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Characterization of pressurized hot water extracts of grape pomace: Chemical and biological antioxidant activity

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ABSTRACT

Pressurized hot water extracts obtained at different temperatures possess different compositions and antioxidant activities and, consequently, different bioactivities. We characterized two pressurized hot water extracts from grape pomace obtained at 100 °C (GPE100) and 200 °C (GPE200) in terms of antioxidant activity and composition, as well as protective effect on cell growth and mitochondrial membrane potential ($\Delta\psi_m$) in a HL-60 cell culture under oxidative conditions. GPE100 extracts were richer in polyphenols and poorer in Maillard reaction products (MRPs) than were GPE200 extracts. Moreover, hydroxymethylfurfural was detected only in GPE200. Both extracts exhibited similar protective effects on cell growth (comparable to the effect of trolox). In addition, GPE100 strongly decreased the $\Delta\psi_m$ loss, reaching values even lower than those of the control culture. This protective effect may be related to its high polyphenols content. At the highest concentration assessed, both extracts showed strong cytotoxicity, especially GPE200. This cytotoxicity could be related to their MRPs content.

1. Introduction

Pressurized hot water extraction (PHWE) is a highly promising energy-efficient and environmentally benign technique for recovering polyphenols from natural materials. Many of water's solvent properties can be manipulated to optimize phytochemical extraction by raising the temperature, while maintaining water in its liquid state by pressurization (Hawthorne, Miller, Lagadec, Hammond, & Clifford, 2002). Water polarity declines dramatically with increasing temperature, due to hydrogen bond breakdown, and reaches values comparable to organic solvent–water mixtures (Hawthorne et al., 2002). The decrease in viscosity and surface tension with rise of temperature improves the mass transfer rates of compounds from the plant tissue matrix (Hawthorne et al., 2002). Both temperature and pressure play significant roles in disrupting water surface equilibrium, thereby lowering the activation energy required for desorption processes (Ong, Cheong, & Goh, 2006).

However, diverse phenomena, highly dependent on temperature, occur during the PHWE of polyphenols from plant materials: polyphenol degradation and formation of polyphenol-derived antioxidants, selective polyphenol extraction, depolymerization, and polymerization (Ju & Howard, 2005; Vergara-Salinas et al., 2013), as well as the generation of antioxidant-potent Maillard reaction products (MRPs), such as melanoidins (Plaza, Amigo-Benavent, Castillo, Ibáñez, & Herrero, 2010) and hydroxymethylfurfural (He et al., 2012). In PHWE the relationship between polyphenol content and antioxidant activity of the extracts is weak or even inverse (Vergara-Salinas et al., 2013). Therefore, depending on the PHWE temperature used, it is possible to obtain extracts with different compositions and antioxidant activities and, consequently, different activities in biological systems.

The ability of chemical antioxidant activity assays to predict the *in vivo* activity is questionable because it does not take into account several physiological aspects, such as bioavailability and metabolism alterations (Liu & Finley, 2005). However, cell cultures are suitable for assessing the potential action of the antioxidants in a biological system, previous to animal and human studies (Liu & Finley, 2005). The human promyelocytic leukaemia line, HL-60, has been widely used to study oxidative stress-related aspects. This cell line is very sensitive to oxidative stress inducers, such as ultraviolet radiation and hydrogen peroxide (Verhaegen, McGowan, Brophy, Fernandes, & Cotter, 1995) and it is a good option for preliminary evaluation of the potential protective effect of antioxidant compounds.

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Grapes (*Vitis* spp.) are one of the largest fruit crops in the world and grape by-products are produced in massive quantities, especially by the winemaking industry (Djilas, Canadanovic-Brunet, & Cetkovic, 2009). After fermentation in wine production, most of the grapes' original polyphenolic content remains in the pomace (Vergara-Salinas et al., 2013). Polyphenols, such as anthocyanins and condensed tannins (proanthocyanidins), play significant roles in the health benefits of wine (Diebolt, Bucher, & Andriantsitohaina, 2001).

It has been reported that grape polyphenols, in cell line culture ranges, reduce oxidative stress, inhibit DNA damage induced by reactive oxygen species (ROS) (Apostolou et al., 2013) and activate the antioxidant response (Xia, Deng, Guo, & Li, 2010), but also exhibit a dose-dependent toxicity (Gao et al., 2009). This toxicity increases in the case of crude extracts (mixtures of many compounds) and it could be even higher in the pressurized hot water extracts due to the degradation and formation of new compounds (e.g. MRPs) at high temperatures. MRPs can suppress oxidative stress and inflammation in human cells cultures (Kitts, Chen, & Jing, 2012). However, these compounds are considered to be toxic and mutagenic (Husøy et al., 2008).

The objective of this study was to assess grape pomace extracts obtained at different extraction temperatures in terms of polyphenol and MRP contents, chemical antioxidant activity, and bioactivity on HL-60 cell line culture under oxidative conditions, including protective effect on cell growth and mitochondrial membrane potential.

2. Materials and methods

2.1. Chemicals and cell line

Reagents and standards used were of analytical grade. Folin–Ciocalteu reagent, methanol and sodium carbonate were purchased from Merck (Germany). Tripyridyl triazine (TPTZ), $FeCl_3(6H_2O)$, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox), 2,5-dihydroxybenzoic acid, ascorbic acid, gallic acid, maleic acid-sodium dodecyl sulphate, triethanolamine, iron(III) chloride, bovine serum albumin, sodium hydroxide, hydrochloric acid (37%), glacial acetic acid, and sodium chloride, 5-hydroxymethylfurfural (5-HMF), RPMI-1640, D-glucose, L-glutamine and Human promyelocytic leukaemia cells HL-60 were obtained from Sigma (USA). Heat inactivated fetal bovine serum was obtained from Gibco (Brazil). 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolyl-carbocyanine iodide (JC-1) was obtained from Life Technologies (USA). Valinomycin was obtained from Santa Cruz Biotechnology (USA).

2.2. Grape pomace

Cabernet Sauvignon pomace was obtained from Carmen Vineyard, Region Metropolitana, Chile. The pre-fermentation process was performed at 18 °C for 10 days and the must was loaded into a 10 m^3 fermentation tank. Fermentation was conducted between 25 °C and 30 °C for 21 days without pectolytic enzymes. The grape pomace sample was taken after the fermentation process had finished. Each sample was reduced to a particle size lower than 1 mm diameter by an Oster® blender (Sunbeam Products, Inc., Boca Raton, FL) and then frozen to −20 °C prior to extraction.

2.3. Pressurized hot water extraction

Grape pomace was subjected to pressurized hot water extraction (PHWE). A 5 g sample (dry weight) of grape pomace was mixed with 100 g of neutral quartz sand to avoid filter clogging in the 100 ml stainless steel extraction cell. The grape pomace was extracted in an Accelerated Solvent Extraction device (ASE® 150, Dionex) with approximately 50 ml of distilled and filtered (0.22 μm) water to obtain a matrix/extractant ratio of 1:10. The extractions were done in triplicate during 5 min at two temperatures: 100 (GPE100) and 200 °C (GPE200). After extraction, the cell contents were rinsed with 100 ml of distilled and filtered (0.22 μm) water and purged for 360 s by applying pressurized nitrogen (10.2 atm). Finally, the collected extracts were freeze-dried and stored in amber vials at −20 °C prior to analysis. Extract solutions of 1 g/l were prepared for analysis.

2.4. Ferric-reducing antioxidant power determination

The ferric reducing ability of plasma (FRAP) test offers a putative index of antioxidant reducing capacity in a sample. A working solution was prepared by mixing 300 mM acetate buffer (pH 3.6), a solution of 10 mM TPTZ in 40 mM HCl, and a freshly prepared 20 mM $FeCl_3(6H_2O)$ solution in 10:1:1 (v/v/v) proportions. For the assay, 3 ml of working reagent were mixed with 100 μl sample or calibration standard (ascorbic acid), and absorbance was measured at 593 nm after a 30 min reaction time (Pulido, Bravo, & Saura-Calixto, 2000). A calibration curve was constructed, using ascorbic acid (0.1–0.8 mM). The regression coefficient of ascorbic acid was 0.9989. Results were expressed as ascorbic acid equivalents (AAE) per gramme of dry extracts (d.e.).

2.5. Total antioxidant determination by Folin assay

Total antioxidants were determined by Folin assay. Although this method is commonly considered for polyphenol analysis, it indeed determines all compounds in the sample with antioxidant capacity and not only polyphenols (Pérez-Jiménez, Neveu, Vos, & Scalbert, 2010). A mixture of 4.25 ml of phenolic extract (1 mg/ml) and 0.25 ml of Folin–Ciocalteu reagent was diluted 1:1 (v/v) with distilled water, and mixed with 0.5 ml of a 10% sodium carbonate solution (w/v). Absorbance was measured at 765 nm after a 1 h reaction time at room temperature. A calibration curve was constructed, using gallic acid as the calibration standard (20–90 mg/l). The regression coefficient of gallic acid was 0.9987. Results were expressed as gallic acid equivalents (GAE) per g of d.e.

2.6. Anthocyanins and tannin determination by Harbertson-Adams assay

Anthocyanin and condensed tannin contents in grape pomace extracts were determined with the Harbertson-Adams assay

Table 1
Characterization of the pressurized hot water extracts from grape pomace.[a]

Extract		GPE100	GPE200
FRAP (mg AAE/g d.e.)		10.2[b]	15.0[c]
Total antioxidants (mg GAE/g d.e.)		10.6[b]	13.6[c]
Anthocyanins (mg M3GE/g d.e.)		10.5	n.d.
Condensed tannins (mg EC/g d.e.)		52.9[b]	18.3[c]
(+)-Catechin (mg/g d.e.)		0.81[b]	0.65[c]
(−)-Epicatechin (mg/g d.e.)		1.05[b]	1.24[c]
Kaempferol (mg/g d.e.)		0.12[b]	0.03[c]
Myricetin (mg/g d.e.)		0.18[b]	0.16[c]
Resveratrol (mg/g d.e.)		0.02	n.d.
Σ Total polyphenols (mg/g d.e.)		65.58	20.38
MRPs by absorbance (AU)	360 nm	0.318[b]	0.371[c]
	420 nm	0.148[b]	0.281[c]
HMF (mg 5-HMF/g d.e.)		n.d.	0.087

M3GE, malvidin 3-*O*-glucoside equivalents; d.e., dry extract; EC, epicatechin equivalents; FRAP, ferric-reducing antioxidant power; AAE, ascorbic acid equivalents; GAE, gallic acid equivalents; MRPs, Maillard reaction products; AU, absorbance units; HMF, Hydroxymethylfurfural; n.d., not detected.

[a] Values with the same letter (b–c) in each row showed no statistically significant difference at the confidence interval of 95%.

adapted from the Hagerman and Butler method (Hagerman & Butler, 1978). Results were expressed as malvidin 3-*O*-glucoside equivalents per g of dp, catechin equivalents (CEs) per g of d.e., and absorbance units for anthocyanins, total tannins, and polymeric pigments, respectively.

2.7. Determination of Maillard reaction products (MRPs) by absorbance measurement

Maillard reaction products (melanoidins) were estimated by means of browning intensity of the grape pomace extracts. These extracts were filtered and the browning intensity was directly measured at 360 and 420 nm (Plaza et al., 2010).

2.8. Determination of phenolic compounds and hydroxymethylfurfural content

Phenolic compounds ((+)-catechin, (−)-epicatechin, kaempferol and myricetin) and hydroxymethylfurfural (5-HMF) were measured on an HPLC system consisting of a 1024 photodiode-array detector model L-2455, a high throughput analysis pump, model L-2130, a column oven model L-2350 and an autosampler model L-2200 (Hitachi LaChrom Elite, Japan). The extracts were re-suspended in a methanol/water (50/50) solution at 20 mg/ml. After injecting 30 μl of sample, the separation was performed using a reverse phase Nova-Pack C18 column (300 mm × 3.9 mm i.d. 4 μm) at 20 °C (Waters Corp.). Two mobile phases were employed

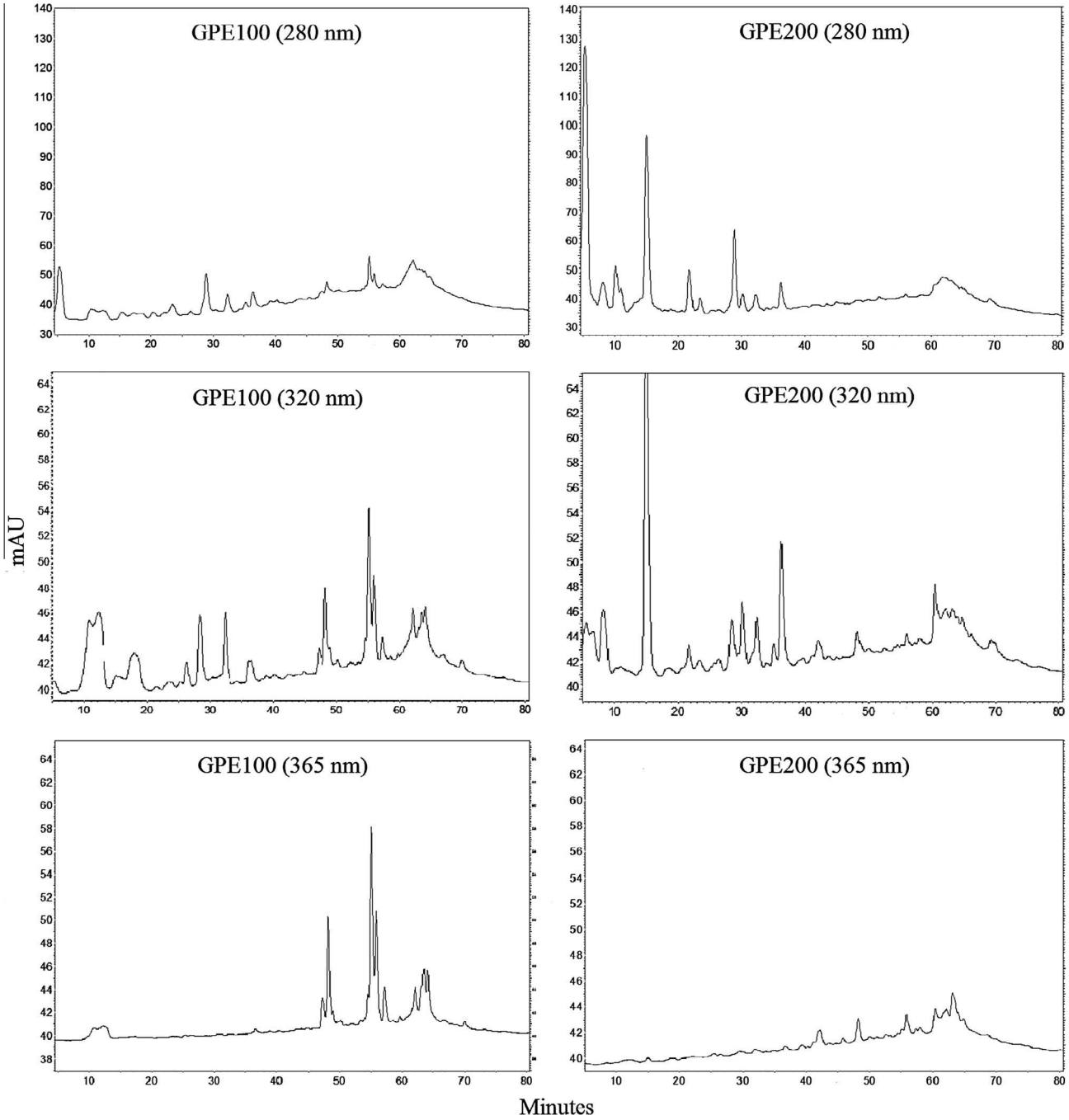

Fig. 1. The chromatograms of the extracts, GPE100 (left) and GPE200 (right), at different wavelengths evidence the differences in their composition due to the different extraction conditions.

for elution: A (water/acetic acid, 98:2, v/v) and B (water/acetonitrile/acetic acid, 78:20:2, v/v/v). The gradient profile was 0–55 min, 100–20% A and 0–80% B; 55–57 min, 20–10% A and 80–90% B; 57–90 min, 10% A and 90% B isocratic, followed by washing with methanol and re-equilibration of the column. The flow rate was 1.0 ml/min from 0 to 55 min and 1.2 ml/min from 55 to 90 min. Detection was performed by scanning from 220 to 600 nm with an acquisition speed of 1 s. Identification and quantification were carried out by comparison (spectrum and retention time) with the standard molecule.

2.9. Cell culture, growth conditions and treatments

Human promyelocytic leukaemia cells HL-60, were obtained from Sigma–Aldrich (Sigma, 98070106). The cells were grown in a RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 g/l of D-glucose and 2 mM L-glutamine. Cells were grown in a CO_2 incubator (Forma Scientific, USA) at 37 °C in an atmosphere of 5% CO_2 with 95% humidity. HL-60 cells were suspended at 2.5×10^5 cell/ml and cultured in 12-well plates (Orange Scientific). To assess antioxidative capability, HL-60 cells were incubated with 0.15 and 0.015 mg/ml of grape extract, using 0.1 mM H_2O_2 as positive control of oxidative environment. As control of antioxidative capability, HL-60 cells were treated with 0.15 mM trolox, using 0.1 mM H_2O_2.

2.10. Loss of mitochondrial membrane potential

A cell sample from a growth assay taken at 24 h was used to detect the loss of mitochondrial membrane potential ($\Delta\psi_m$). HL-60 cells were incubated with 10 μg/ml of JC-1 at 37 °C. Loss of $\Delta\psi_m$, was assayed by observing a shift in fluorescence emission from red (~590 nm) to green (~525 nm), using flow cytometry (Beckman Coulter Cytomics, FC 500) (Prince et al., 2008). HL-60 cells were treated with 100 nM valinomycin as a positive control of membrane potential loss (approximately 98%).

2.11. Statistical analyses

Extractions and analyses were performed in triplicate with the data presented as mean values ± SD. Statgraphics® Plus for Windows, version 4.0 (StatPoint Technologies, Inc., Herndon, VA), was used for statistical analyses. Tests were applied to the response variables with p-values $\leqslant 0.05$, which were considered to be indicative of statistically significant differences between comparison groups; these tests allow us to study the effects of extraction temperature, extract composition and activity, factorial variance analysis and least significant difference.

3. Results and discussion

3.1. Characterization of the extracts

3.1.1. General

The extracts were characterized in terms of antioxidant activity (AA), total antioxidant content (TAC), polyphenol content, and content of Maillard reaction products (MRPs) and 5-HMF in particular, in an attempt to relate their composition to their protective antioxidant bioactivity in HL-60 cell culture.

3.1.2. Antioxidant activity and total antioxidants content

The grape pomace extracts obtained at 100 °C (GPE100) and at 200 °C (GPE200) showed significant differences in their AA and TAC. GPE200 had 50% more AA and 30% more TAC than had GPE100. These results are in agreement with the findings of similar research in which the TAC of the extracts and their AA increased with the extraction temperature (Plaza et al., 2010).

In the PWHE of plant materials, temperature has a positive effect on the extract's TAC and on its ability to reduce antioxidants; this is due to several chemical reactions that occur at high temperatures. The thermal degradation of cell wall facilitates the release of phenolic cell wall-associated compounds (Pérez-Jiménez & Torres, 2011), resulting in extracts with higher antiradical activity. Furthermore, it has been reported before that, in high temperature extraction processes, compounds with high antioxidant capacity (e.g. melanoidins) are formed from the Maillard reaction (Plaza et al., 2010).

3.1.3. Absorbance measurement of MRPs

The GPE200 had a significantly higher content of MRPs than did GPE100, based on the browning measurement at 360 and 420 nm (Table 1). These absorbance values are employed as an indicator of caramelization and formation of brown advanced MRPs in thermally processed foods. Our data show an increase in the formation of MRPs with the increase of the extraction temperature in the PHWE of grape pomace. The formation of MRPs during the PHWE of several plant samples has also been reported by other researchers (He et al., 2012; Plaza et al., 2010). The MRPs include a wide range of compounds of significant importance for the nutritional value of food and beverages. Some of these compounds present strong antioxidant activities but others, such as hydroxymethylfurfural (5-HMF), could be toxic and mutagenic (Husøy et al., 2008).

3.1.4. 5-HMF content

5-HMF was detected only in GPE200 with a concentration of 0.087 mg per g of extract (Table 1). The formation of 5-HMF is favoured at high temperatures and at pH 7 or below (Martins, Jongen, & van Boekel, 2000). The increase in the extraction temperature reduces the pH of the extracts due to a decrease in the amino groups available as Maillard reactions progress (Liu, Yang, Jin, Hsu, & Chen, 2008). Plaza et al. (2010) reported pH values between 5 and 7 in pressurized hot water extracts from several plant samples.

3.1.5. Polyphenols content

The increase of the extraction temperature significantly alters the polyphenolic composition of the grape pomace extracts

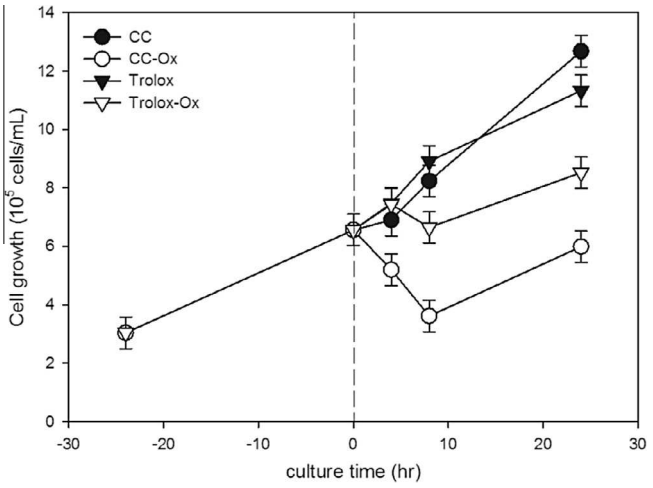

Fig. 2. Evaluation of protective effect of trolox on HL-60 cell growth (concentration of viable cells, Xv) under oxidative conditions. Control culture (CC) under optimal growth conditions and culture under oxidative conditions, 0.1 mM H_2O_2 (Ox), are used as reference curves to evaluate the protective effect of trolox. Trolox: culture with 0.15 mM trolox, and trolox-Ox: culture with 0.15 mM trolox and 0.1 mM H_2O_2. Bars represent the upper and lower limits of the 95% confidence interval.

(Fig. 1). The GPE200 extract, measured at 280 and 320 nm, shows peaks not present in the GPE100 extract, which may correspond to products of the thermal degradation of polyphenols. This degradation is evident in the reduction of the GPE200 chromatogram peaks at 365 nm.

Anthocyanins, condensed tannins and total polyphenol contents in GPE100 were significantly higher than those in GPE200 (Table 1). Moreover, the contents of (+)-catechin, kaempferol and myricetin are higher in GPE100, whereas its (−)-epicatechin content was slightly lower.

Temperature has a strong influence on polyphenol stability, especially on anthocyanins. In water, at temperatures of 100 °C and above, simple flavonoids are degraded and the formation of derived antioxidant compounds is favoured. Previous studies have reported that PHWE temperatures above 110 °C decrease individual and total anthocyanin contents in grape skin extracts (Ju & Howard, 2005). Antioxidant compounds, such as chalcone glycosides, phenolic acids, and phloroglucinaldehyde, are the major anthocyanin thermal degradation products (Sadilova, Carle, & Stintzing, 2007). To our knowledge, there are few studies of tannin stability at temperatures above 100 °C. However, Gaugler and Grigsby (2009) reported that the onset temperature of degradation of these polyphenols is approximately 150 °C, and it is dependent on factors such as the degree of acetylation and content of carbohydrates in the extract. The breakdown of tannins at 200 °C (with the consequent release of subunits) could be the reason for the higher (−)-epicatechin content in GPE200.

3.2. Bioactivity of the grape pomace extracts

The protective antioxidant bioactivities of the GPE100 and GPE200 extracts were assessed in HL-60 cell culture under oxidative conditions conferred by the presence of an oxidant (H_2O_2). Their activity was compared with the protective activity of trolox (the reference antioxidant compound) at 24 h of cell growth. Both extracts exhibited potent protective activities on HL-60 cell growth and mitochondrial membrane potential ($\Delta\psi_m$), comparable to the activity of trolox, but also showed cytotoxic activity.

In the presence of the oxidant, trolox and both extracts, at the highest concentration assessed (0.15 mg/ml), exhibited protective

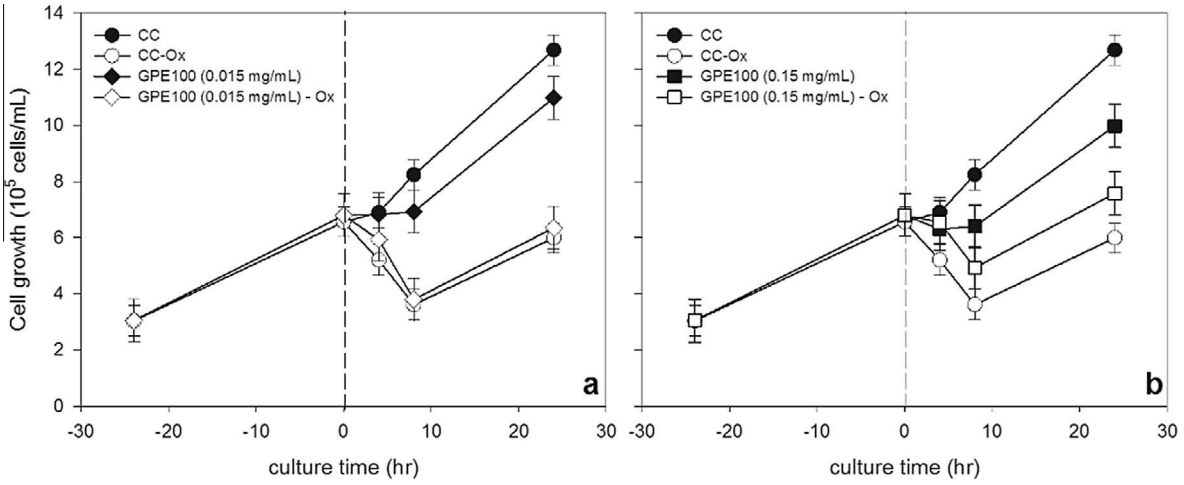

Fig. 3. Evaluation of protective effect of GPE100 at two concentrations on HL-60 cell growth under oxidative conditions compared with the protective effect of trolox. Control culture (CC) in optimal growth conditions and culture under oxidative conditions, 0.1 mM H_2O_2 (Ox), are used as reference curves to evaluate the protective effect of the extract. (a) HL-60 cell culture treated with GPE100 at 0.015 mg/ml and GPE100 at 0.015 mg/ml plus 0.1 mM H_2O_2 (Ox). (b) HL-60 cell culture treated with GPE100 at 0.15 mg/ml and GPE100 at 0.15 mg/ml plus 0.1 mM H_2O_2 (Ox). Bars represent the upper and lower limits of the 95% confidence interval.

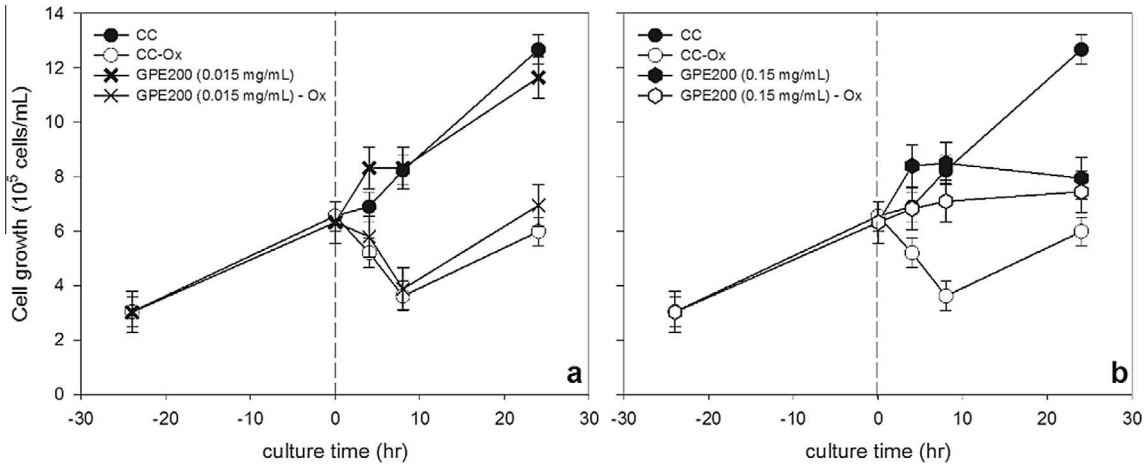

Fig. 4. Evaluation of protective effect of GPE200 at two concentrations on HL-60 cell growth under oxidative conditions compared with the protective effect of trolox. Control culture (CC) under optimal growth conditions and culture under oxidative conditions, 0.1 mM H_2O_2 (Ox), are used as reference curves to evaluate the protective effect of the extract. (a) HL-60 cell culture treated with GPE200 at 0.015 mg/ml and GPE200 at 0.015 mg/ml plus 0.1 mM H_2O_2 (Ox). (b) HL-60 cell culture treated with GPE200 at 0.15 mg/ml and GPE200 at 0.15 mg/ml plus 0.1 mM H_2O_2 (Ox). Bars represent the upper and lower limits of the 95% confidence interval.

antioxidant activity on cell growth of HL-60. Trolox showed the highest protective effect, increasing the cell growth by 42%, compared to the control culture with the oxidant (Fig. 2). Likewise, GPE100 and GPE200 increased the cell growth by 27% and 24%, respectively (Figs. 3 and 4). Although the two crude extracts had different compositions, they showed similar protective effects. The level of this protective activity was close to that exhibited by trolox, despite the low purity of the grape polyphenolic extracts. At the lowest concentration (0.015 mg/ml), no effect on HL-60 growth was observed.

The positive effect of the extracts on the $\Delta\psi_m$ was observed in both the absence and the presence of the oxidant (Fig. 5). In the absence of the oxidant, GPE100 and GPE200 showed a protective effect at both concentrations, decreasing the $\Delta\psi_m$ loss to lower values than that of the control culture, especially at the highest concentration. trolox showed no effect on $\Delta\psi_m$ in the absence of the oxidant. In the presence of the oxidant, the HL-60 cells showed a $\Delta\psi_m$ loss three times higher than that of the control culture. This negative effect of the oxidant was strongly inhibited by both GPE100 and GPE200 at 0.15 mg/ml, reaching values of $\Delta\psi_m$ loss of 1% and 5%, respectively, which are lower than those of trolox and the control culture. At 0.015 mg/ml, no effect on $\Delta\psi_m$ of HL-60 was observed.

GPE100 had higher content of polyphenols, especially anthocyanins and condensed tannins, than had GPE200, which may explain its greater protective effect against oxidation. The lipid peroxidation induced by oxidative stress causes the loss of $\Delta\psi_m$ and the release of cytochrome c into the cytosol, triggering the caspase cascade leading to apoptotic cell death in HL-60 cells (Anuradha, Kanno, & Hirano, 2001). Some phenolic compounds prevent the decrease in mitochondrial membrane potential and suppress the accumulation of ROS (Guo, Bezard, & Zhao, 2005). Condensed tannins or proanthocyanidins from grape have shown a protective effect on mitochondrial membrane reducing and restoring the $\Delta\psi_m$ altered by oxidative stress (Li, Jang, Sun, & Surh, 2004). Also, melanoidins have antioxidant activity at a concentration of 2500 mg/ml, protecting the DNA from damage caused by ROS in HL-60 (Miwa, Watanabe, Kawasumi, & Hayase, 2002).

Additionally, the grape extracts (also trolox) showed no negative effect on the $\Delta\psi_m$ in the absence of the oxidant (Fig. 5), even

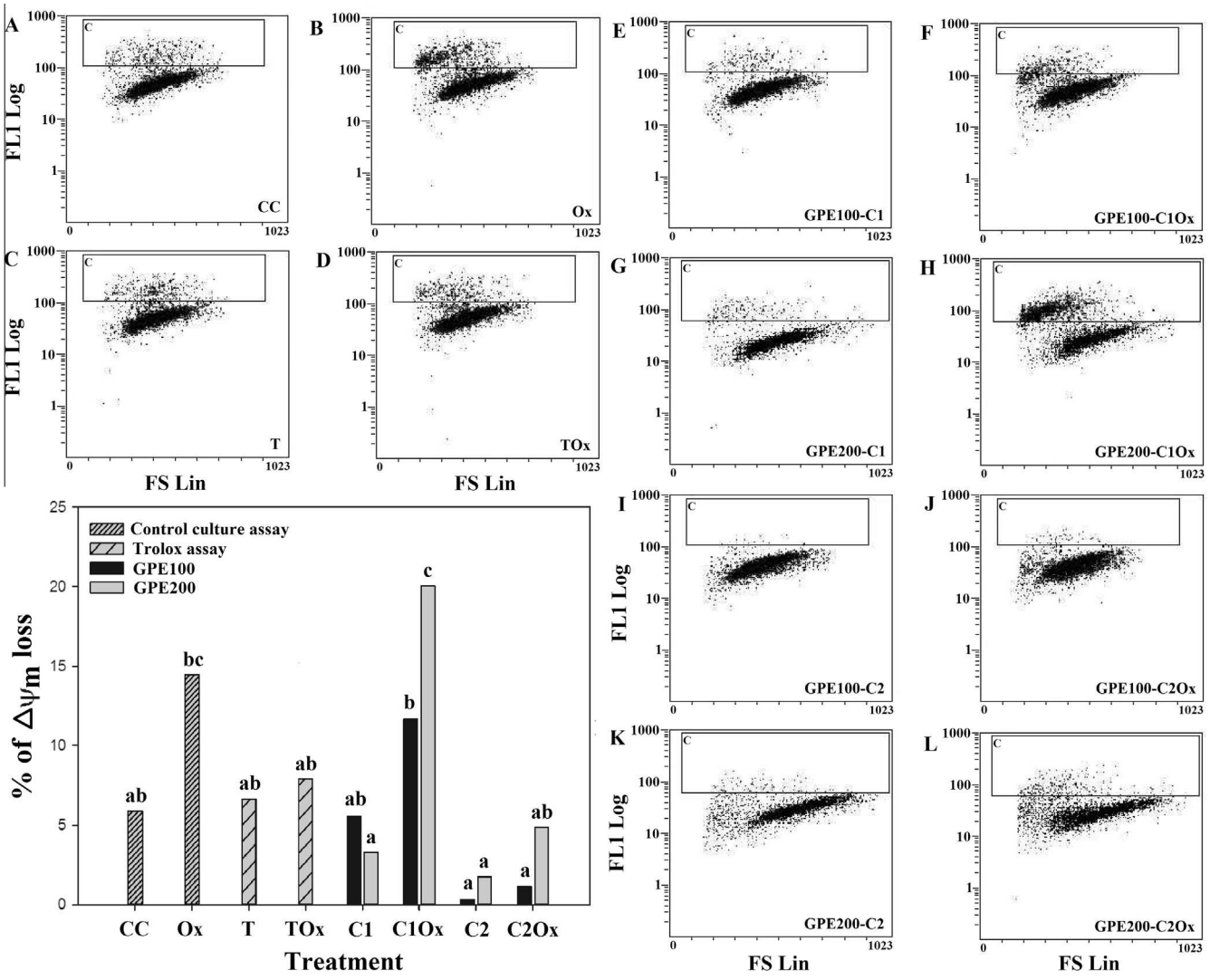

Fig. 5. Evaluation of protective effect of GPE100 and GPE200 at two concentrations on mitochondrial membrane potential ($\Delta\psi_m$) of HL-60 cells under oxidative conditions compared with the protective effect of trolox. $\Delta\psi_m$ was determined by fluorescent dye JC-1 and measured by flow cytometry. Representative dot plots show the distribution of stained cells after the different treatments at 24 h. (A) Control culture (CC) under optimal growth conditions and culture under oxidative conditions, (B) 0.1 mM H_2O_2 (Ox), are used as references to evaluate the protective effect of trolox and extracts. (C) T: culture with 0.15 mM trolox, (D) TOx: culture with 0.15 mM trolox and 0.1 mM H_2O_2, C1: culture with GPE100 (E) or GPE200 (G) at 0.015 mg/ml, C1Ox: culture with GPE100 (F) or GPE200 (H) at 0.015 mg/ml and 0.1 mM H_2O_2, C2: culture with GPE100 (I) or GPE200 (K) at 0.15 mg/ml, C2Ox: culture with GPE100 (J) or GPE200 (L) at 0.15 mg/ml and 0.1 mM H_2O_2. The bar chart shows the percentage of $\Delta\psi_m$ loss after different treatments. Bars with the same letter showed no statistically significant difference at the confidence interval of 95%.

though the oxidized forms of polyphenols may cause mitochondrial toxicity by collapsing the $\Delta\psi_m$. However, this negative effect is highly dependent on the concentration. For example, Wang, Lin-Shiau, and Lin (1999) reported that increasing concentrations of apigenin, quercetin, myricetin, and kaempferol increase the loss of $\Delta\psi_m$, the ROS production, the release of mitochondrial cytochrome c into the cytosol and the caspase activity. Therefore, the concentrations assessed in this work produced no negative effect on the $\Delta\psi_m$.

Conversely, trolox, GPE200 and GPE100 showed cytotoxic effect on the HL-60 cell growth in the absence of the oxidant. trolox decreased the cell growth by about 11% compared to the control culture (Fig. 2). At the lowest concentration (0.015 mg/ml), only a cytotoxic effect of GPE100 was observed, reducing the growth by about 13% (Fig. 3). At 0.15 mg/ml, GPE100 and GPE200 showed a significant negative effect on the cell growth; however, the strengths of their cytotoxic effect were different, GPE100 reduced the growth by 21% while GPE200 reduced the growth by 37% (Figs. 3 and 4). The higher cytotoxic effect exhibited by GPE200 may be due to its higher MRPs content and the presence of 5-HMF. It has been reported that MRPs show dose-dependent antiproliferative activity (above 100 μg/ml) in HL-60 cells (Usui, Shizuuchi, Watanabe, & Hayase, 2004).

Additionally, grape polyphenols have shown anticancer activity, inhibiting the growth of different cancer cell lines. Gao et al. (2009) demonstrated a strong dose-dependent cytotoxic effect of grape seed extract in HL-60, Jurkat and U937 cells at a concentration of 0.050 mg/ml and above. This result agrees with that observed in the present work, in which a high concentration of grape extracts (0.150 mg/ml) results in a strong negative effect on cell growth. Moreover, polyphenolic grape stem extracts inhibit (at low concentrations) the growth of HepG2 and HeLa, with activities comparable to those of grape seed extracts (Apostolou et al., 2013). The results presented in this article, along with previous work in the area, would suggest the use of these bioactive extracts and their principal bioactive compounds as cancer preventive agents.

Finally, these results illustrate the complex action of antioxidants in a biological system. Polyphenols and other antioxidants could present protective antioxidant or prooxidant/cytotoxic activities in cell cultures, depending on several factors, such as their concentration, their ability to oxidize, their lipophilicity, the content of other antioxidants and metals, and the oxidative stress level of the cell culture (Halliwell, 2008; Kitts et al., 2012).

4. Conclusions

Our results evidence the effects of extraction temperatures on the chemical composition, antioxidant activity and bioactivity of pressurized hot water extracts. High extraction temperature (200 °C) yield an extract (GPE200) with higher AA, higher MRPs and lower polyphenols content than does an extract obtained at 100 °C (GPE100). In addition, 5-HMF was only detected in the GPE200 extract. Under oxidative conditions, both extracts exhibited a high protective bioactivity on HL-60, similar to or higher than that exhibited by trolox. Under normal culture conditions, GPE200 exhibited higher cytotoxicity on HL-60 cancer cells than did GPE100, possibly due to its higher MRPs content.

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References

Anuradha, C. D., Kanno, S., & Hirano, S. (2001). Oxidative damage to mitochondria is a preliminary step to caspase-3 activation in fluoride-induced apoptosis in HL-60 cells. *Free Radical Biology and Medicine, 31*, 367–373.

Apostolou, A., Stagos, D., Galitsiou, E., Spyrou, A., Haroutounian, S., Portesis, N., et al. (2013). Assessment of polyphenolic content, antioxidant activity, protection against ROS-induced DNA damage and anticancer activity of Vitis vinifera stem extracts. *Food and Chemical Toxicology, 61*, 60–68.

Diebolt, M., Bucher, B., & Andriantsitohaina, R. (2001). Wine polyphenols decrease blood pressure, improve NO vasodilatation, and induce gene expression. *Hypertension, 38*, 159–165.

Djilas, S., Canadanovic-Brunet, J., & Cetkovic, G. (2009). By-products of fruits processing as a source of phytochemicals. *Chemical Industry and Chemical Engineering Quarterly, 15*, 191–202.

Gao, N., Budhraja, A., Cheng, S., Yao, H., Zhang, Z., & Shi, X. (2009). Induction of apoptosis in human leukemia cells by grape seed extract occurs via activation of c-Jun NH2-terminal kinase. *Clinical Cancer Research, 15*, 140–149.

Gaugler, M., & Grigsby, W. J. (2009). Thermal degradation of condensed tannins from radiata pine bark. *Journal of Wood Chemistry and Technology, 29*, 305–321.

Guo, S., Bezard, E., & Zhao, B. (2005). Protective effect of green tea polyphenols on the SH-SY5Y cells against 6-OHDA induced apoptosis through ROS–NO pathway. *Free Radical Biology and Medicine, 39*, 682–695.

Hagerman, A. E., & Butler, L. G. (1978). Protein precipitation method for the quantitative determination of tannins. *Journal of Agricultural and Food Chemistry, 26*, 809–812.

Halliwell, B. (2008). Are polyphenols antioxidants or pro-oxidants? What do we learn from cell culture and in vivo studies? *Archives of Biochemistry and Biophysics, 476*, 107–112.

Hawthorne, S. B., Miller, D. J., Lagadec, A. J.-M., Hammond, P. J., & Clifford, A. A. (2002). Method of manipulating the chemical properties of water to improve the effectiveness of a desired process. in: *United States Patent*, Vol. US 6,352,644 B1, Energy and Environmental Research Center Foundation. United States of America.

He, L., Zhang, X., Xu, H., Xu, C., Yuan, F., Knez, Ž., et al. (2012). Subcritical water extraction of phenolic compounds from pomegranate (*Punica granatum* L.) seed residues and investigation into their antioxidant activities with HPLC–ABTS+ assay. *Food and Bioproducts Processing, 90*, 215–223.

Husøy, T., Haugen, M., Murkovic, M., Jöbstl, D., Stølen, L. H., Bjellaas, T., et al. (2008). Dietary exposure to 5-hydroxymethylfurfural from Norwegian food and correlations with urine metabolites of short-term exposure. *Food and Chemical Toxicology, 46*, 3697–3702.

Ju, Z., & Howard, L. R. (2005). Subcritical water and sulfured water extraction of anthocyanins and other phenolics from dried red grape skin. *Journal of Food Science, 70*, S270–S276.

Kitts, D. D., Chen, X.-M., & Jing, H. (2012). Demonstration of antioxidant and anti-inflammatory bioactivities from sugar-amino acid Maillard reaction products. *Journal of Agricultural and Food Chemistry, 60*, 6718–6727.

Li, M.-H., Jang, J.-H., Sun, B., & Surh, Y.-J. (2004). Protective effects of oligomers of grape seed polyphenols against β-amyloid-induced oxidative cell death. *Annals of the New York Academy of Sciences, 1030*, 317–329.

Liu, R. H., & Finley, J. (2005). Potential cell culture models for antioxidant research. *Journal of Agricultural and Food Chemistry, 53*, 4311–4314.

Liu, S.-C., Yang, D.-J., Jin, S.-Y., Hsu, C.-H., & Chen, S.-L. (2008). Kinetics of color development, pH decreasing, and anti-oxidative activity reduction of Maillard reaction in galactose/glycine model systems. *Food Chemistry, 108*, 533–541.

Martins, S. I. F. S., Jongen, W. M. F., & van Boekel, M. A. J. S. (2000). A review of Maillard reaction in food and implications to kinetic modelling. *Trends in Food Science & Technology, 11*, 364–373.

Miwa, M., Watanabe, T., Kawasumi, T., & Hayase, F. (2002). Protective effects of melanoidins derived from soy sauce and soy paste on NO-induced DNA damage. *Food Science and Technology Research, 8*, 231–234.

Ong, E. S., Cheong, J. S. H., & Goh, D. (2006). Pressurized hot water extraction of bioactive or marker compounds in botanicals and medicinal plant materials. *Journal of Chromatography A, 1112*, 92–102.

Pérez-Jiménez, J., Neveu, V., Vos, F., & Scalbert, A. (2010). Identification of the 100 richest dietary sources of polyphenols: An application of the Phenol-Explorer database. *European Journal of Clinical Nutrition, 64*, S112–S120.

Pérez-Jiménez, J., & Torres, J. L. (2011). Analysis of nonextractable phenolic compounds in foods: The current state of the art. *Journal of Agricultural and Food Chemistry, 59*, 12713–12724.

Plaza, M., Amigo-Benavent, M., Castillo, M. D. d, Ibáñez, E., & Herrero, M. (2010). Facts about the formation of new antioxidants in natural samples after subcritical water extraction. *Food Research International, 43*, 2341–2348.

Prince, L. R., Bianchi, S. M., Vaughan, K. M., Bewley, M. A., Marriott, H. M., Walmsley, S. R., et al. (2008). Subversion of a lysosomal pathway regulating neutrophil apoptosis by a major bacterial toxin, pyocyanin. *The Journal of Immunology, 180*, 3502–3511.

Pulido, R., Bravo, L., & Saura-Calixto, F. (2000). Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. *Journal of Agriculture and Food Chemistry, 48*, 3396–3402.

Sadilova, E., Carle, R., & Stintzing, F. C. (2007). Thermal degradation of anthocyanins and its impact on color and in vitro antioxidant capacity. *Molecular Nutrition & Food Research, 51*, 1461–1471.

Usui, T., Shizuuchi, S., Watanabe, H., & Hayase, F. (2004). Cytotoxicity and oxidative stress induced by the glyceraldehyde-related Maillard reaction products for HL-60 cells. *Bioscience, Biotechnology, and Biochemistry, 68*, 333–340.

Vergara-Salinas, J. R., Bulnes, P., Zúñiga, M. C., Pérez-Jiménez, J., Torres, J. L., Mateos-Martín, M. L., et al. (2013). Effect of pressurized hot water extraction on antioxidants from grape pomace before and after enological fermentation. *Journal of Agricultural and Food Chemistry, 61*, 6929–6936.

Verhaegen, S., McGowan, A. J., Brophy, A. R., Fernandes, R. S., & Cotter, T. G. (1995). Inhibition of apoptosis by antioxidants in the human HL-60 leukemia cell line. *Biochemical Pharmacology, 50*, 1021–1029.

Wang, I. K., Lin-Shiau, S. Y., & Lin, J. K. (1999). Induction of apoptosis by apigenin and related flavonoids through cytochrome c release and activation of caspase-9 and caspase-3 in leukaemia HL-60 cells. *European Journal of Cancer, 35*, 1517–1525.

Xia, E.-Q., Deng, G.-F., Guo, Y.-J., & Li, H.-B. (2010). Biological activities of polyphenols from grapes. *International Journal of Molecular Sciences, 11*, 622–646.