of OH causes destruction of the cell membrane making it permeable and thus, raising the release of phenolic compounds from plant tissues (Kulshrestha & Sastry, 2010; Lebovka, Shynkaryk, & Vorobiev, 2007). The

permeabilization increases with the rise of the intensity and temperature of the electric field (El Darra, Grimi, Vorobiev, Louka, & Maroun, 2013). Previous studies showed that the use of ethanol/water blends in pulsed ohmic heating accelerates the extraction kinetics of this type of compounds from grape marc (El Darra et al., 2013).

Currently, studies of bioactive compound extractions performed from VPR have been concentrated on the qualitative/quantitative evaluation of polyphenols and on their antioxidant activity and antimicrobial capacity onto bacteria and yeasts. However, to the best of our knowledge, there are no studies that use the sustainable and environmentally friendly ohmic heating to recovery bioactive compounds from VPR. In addition, there is no study in the literature that reports the antimicrobial inhibition of VPR extracts onto filamentous fungi, as well as the anti-carcinogenic activity when used a VPR integral extract in order to evaluate the synergistic effects of the set of bioactive compounds present in that extract. Therefore, the aim of this study was to investigate the levels of polyphenolic compounds and their profile, as well as the functional properties, including antioxidant and antimicrobial capacities, and cytotoxic activity in human tumor cell lines, in produced extracts from VPR by using the emerging OH extraction technique at different intensities: Intermediate electric field (IEF) and Low electric field (LEF).

2. Materials and methods

2.1. Raw material and analysis of the chemical composition

Vine pruning residue (VPR) from *V. vinifera* variety Loureiro were collected randomly from the Portuguese region of Minho (Amares, Braga-PT) in January of 2015. Samples were dried at room temperature, milled to pass an 8 mm mesh, homogenized in a single batch and stored at room temperature into a dark and dry place until use. The VPR used in this work was previously studied and characterized (Jesus et al., 2019; Jesus et al., 2017) and presents the following chemical composition: 32.9 ± 0.6 of cellulose (as glucan); 14.9 ± 0.2 of xylan; 0.4 ± 0.01 of arabinan; 3.9 ± 0.5 of acetyl groups; 29.5 ± 1.2 of Klason lignin; 13.7 ± 1.0 of extractives in water; 2.9 ± 0.9 of extractives in ethanol and 3.3 ± 0.5 of ashes, expressed in g per 100 g VPR on oven-dry basis ± standard deviation based in three replicate determinations.

2.2. Extraction process

The extraction of phenolic compounds from VPR was performed by conventional heating (CH) and OH techniques. The extraction parameters defined in this study were based on previous results (Jesus et al., 2019). Briefly, the extractions were carried out in a glass cylindrical reactor of 30 cm total length and 2.3 cm of internal diameter containing two electrodes of stainless steel insulated with polytetrafluoroethylene, which kept constant at 7 cm of distance. Voltages were controlled using a function generator (Agilent 33.220 A, Bayan Lepas, Malaysia, 1 Hz-25 MHz, and 1–10 V) with a sinusoidal wave at a 25 kHz frequency, connected to an amplifier (Peavey CS3000, Meridian, MS, USA, 0.3–170 V). The electric fields ranged from 400 to 1600 V/cm, and the temperature was monitored by a k-type thermocouple (precision temperature ± 1 °C, Omega, 709, USA) placed in the geometric center of the sample volume and connected to a data logger software (National Instruments, USB-9161, USA), according to previous reports (Pereira et al., 2010; Rodrigues, Vicente, Petersen, & Pereira, 2019).

For the extractions, 1 g of VPR was suspended in 40 mL of a hydroalcoholic solution at 45% (v/v), put inside the reactor and maintained to a constant agitation with a magnetic stirrer. NaCl was used to adjust the electrical conductivity of the solution (2.3 mS/cm) in order to ensure a homogeneous current flow. The power was controlled to reach a temperature of 80 °C, and the extraction time ranged from 20 to 90 min. Extraction of polyphenolic compounds was performed for samples subjected at four different treatments, all of them carried out into the glass cylindrical reactor. Thus, an IEF (840.0 V/cm) and LEF (496.0 V/cm) treatment (using an extraction time of 60 min) were applied. For the IEF only the electrodes were used to achieve 80 °C, while for LEF, an additional thermo-stabilized water bath circulating in the reactor jacket was used to reach the temperature. On the other hand, CH extraction treatment was made using a circulating thermo-stabilized water bath in the reactor jacket, and finally, an extraction treatment at room temperature, used as a control, was performed (RT). All experiments were made in triplicate.

At the end of each treatment, the obtained extracts were immediately cooled down in an ice bath in order to stop the reaction, and then, filtered with a paper filter of cellulose. The ethanol was evaporated on an orbital shaker at 40 °C and 150 rpm, and the extracts lyophilized and stored for further analysis.

2.3. Analytical methodology

2.3.1. Total phenolic compounds analysis

The total phenolic compounds (TPC) of the VPR extracts were determined by using the Folin-Ciocalteu reagent, adapted to 96-well microplate, as described by Ballesteros, Cerqueira, Teixeira, and Mussatto (2015). In detail, to a sample of 10 μL were added 60 μL of sodium carbonate solution (7.5%, w/v), 15 μL of Folin-Ciocalteu reagent (Sigma-Aldrich Co., St. Louis, MO, USA) and 200 μL distilled water. Finally, the plate was heated at 60 °C for 6 min, and the absorbance was measured at 700 nm against a blank sample using UV–Vis (Synergy HT-BIOTEK).

The total phenolic content was calculated as equivalents of gallic acid from a standard curve ($Y = 0.0007x + 0.0671$, $r = 0.99433$). The results were expressed in grams of equivalent gallic acid per 100 g dry matter (g GAE/100 g VPR).

2.3.2. UHPLC analyses

In addition, phenolic compounds were also identified and quantified by Shimatzu Nexpera X2 UHPLC chromatograph equipped with Diode Array Detector (Shimadzu, SPD-M20A). The separation was performed on a reversed-phase Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm particle size; from Waters) at 40 °C. The flow rate was 0.4 mL/min. The solvents used were water/formic acid (0.1%) as solvent A and acetonitrile as solvent B, the elution gradient used was according Jesus et al. (2019). The phenolic compounds were identified by comparing their UV/Vis spectra and retention times, at the most suitable wave-length for each compound, with that of corresponding standards (Jesus et al., 2019).

2.3.3. Antioxidant activity analysis

The effect of OH and CH techniques on the antioxidant activity of the extracted bioactive compounds was evaluated by using different antioxidant assays as described below.

Ferric reducing antioxidant power (FRAP) assay, was carried out according the methodology described by (Ballesteros, Cerqueira, Teixeira, & Mussatto, 2015; Meneses, Martins, Teixeira, & Mussatto, 2013). Thus, FRAP reagent was prepared from a 0.3 M acetate buffer solution, 10 mM 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ) and a 40 mM aqueous ferric chloride solution in a ratio of 10:1:1 (v/v/v). For the analysis, 10 μL of the filtered samples were added to 290 μL of the FRAP reagent into a 96-well microplate and then, the samples were incubated at 37 °C for 15 min. The absorbance was recorded at 593 nm (Synergy HT-BIOTEK). The $FeSO_4$ (10 to 275 mg/L) was used as a standard and water used as a blank. The results were expressed in grams of ferrous equivalent per 100 g of VPR extract (g FE/100 g VPR).

DPPH radical scavenging assay was carried out preparing a solution of 2,2-diphenyl-1-picrylhydrazine (DPPH) radical in methanol (6×10^{-5} M to an absorbance of 0.700 at 515 nm) as described by (Meneses et al., 2013; Sánchez-Gómez, Zalacain, Pardo, Alonso, &

Salinas, 2017). Briefly, aliquots of 10 μL of each sample were added to 290 μL of DPPH solution into a 96-well microplate, and the samples were kept in dark for 1 h at room temperature, and then, measured at 515 nm using detector UV–Vis (Synergy HT-BIOTEK). Negative and positive controls were made with methanol, and the standard curve was linear between 48 and 719 μM Trolox.

On the other hand, radical cation decolorization (ABTS) assay was determined according to Ballesteros and co-workers (2015). ABTS radical cation was prepared by mixing 7.4 mM 2.2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) dissolved in ethanol with potassium persulfate (2.6 mM) in a ratio of 1:1 (v/v). The mixture was kept for 16 h at room temperature in the dark. After 16 h, 1 mL of ABTS radical cation solution was added to approximately 50 mL of solution absolute ethanol and then adjusted to an absorbance of 0.700 at 734 nm using detector UV–Vis (Synergy HT-BIOTEK). In a 96-well microplate, aliquots of 10 μL of each sample were added to 200 μL of the ABTS radical cation solution and the samples were kept in dark for 6 min at 30 °C.

The inhibition activity (%) of DPPH and ABTS were calculated following the equation:

$$\%Inhibition\ activity = \frac{A_0 - A_1}{A_1} \times 100 \quad (1)$$

where A_0 is the absorbance of the negative control and A_1 is the absorbance of the extracts. The sample concentration for the inhibition concentration at 50% (IC_{50}) for both methodologies was calculated by interpolation. The antioxidant activity was expressed as the amount of antioxidant able to reduce the initial concentration of DPPH and ABTS by 50%. The IC_{50} values were expressed as g of Trolox equivalent per 100 g of dry weight material (g TE/100 g VPR).

2.3.4. Antimicrobial activity analysis

2.3.4.1. Microbial strains. Antimicrobial evaluation was performed against five food pathogenic fungi that drastically influence the quality and safety of postharvest fruits including *Alternaria sp*. (MUM 02.42), *Cladosporium cladosporioides* (MUM 97.06), *Phoma violacea* (MUM 97.08), *Penicillium italicum* (MUM 02.25) and *Penicillium expansum* (MUM 02.14), being obtained from the collection of the Mycology Laboratory of the Minho University (MUM), Portugal. All strains were cultured into potato dextrose agar (PDA) and incubated at 25 ± 2 °C during 15 days before the antimicrobial test.

2.3.4.2. Micro-dilution methodology for filamentous fungi. The determination of the optimal inhibitory concentration of the obtained extracts from VPR against microbial strains was performed using the micro-dilution methodology for filamentous fungi described by the Clinical and Laboratory Standards Institute (CLSI, 2002). Thus, the fungi cell number was adjusted to approximately 10^6 CFU (colony forming unit)/mL (0.5 on the McFarland scale). Additionally, a lyophilized fraction (2 mg/mL) of the VPR extracts obtained from each treatment were serially two-fold diluted in the synthetic culture medium RPMI 1640 (with glutamine and without sodium bicarbonate buffered with bicarbonate 3-(*N*-morpholino) propanesulfonic acid - MOPS), in order to obtain final concentrations from 1000 to 1.95 μg/mL. Experiments were carried out in a sterile 96-well microplate, in which 100 μL of inoculum suspension was added to 100 μL sample. The microplate was incubated at 25 ± 2 °C for 96 h and the absorbance was measured at 530 nm using a spectrophotometric microplate reader (Sunrise Tecan, Grödig, Austria). The behavior of the samples was evaluated against growth and sterility controls, which consisted in using 100 μL of medium RPMI 1640 plus 100 μL of inoculum suspension as a microbial growth control (negative control) and 200 μL of medium RPMI 1640 as a sterility control (positive control). Moreover, fluconazole solutions (concentrations from 0.19 to 100 μg/mL) were used as standard controls. All the treatments were analyzed against the 5 fungi, and the concentrations of each treatment capable of inhibiting growth when compared with the negative control were considered as the optimal conditions.

2.3.5. Anticancer activity analysis

2.3.5.1. Cell culture and experimental conditions. Caco2 human colon carcinoma cells, MDA-MB-231 and MCF-7 human breast cancer cells were kindly provided by Dr. Raquel Seruca (Ipatimup, University of Porto, Portugal). HepG2 human hepatocellular carcinoma cells (HB-8065) and CCD841 CoN normal human colonic cells (CRL-1790) were purchased from American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained at 37 °C in a humidified 5% CO_2/95% air (v/v) atmosphere in the appropriate culture medium. Caco-2 cells were maintained in DMEM supplemented with 10 mM HEPES, 10% FBS, 1% antibiotic/antimycotic solution, and 1% non-essential amino acids solution. On the other hand, MDA-MB-231cells were cultivated in DMEM with 10 mM HEPES, 1.5 g/L sodium bicarbonate, 10% FBS, and 1% antibiotic/antimycotic solution, while MCF-7 in RPMI-1640 supplemented with 10 mM HEPES, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, 10% FBS, and 1% antibiotic/antimycotic solution. Finally, HepG2 and CCD841 CoN cells were maintained in MEM supplemented with 10 mM HEPES, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, 10% FBS, and 1% antibiotic/antimycotic solution.

For cell experiments, Caco2 cells were seeded at 50,000 cells/mL, MDA-MB-231 and MCF-7 at 75,000 cells/mL, HepG2 at 200,000 cells/mL, and CCD841 CoN at 75,000 cells/mL, 24 h before incubation with the different extracts. Stock solutions of lyophilized extracts were prepared in dimethyl sulfoxide (DMSO) and kept in aliquots at −20 °C. Extracts were then added to the culture medium just before incubation, keeping the DMSO concentration not higher than 0.5% (v/v). Controls contained only DMSO.

2.3.5.2. Assessment of anticancer effect on cancer cell lines. The effect of varying concentrations of the tested extracts obtained from the distinct extraction procedures (in 24 and 48 h incubations) on cell proliferation was evaluated by the methylthiazolyldiphenyl-tetrazolium bromide (MTT) reduction assay as previously described (Lima, Pereira-Wilson, & Rattan, 2011). Briefly, 2 h before the end of the treatment period, the cells were incubated with MTT to a final concentration of 0.5 mg/mL. After removing the medium, the formazan crystals formed by the cell's capacity to reduce MTT were dissolved with a DMSO: ethanol solution 50:50 (v/v), and measured at an absorbance of 570 nm. The results were expressed as a percentage relative to the control (cells without any test extract). The extract concentration that inhibited cell growth by 50% (IC_{50}) relative to control was calculated using GraphPad Prism 7.0 software (San Diego, CA, USA).

2.4. Statistical analysis

All experiments were conducted in triplicate. Statistical significances were assessed by two-way ANOVA and Tukey post hoc test (95% confidence interval), using Statistica software (version 10) and GraphPad Prism 7.0. Differences between groups were considered to be significant when $p \leq 0.05$.

3. Results and discussion

3.1. Determination of ohmic heating extraction conditions: Preliminary assays

The extraction time can significantly influence the concentration of TPC, impacting on the antioxidant activity of the extracts (Jesus et al., 2019), besides reducing the energy consumption and cost of the extraction process. Therefore, it is important to evaluate the extraction time to optimize the recovery of the bioactive compounds. For defining the extraction process conditions, preliminary tests were performed in order to evaluate the optimum extraction time (20–90 min) for OH

treatments in comparison to the tests carried out by the CH and RT methods, being the last one used as a control. The temperature (80 °C) and the liquid/solid ratio (40:1 mL/g of VPR) were defined according to results obtained in previous CH extractions (Jesus et al., 2019). The extraction time efficiency was evaluated in terms of the recovered TPC and the antioxidant activity evaluated by different methods, including FRAP, DPPH and ABTS (Fig. 1). For evaluating the treatments, the TPC concentration and the antioxidant activity of the samples obtained by OH and CH were compared with the control extraction (RT) method, inferred by two-way ANOVA.

The results showed no significant differences ($p > 0.05$) for the polyphenol content in the extracts obtained by CH and OH (3.2 ± 0.1 and 3.1 ± 0.2 g GAE/100 g, respectively, at 60 min) for all evaluated times, correlating with the values previously reported by other authors (Çetin, Altinöz, Tarçan, & Baydar, 2011; Jesus et al., 2019; Moreira et al., 2018). However, there were significant differences between treatments with respect to the antioxidant activity where OH treatment presented the best FRAP value (3.1 ± 0.3 g FE/100 g), and also the highest values when DPPH (3.2 ± 0.4 g TE/100 g) and ABTS (1.50 ± 0.2 g TE/100 g) assays were determined. In general, it was possible to observe significant differences ($p < 0.05$) between the extraction treatments and the time of extraction in the OH treatment, with the better result obtained with 60 min of extraction. Therefore, 60 min may be considered an adequate time to be used in all the extraction processes, which is an advantage, since the use of the extraction times shorter result in important economic viewpoints.

3.2. Experimental TPC and antioxidant extraction

Bioactive compounds from VPR, were extracted with a 40% hydroalcohol solution, at 80 °C, for 60 min. The VPR were subjected to three methods of extracting, including CH, OH with two different electric field intensities (LEF- Low Electric Field and IEF-Intermediate Electric Field), and a treatment at RT, which was used as controls Table 1. The total content of phenolic compounds extracted by OH using IEF was 23% higher than that obtained in previous works from high electrical voltage (2.0 g GAE/100 g VPR) (Rajha et al., 2018). In addition, significant ($p < 0.05$) variations of TPC in the extracts obtained through the different extraction methods were observed, being IEF and LEF the extract with the highest TPC values (3.4 ± 0.1 and 3.1 ± 0.2 g GAE/100 g VPR, respectively). The extract obtained through RT treatment (1.2 ± 0.1 g GAE/100 g VPR), as expected, exhibited the lowest TPC values, which is in agreement with other authors, who observed a marked reduction of TPC in extracts obtained at low temperatures (Jesus et al., 2019; Sánchez-Gómez, Sánchez-Vioque, et al., 2017). The temperature directly influences the TPC concentration, since mild treatments are not able to solubilize the phenolic compounds present in the lignicellulosic materials. The extracts produced by the IEF treatments had statistically higher concentrations ($p > 0.05$) compared to the RT, CH and LEF treatments indicating that the electric field strength could positively affect the final TPC concentration (Rajha et al., 2015b). Althought there are no significant difference between the extracts produced with CH ans LEF treatments.

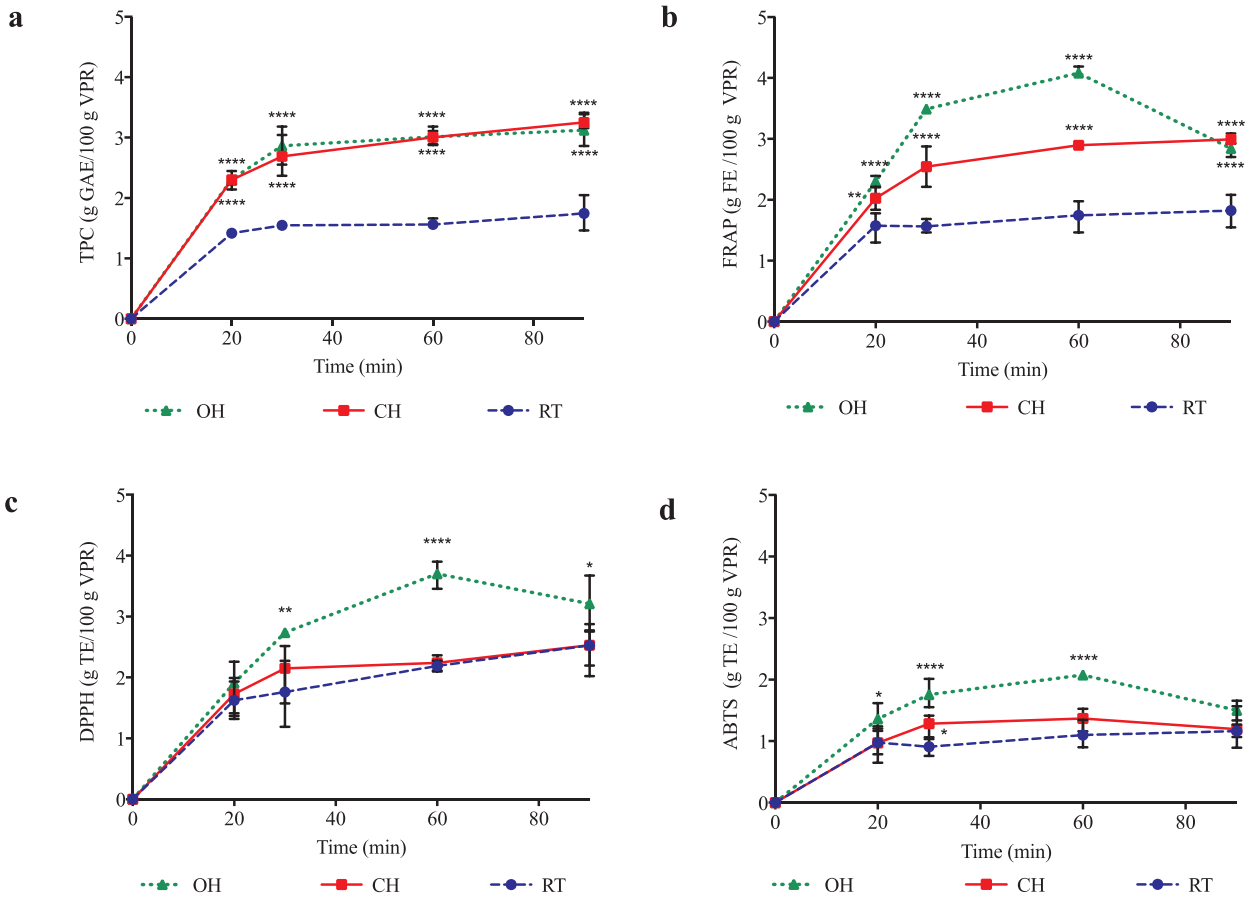

Fig. 1. Extraction of phenolic compounds from vine pruning residue using different extraction times (20, 30, 60 and 90 min) and methods of heating: room temperature (RT), conventional heating (CH) and ohmic heating (OH). (a) Total phenolic compounds; (b) FRAP; (c) DPPH; (e) ABTS. Fixed conditions: solid liquid ratio (1:40 w/v), ethanol concentration (45%) and temperature (80 °C). All experiments were done in triplicate and the results expressed as mean ± SD. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Table 1
Total phenolic compounds (TPC) and antioxidant activity FRAP, DPPH and ABTS of the extracts produced from vine pruning residue by using different methods of heating: room temperature (RT), conventional heating (CH) and ohmic heating at different electric fields (LEF: Low Electric field; IEF: Intermediate Electric field).

Runs	TPC	FRAP	DPPH		ABTS	
	g GAE/100 g VPR	g FE/100 g VPR	g TE/100 g VPR	IC_{50}	g TE/100 g VPR	IC_{50}
IEF	3.4 ± 0.1^{c}	4.6 ± 0.2^{c}	4.1 ± 0.1^{d}	0.76^{a}	3.1 ± 0.1^{c}	0.34^{a}
LEF	3.1 ± 0.2^{b}	4.1 ± 0.3^{b}	3.2 ± 0.1^{c}	0.90^{b}	1.9 ± 0.2^{b}	0.44^{b}
CH	3.0 ± 0.2^{b}	3.7 ± 0.1^{d}	2.7 ± 0.2^{b}	0.95^{b}	1.9 ± 0.1^{b}	0.40^{b}
RT	1.2 ± 0.1^{a}	1.7 ± 0.2^{a}	2.2 ± 0.1^{a}	1.25^{c}	1.01 ± 0.1^{a}	0.94^{c}

*The averages followed by the same letters within a column do not differ by the Tukey test ($p < 0.05$). GAE: gallic acid equivalents; FE ferrous equivalents; TE (II): Trolox equivalents. Antiradical activity is expressed as a mean ($n = 3$) of IC_{50} values (g of extract/L of solution).

The experimental results showed that the highest antioxidant activities evaluated by FRAP (4.6 ± 0.2 g FE/100 g), DPPH (4.0 ± 0.1 g TE/100 g) and ABTS (3.1 ± 0.1 g TE/100 g) assays were achieved when the ohmic IEF heating was used. The LEF treatment presented higher antioxidant activities for FRAP (4.1 ± 0.3 g FE/100 g) and DPPH (3.2 ± 0.1 g TE/100 g) assays compared with the obtained values when CH and RT methods were carried out. However, the ABTS values presented no significant differences ($p > 0.05$) (1.9 ± 0.1 g TE/100 g) between both, LEF and CH treatments. Previous works have shown that there is a great influence of the applied electric field on the antioxidant activity values when FRAP and DPPH assay were determined, showing better results with respect to other extraction methodologies (Cappato et al., 2018; Loypimai, Moonggarm, & Chottanom, 2009). When the comparison based on the extraction method is conducted, (Table 1), it becomes evident that RT and CH were the worst methods for obtaining extracts with relevant antioxidant activity. The increase of the intensity of the electric discharges in the IEF treatment raises the TPC and the antioxidant activity in the VPR extracts, probably due to the microscopic damages induced by shock waves of pressure that lead to the deterioration of the structural components of the residue, facilitating the rupture of the tissues by cavitation bubbles (Boussetta et al., 2012). These results highlight the significant effects of using IEF to extract antioxidant phenolic compounds in relation to other extraction methods.

Additionally, half of the maximal inhibitory concentration (IC_{50}) was also calculated by the DPPH and ABTS assays. The highest value was obtained in the extracts obtained by the CH and RT treatments, which means that the electric fields have influenced positively the extraction of bioactive compounds in VPR.

3.3. Chemical composition of VPR extracts

The samples extracted using OH at two different intensities (LEF and IEF), CH and RT techniques were analyzed by UHPLC (Ultra high-performance liquid chromatography) and the identification and quantification of the phenolic compounds present in the extracts were carried out. The compounds identified in VPR extracts (results expressed in mg/100 g of VPR) are listed in Table 2.

Twelve different types of polyphenolic compounds including phenolic acid, flavonoids, phenylethanoids and stilbenes, were identified and quantified using their corresponding standards. The LEF and IEF extracts showed the highest concentrations of flavonoids. However, apigenin and hesperidin flavonoids, were more abundant in IEF treatment (287.2 and 180.3 mg/100 g VPR, respectively) than LEF, and were not identified in the extracts obtained by CH and RT treatments. The concentration of apigenin in LEF was higher than that previously described (Jesus et al., 2019), which was 207.9 mg of apigenin per 100 g of VPR when a conventional liquid-solid method using a 45% hydroalcoholic solution and 120 min of extraction was applied. Other authors employed a conventional heating method to extract the same compound from grape pomace, using an 80% hydroalcoholic solution and acetone as extraction solvents, obtaining extracts with 9.1 mg apigenin /100 g of grape pomace (Pintać et al., 2018; Dutra et al., 2018). Hesperidin was also found analyzed the extraction of phenolic compounds from conventional grape juice (4.04 mg/L^{-1}), conventional wines (4.9 mg/L^{-1}) and organic wines (4.3 mg/L^{-1}). On the other hand, quercetin was present in all extracts, IEF, LEF and CH (287.2, 286.8 and 281.6 mg/100 g VPR, respectively) with significantly higher concentrations ($p < 0.05$) than in RT (132.8 mg/100 g VPR). These values were higher than those obtained in previous works when used CH extraction (27.7 mg/100 g VPR) (Jesus et al., 2019) and microwave extraction (82.1 mg/100 g VPR) (Moreira et al., 2018). The identification of this compound in the VPR extracts is relevant, since quercetin is the main flavonol present in vine leaves of white and red grape varieties, representing more than 70% of total flavonols (Flamini, Mattivi, De Rosso, Arapitsas, & Bavaresco, 2013).

Single phenols were identified as hydroxytyrosol and tyrosol. Hydroxytyrosol was not identified in the RT extracts and did not present statistically significant ($p > 0.05$) differences in the concentrations found in the extracts obtained by CH, LEF and IEF (149.6, 151.6, and 152.4 mg/100 g VPR, respectively). IEF extract presented the highest tyrosol concentration (142.3 mg/100 g VPR), followed by LEF and CH (139.8 and 137.1 mg/100 g VPR, respectively), which showed no statistically significant differences ($p > 0.05$) and RT extract (64.2 mg/100 g VPR). Hydroxytyrosol and tyrosol were also identified in samples of commercial white Greek wines of *Vitis vinifer*a L. cv. Malagusia (Tourtoglou, Nenadis, & Paraskevopoulou, 2014).

Although stilbenes are extensively explored in VPR studies, in this study *trans*-resveratrol was the unique compound identified in the extracts. The highest *trans*-resveratrol concentration was obtained in the LEF treatment (137.3 mg/100 g VPR), where it was twice as high as the

Table 2
Polyphenolic composition of the VPR extracts (Expressed as mg/100 g VPR) obtained by different methods of heating: room temperature (RT), conventional heating (CH) and ohmic heating at different electric fields (LEF: Low Electric field; IEF: Intermediate Electric field).

Polyphenols (mg/100 g VPR)	IEF	LEF	CH	RT
Phenolics acid				
Gallic acid	2.9^{b}	3.5^{a}	ND	ND
o-Cumaric acid	15.8^{b}	26.5^{c}	14.2^{b}	6.6^{a}
Ferulic acid	46.6^{a}	46.1^{a}	ND	ND
Ellagic acid	222.9^{b}	77.7^{a}	ND	ND
Vanillic acid	68.4^{bc}	70.3^{c}	67.2^{b}	31.2^{a}
Flavonoids				
Hesperidin	180.3^{b}	149.0^{a}	ND	ND
Apigenin	384.2^{b}	157.5^{a}	ND	ND
Quercetin	287.2^{bc}	286.8^{bc}	281.6^{b}	132.8^{a}
Taxifolin	23.7^{c}	21.8^{bc}	19.8^{a}	ND
Simple phenols				
HidroxiTyrosol	152.4^{c}	151.6^{bc}	149.6^{bc}	ND
Tyrosol	142.3^{c}	139.8^{b}	137.1^{b}	64.2^{a}
Stilbenes				
trans-resveratrol	65.4^{a}	137.3^{b}	ND	ND

*Where The averages followed by the same letters within a file do not differ by the Tukey test ($p < 0.05$). ND: not detected.

IEF extract value (65.4 mg/100 g VPR). These values are in agreement with those obtained by other authors (Moreira et al., 2018), who evaluated the extraction of *trans*-resveratrol (136 mg/100 g VPR) from two varieties of Portuguese grapes by using microwave extraction.

As it is well known, residues of the wine industry have been extensively studied for the extraction of bioactive compounds. However, the different cultivars, parts of the plant, geographical location, climatic conditions and storage time can influence on the properties and chemical composition of the recovered compounds (Cebrián-Tarancón et al., 2019). The content in flavonols in VPR is the one that varies most according to the grape varieties (Cebrián, Sánchez-Gómez, Salinas, Alonso, & Zalacain, 2017). The main phenolic compounds found in this work were apigenin, quercetin, ellagic acid and hesperidin. According to Delgado-Torre and co-workers, catechin was the main phenolic compound isolated in VPR extract (Delgado-Torre et al., 2012;), while in other studies, (E)-resveratrol followed by (E)-ε-viniferine were also identified as the main phenolic compounds in VPR extracts (Gabaston et al., 2018). Other authors found in VPR, after toasting treatment, high concentrations of proanthocyanidins (Cebrián-Tarancón et al., 2018). On the other hand, studies based on the polyphenolic composition extracted from VPR by using heating by electric fields are scarce, and most of them use pulsed electro technologies and water as the extraction solvent followed by other pre-treatments. In addition, these studies have described the presence of kaempferol, epicatechin, resveratrol, hydrodobenzoic acid, *p*-coumaric acid and ferulic acid (Rajha, Boussetta, Louka, Maroun, & Vorobiev, 2014; Rajha et al., 2015a, 2018). In other varieties of vine-shoots the majority of the flavonols of the present study were not detected, instead the flavanols content (catechin and epicatechin) was the most important (Cebrián et al., 2017). It was proven that the content of flavanols varied significantly according to the time elapsed since pruning (Cebrián-Tarancón et al., 2018; Cebrián et al., 2017).

3.4. Antimicrobial activity of VPR extracts

The VPR extracts obtained in this study through LEF, IEF, CH and RT treatments were screened for antimicrobial activity against five fungi using a micro-dilution methodology. All fungi were evaluated as a function of the incubation time (assessing the growth rate at 24, 48, 72 and 96 h) at 25 ± 2 °C. Thus, different concentrations of each extract (1000, 500, 250, 125, 62.5, 31.3, 15.6, 7.8, 3.9, 1.95 μg/mL) were tested against *Alternaria* sp., *Cladosporium cladosporioides, Phoma violacea, Penicillium italicum* and *Penicillium expansum* in order to observe the fungal growth and determine the optimal inhibitory extract concentrations for each treatment. The main results are summarized in Table 3, where are presented the two best concentrations found of each extract (LEF, IEF, CH and RT) to inhibit the growth of the evaluated fungi. In a general way, all the extracts exhibited antifungal activity against the studied fungi, showing greater activity after 96 h of exposure at the different extract concentrations. Antifungal activity varied according to the concentrations and extracts, although none of the concentrations used presented 100% inhibition. Some authors suggest plant extracts as potential natural antifungal agents because of the need for low dosages to achieve antimicrobial bioactivity and reducing negative sensory impact on food (Kumar, Kujur, Singh, & Prakash, 2019).

The highest antimicrobial inhibition was obtained when the maximum extract concentration (1000 μg/mL) was tested after 96 h of incubation. In addition, IEF, LEF and CH extracts expressed much more significant inhibitory effects against *P. italicum, P. expansum and C. cladosporioides* when the same concentration (1000 μg/mL) was used, in contrast to RT extract that only presented a high percent inhibition against *P. italicum*. In general, VPR extracts showed a growth inhibition from 18.1 to 68.2% (Table 3) when the maximum concentration was applied. On the other hand, the minimum inhibitory concentration for CH and RT extracts was 15.6 μg/mL, showing inhibition percentages between 12.3 and 38.5 and 18.1–37.3%, respectively. The results showed that the IEF extract presented a significant inhibitory power against *C. cladosporioides* (38.4%) and *Phoma violacea* (24.5%) when 3.9 μg/mL extract was used, as well as against *P. italicum* (30.5%) and *P. expansum* (21.3%)when 7.8 μg/mL extract was tested. As regards the LEF extract, the minimum inhibitory concentration was 3.9 μg/mL for *P. expansum*, which was able to inhibit 13.1%, whereas for P. *italicum* and *Phoma violacea* the concentration 7.8 μg/mL was able to inhibit 38.8% and 14.2% of the fungal growth, respectively. Finally, *Alternaria* sp. and *C. cladosporioides* showed a percent growth inhibition of 25.8% and 27.1%, respectively, to 15.6 μg/mL of LEF extract. The LEF and IEF extracts showed minimal inhibitory concentrations that expressed stronger antifungal activities for *Alternaria* sp., *C. cladosporioides, P. violacea, P. italicum* and *P. expansum*. These results are related to the phenolic profile and antioxidant activity previously provided.

Some authors claim that the antifungal effects of plant extracts and essential oils rich in bioactive compounds such as flavonoids are related to the disruption of the fungal cell endomembrane system (plasma membrane and mitochondria) and mitochondrial dysfunction, inducing metabolic stagnation (Hu, Zhang, Kong, Zhao, & Yang, 2017; Yang et al., 2017). Inhibition of fungal growth and germination can be explained by the presence of flavonoids such as quercetin, hesperidin and apigenin, and phenolic acid such as ellagic acid, which have the capacity to reduce microbial growth, making a preferential alternative to synthetic antimicrobial agents, as these are environment friendly and

Table 3
Percent inhibition of the VPR extracts by using different methods of heating: room temperature (RT), conventional heating (CH) and ohmic heating at different electric fields (LEF: Low Electric field; IEF: Intermediate Electric field) against the growth of different fungi calculated after 96 h of exposure.

Extraction technique	IEF		LEF		CH		RT	
Fungi	Extracts concentration (μg/mL)	Inhibition (%)	Extracts concentration (μg/mL)	Inhibition (%)	Extracts concentration (μg/mL)	Inhibition (%)	Extracts concentration (μg/mL)	Inhibition (%)
Penicillium italicum	7.8	30.5 ± 1.9	7.8	38.8 ± 3.4	15.6	38.5 ± 8.1	15.6	37.3 ± 2.5
	1000	68.2 ± 1.2[a]	1000	66.5 ± 1.4[a]	1000	67.8 ± 4.5[a]	1000	64.3 ± 2.9[a]
Penicillium expansum	7.8	21.3 ± 9.6	3.9	13.1 ± 5.9	15.6	24.6 ± 3.4	15.6	18.1 ± 6.1
	1000	53.3 ± 10.3[a]	1000	54.5 ± 13.7[a]	1000	34.7 ± 3.4[b]	1000	25.0 ± 8.3[c]
Alternaria sp.	15.6	20.9 ± 2.1	15.6	25.8 ± 5.4	15.6	24.9 ± 11.7	15.6	27.8 ± 6.0
	1000	40 ± 5.2[d]	1000	45.4 ± 7.5[c]	1000	32.1 ± 1.6[a]	1000	35.1 ± 1.2[b]
Phoma violacea	3.9	24.5 ± 2.1	7.8	14.2 ± 3.1	15.6	12.3 ± 6.4	15.6	21.4 ± 11.5
	1000	41 ± 5.9[c]	1000	33.9 ± 4.5[a]	1000	31.6 ± 2.1[a]	1000	28.5 ± 5.8[b]
Cladosporium Cladosporioides	3.9	38.4 ± 3.3	15.6	27.1 ± 2.7	15.6	30.3 ± 17.9	15.6	26.0 ± 10.4
	1000	62.4 ± 7.9[a]	1000	59.9 ± 2.7[a]	1000	43.8 ± 5.4[b]	1000	18.1 ± 9.2[c]

*The statistic was carried out only for the concentration of 1000 μg/mL. The averages followed by the same letters within a line do not differ by the Tukey test ($p < 0.05$).

easy to use (Kumar et al., 2019; Yang et al., 2017).

3.5. Anticancer activity of VPR extracts

The anticancer potential of VPR extracts was evaluated by their capacity to decrease the cell proliferation of four different cancer cell lines (MDA-MB-231, MCF-7, HepG2, and Caco2) and a non-carcinogenic cell line (CCD 841 CoN) being assessed by the MTT assay. As shown in Table 4, all VPR extracts inhibited cell growth in a concentration- and time-dependent manner in all cell lines tested. In recent years, several studies have attributed to flavonoids the selective reduction of cell viability of cancer cells (Mahmoudi et al., 2019; Tavsan & Kayali, 2019; Zhu, Wang, Jia, & Xie, 2019). These results can be attributed, among other compounds, to the abundant presence of quercetin in all extracts, which is considered to have anticancer potential due to its ability to induce apoptosis and inhibit the progression of numerous human cancer cell lines (Rauf et al., 2018). The IEF extract showed the most potent activity against MDA-MB-231, MCF-7, HepG2, Caco2, and CCD 841 CoN cells with IC_{50} values after 48 h of exposure of 62.8, 54.7, 89.7, 49.7, 71.0 μg/mL, respectively (Table 4). The distinct polyphenolic composition of the VPR extracts may explain the different growth inhibitory capacity between the VPR extracts. Taking into account the chemical characterization of the VPR extracts, apigenin, ellagic acid, hesperidin and quercetin are more abundant in the IEF extract than in the other extracts. Some authors recognize apigenin as a potential inhibitor of viability of human lung carcinoma cells (A549), while not showing significant side effects in healthy human umbilical vein endothelial cells (HUVEC) (Mahmoudi et al., 2019). Apigenin is considered an efficient inhibitor of the expression of nuclear factor E2-related factor 2 important contributor to chemoresistance in cancer therapy (Gao, Ke, Shi, Sun, & Chen, 2013; Paredes-Gonzalez et al., 2015). Over the years, studies with hesperidin have shown potent anti-inflammatory effects, anticancer and chemopreventive antioxidants (Kamaraj et al., 2019; Roohbakhsh, Parhiz, Soltani, Rezaee, & Iranshahi, 2015). In earlier studies hesperidin significantly inhibited the cell viability of human lung cancer A549 cells (Kamaraj et al., 2019), Caco2 cells (El-Readi, Hamdan, Farrag, El-Shazly, & Wink, 2010) and MCF-7 breast cancer cells (Febriansah, Dyaningtyas, Sarmoko, Meiyanto, & Nugroho, 2014), these works suggest hesperidin as a new agent in carcinoma therapy. Other authors show that keratin enhances anticancer and proapoptotic effects in colon CO115 and HCT1 cell lines and has also been shown to act on KRAS and PI3K (Xavier et al., 2009; Xavier, Lima, Rohde, & Pereira-Wilson, 2011). Previous publications showed that ellagic acid isolated from *Phyllanthus emblica* L. exert a dose-dependent anticancer activity against MCF-7 cells in a concentration range between 5 and 50 μg/mL (Luo et al., 2011).

The cancer cell lines MDA-MB-231, MCF-7, HepG2, and Caco2 were more sensitive to VPR extracts than CCD 841 CoN non-carcinogenic cells under the same treatment conditions. A chemopreventive agent should have strong inhibitory effect on cell proliferation and carcinogenic pathways of cancer cells however, minimal effect on healthy cells. For the treatment of cancer, the natural therapies by plant extracts may reduce the side effects and toxic effects of therapies (Liu, Wang, Tang, Bowater, & Bao, 2019; Ramadan et al., 2017). However, HepG2 cancer cells were less sensitive to VPR extracts than CCD 841 CoN cells. This may be explained by the fact that HepG2 cells retain many of the specialized function of normal hepatocytes (Liu et al., 2019).

These results demonstrated that the IEF extract inhibits cell growth and its effect may be related to its chemical composition. Since, the inhibition of cell growth evaluated by the MTT assay could be attributed to decreased cell proliferation, induction of cell death or both, it would be interesting in the future to evaluate the effects of the VPR extracts on cell cycle and death. The results of this study represent preliminary results that will be beneficial for the evaluation of the bioactive potential of extracts of VPR and the development of novel chemotherapeutic agents.

4. Conclusions

In this study, the extraction of polyphenolic compounds from Vine Pruning Residue (VPR) was evaluated through different electric field intensities (LEF electric field and intermediate electric field - IEF) using the environmentally friendly OH extraction procedure and compared to two conventional extractions (Room Temperature- RT and Conventional Heating-CH). Overall, VPR was validated as a good source for phenolic compounds that can be exploited as ingredients for application on the food, pharmaceutical or cosmetic industries. The extracts obtained by the environmentally friendly OH technique presented better results in comparison to the other treatments most probably due to the rupture of the cells by the electric current passage. These results suggest that OH in IEF is an effective technique for extracting phenolic compounds from VPR. In addition, the IEF extract, which used higher electrical intensity, showed greater enrichment in the phenolic compounds, antioxidant activity, antimicrobial activity, and cancer cell growth inhibitory activity. Besides its enhanced biological activity, these extracts have additional advantages for application in food, pharmaceutical or cosmetic industries in comparison with other extraction techniques as no toxic solvents have to be removed.

CRediT authorship contribution statement

Meirielly S. Jesus: Formal analysis, Investigation. **Lina F. Ballesteros:** Conceptualization, Methodology. **Ricardo N. Pereira:** Conceptualization, Methodology. **Zlatina Genisheva:** Conceptualization, Methodology, Validation. **Ana S. Carvalho:** Investigation. **Cristina Pereira-Wilson:** Conceptualization, Methodology, Resources. **José A Teixeira:** Supervision, Funding acquisition, Resources. **Lucília Domingues:** Supervision, Funding

Table 4
IC_{50} values (μg/mL) of VPR extracts obtained by different methods of heating: room temperature (RT), conventional heating (CH) and ohmic heating at different electric fields (LEF: Low Electric field; IEF: Intermediate Electric field) against different cancer cell lines (MDA-MB-231- human breast, MCF-7- human breast, HepG2- human hepatocellular, and Caco2- human colon) and a non-carcinogenic cell line (CCD 841 CoN– human colon), calculated after 24 h and 48 h of exposure.

Cell lines	IC_{50} (μg/mL)							
	IEF		LEF		CH		RT	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
MDA-MB-231	91.7 ± 12.7^{ab}	62.8 ± 6.5^{a}	116.1 ± 6.5^{a}	74.2 ± 5.6^{a}	119.9 ± 14.8^{a}	79.1 ± 8.2^{a}	137 ± 4.8^{ab}	86.97 ± 14.2^{a}
MCF-7	154.6 ± 21.7^{bc}	54.7 ± 5.3^{a}	167.2 ± 76.5^{bc}	62.7 ± 4.9^{a}	186 ± 14.6^{b}	65.7 ± 6.1^{a}	222.9 ± 64.6 ^{cd}	70.8 ± 10.1^{a}
HepG2	134.3 ± 27.7^{ab}	89.7 ± 13.7^{ab}	193.4 ± 51.1^{a}	117.7 ± 15.3^{a}	198.8 ± 29.9^{a}	117.2 ± 16.1^{a}	216.2 ± 28.7^{bc}	122.7 ± 25.0^{a}
Caco2	76.2 ± 4.0^{ab}	49.7 ± 6.0^{a}	104.3 ± 12.6^{a}	62.7 ± 3.7^{a}	105.2 ± 8.5^{b}	65.9 ± 6.3^{a}	115.2 ± 9.5^{b}	83.8 ± 7.4^{ab}
CCD 841 CoN	283.4 ± 5.6^{c}	71.0 ± 0.3^{a}	337.9 ± 5.4^{b}	78.1 ± 2.1^{a}	435.6 ± 8.1^{b}	81.6 ± 1.1^{a}	430.9 ± 8.8^{b}	99.0 ± 2.1^{a}

*Where Values are expressed as mean ± SEM of three independent experiments. The averages followed by the same letters within a line do not differ by the Tukey test ($p < 0.05$).

acquisition.

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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Declaration of interests

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