Wine pomace seasoning attenuates hyperglycaemia-induced endothelial dysfunction and oxidative damage in endothelial cells.

Short title: Wine pomace seasoning attenuates endothelial cell dysfunction and oxidative damage.

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This document is the Submitted Manuscript version of a Published Work that appeared in final form in Journal of Functional Foods. To access the final edited and published work see http://dx.doi.org/10.1016/j.jff.2016.02.001.”

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Abstract

Dietary antioxidants such as plant phenolic compounds have been proposed to prevent/improve endothelial function impairment observed in diabetes and hypertension. This study investigates the specific protective effects of a vegetal seasoning obtained from seed-free wine pomace against hyperglycaemia-induced oxidative damage and endothelial dysfunction in HUVECs EA.hy926. Non-cytotoxic doses of the bioactive compounds obtained following in vitro digestion of the seasoning were used to treat endothelial cells. Digested compounds, especially colonic bacterial metabolites, restored a more balanced redox environment, prevented lipid peroxidation and cell membrane damage, ameliorated protein oxidation, and improved the balance between endothelial reactive oxygen species and nitric oxide production in hyperglycaemic cells. Reduction of ACE activity and modulation of certain genes (HO-1 up-regulation; NOX4, eNOS and SOD2 down-regulation) were found as probable mechanisms implicated. These results support the potential beneficial effects of functional seasonings obtained from wine pomace in vascular complications associated with oxidative stress and endothelial dysfunction.

Keywords:
Colonic metabolites; Dietary antioxidants; Endothelial function; Hyperglycaemia; Redox signalling; Wine pomace

Abbreviations:
ABTS, 2,2′-azinobis 3-ethylbenzothiazoline-6-sulfonic acid; ACE, angiotensin I-converting enzyme; Ang, angiotensin; AREs, antioxidant responsive elements; BCF, potentially bioavailable samples obtained after simulated colonic fermentation; BGID, potentially bioavailable samples obtained after simulated gastrointestinal digestion; CGs, carbonyl groups; eNOS, endothelial nitric oxide synthase; FC, Folin-Ciocalteu; GAE, gallic acid equivalents; GL, glucose; GSH, glutathione reduced; GSSG, glutathione oxidized; HO, heme oxygenase; HUVECs, human umbilical vein endothelial cells; LDH, lactate dehydrogenase; MDA, malondialdehyde; mDP, mean degree of polymerization; NAD(P)H, β-nicotinamide adenine dinucleotide phosphate; NF-κB, nuclear factor-kappa B; NO, nitric oxide; NOX, NAD(P)H oxidase; Nrf2, nuclear factor-erythroid 2-related factor 2; Q-, QUENCHER; RAAS, renin-angiotensin aldosterone system; ROS, reactive oxygen species; SOD, superoxide dismutase; TE, Trolox equivalents.
1. Introduction

Oxidative stress plays a critical role in the pathogenesis and development of cardiovascular disorders, with a growing body of evidence indicating that impaired endothelial function is a common cause and/or consequence of diverse risk factors relating to cardiovascular disease such as hypertension, diabetes mellitus, and hypercholesterolemia (Versari, Daghini, Virdis, Ghiadoni, & Taddei, 2009). An increased production of reactive oxygen species (ROS) and the subsequent decrease in vascular bioavailability of nitric oxide (NO) have long been proposed as one of many complex and multifactorial causes that contribute to endothelial dysfunction (Higashi, Noma, Yoshizumi, & Kihara, 2009).

The most relevant sources of ROS in vascular cells, mainly superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$), are NAD(P)H oxidase (NOX), uncoupled endothelial NO synthase (eNOS), xanthine oxidase, and the enzymes of the mitochondrial electron transport chain (Pennathur & Heinecke, 2007). The rapid reaction of O$_2^-$ with NO leads to an excessive formation of peroxynitrite (ONOO$^-$), a reactive nitrogen specie that may act as a vasoconstrictor, and most importantly, as a cytotoxic molecule, causing direct structural damage to proteins, lipids, and DNA, as well as further ROS generation (Münzel, Gori, Bruno, & Taddei, 2010). A reduction in NO production has also been described as one of the mechanisms leading to the lower bioavailability of NO observed in the pathogenesis of several vascular complications. The main causes reported to underlie this decreased formation of NO are reduced eNOS mRNA levels and/or stability, and decreased synthesis, activation, and/or activity of eNOS enzymes (Schmitt & Dirsch, 2009; Versari et al., 2009).

Another factor implicated in the development of oxidative stress and injury in the vascular system is the over-activation of the renin-angiotensin aldosterone system (RAAS). Angiotensin I-converting enzyme (ACE) is crucial in the regulation of RAAS, as it cleaves angiotensin I (Ang I) to produce the potent vasoconstrictor angiotensin II (Ang II) (Hsueh & Wyne, 2011; McFarlane, Kumar, & Sowers, 2003). The major mechanisms by which Ang II causes vascular damage include ROS generation and the stimulation of redox-dependent signalling pathways in endothelial and other vascular cells (Virdis, Duranti, & Taddei, 2011). Therefore, RAAS is also involved in the control of NO bioavailability in vasculature. However, the inhibition of ACE activity has been suggested as a promising therapeutic approach for the prevention and treatment of several cardiovascular diseases and associated complications (Balasuriya & Rupasinghe, 2011; McFarlane et al., 2003).
Living organisms have developed several effective mechanisms to protect themselves from the detrimental effects of ROS. The endogenous antioxidant-defence system includes enzymes such as superoxide dismutases (SOD), catalase, glutathione peroxidases, heme oxygenases (HO), and others, as well as non-enzymatic antioxidants such as glutathione (GSH) (Lee, Margaritis, Channon, & Antoniades, 2012). In addition, several exogenous interventions using antioxidant dietary compounds have been proposed to prevent endothelial dysfunction and to restore the redox status in the vascular microenvironment, with certain phenolic compounds being highlighted among the most promising natural antioxidants with beneficial cardiovascular effects (Stoclet et al., 2004). In this regard, a number of studies have been initiated dealing with the antioxidant, hypotensive, and vascular health-promoting effects of (poly)phenols in grapes, wine and wine pomace. The antioxidant capacity of these phytochemicals is not simply related to their direct ROS scavenging ability, but also to the inhibition of enzymatic sources of oxidative stress, such as NOX and ACE, as well as the stimulation of endogenous antioxidant enzymes (Eriz, Sanhueza, Roeckel, & Fernández, 2011; Münzel et al., 2010; Nijveldt et al., 2001).

However, one of the principal topics concerning the beneficial effects of phenolic compounds is their bioavailability and metabolic fate, which are dependent upon their digestive stability, their bioaccessibility (release from the food matrix), the efficiency of their trans-epithelial passage, and their further metabolism in enteric and hepatic tissue, as well as by gut microbiota (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004; Scalbert & Williamson, 2000a; Tagliazucchi, Verzelloni, Bertolini, & Conte, 2010). Therefore, despite the limitations of simulated digestion systems (Alminger et al., 2014), the combination of in vitro digestion protocols with cell assays has recently been proposed as a suitable approach to determine the antioxidant activity of (poly)phenols once they have been ingested (Huang, Sun, Lou, & Ye, 2014).

Hyperglycaemia-induced oxidative stress is one cause of the vascular complications that characterize diabetes (Fatehi-Hassanabad, Chan, & Furman, 2010; Hadi & Suwaidi, 2007). As such, several studies have investigated endothelial function impairment and associated pro-oxidative environment ex vivo, by using vascular cells such as human umbilical vein endothelial cells (HUVECs) grown under high glucose concentrations (Inoguchi et al., 2000; Koziel, Woyda-Ploszczyca, Kicinska, & Jarmuszkiewicz, 2012; Patel, Chen, Das, & Kavdia, 2013; Zhou, Xie, Zhou, & Li, 2012).

Recently, new powdered seasonings obtained from wine pomace have been developed (García-Lomillo, González-Sanjose, Del Pino-García, Rivero-Pérez, & Muñiz, 2014). These (poly)phenol-rich products have demonstrated significant antioxidant properties in vitro (Del Pino-García, García-Lomillo, Rivero-Pérez,
González-Sanjosé, & Muñiz, 2015), so their use as additives or ingredients in functional foods could enhance exogenous antioxidant dietary pool and contribute to maintaining/restoring a healthy internal redox status. This paper is focused on the potential protective effects against endothelial dysfunction and oxidative damage in the vasculature of vegetal products proposed as functional food ingredients. Its major aim was to evaluate the specific effects of a red wine pomace-derived seasoning under hyperglycaemia-induced oxidative stress in the endothelial cell line HUVEC EA.hy926. The cell treatments used to achieve this objective consisted of those compounds released after a simulated gastrointestinal digestion and colonic fermentation of the powdered seasoning under study.

2. Materials and methods

2.1. Chemicals

2,2'-Azinobis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS), porcine bile extract, 2',7'-dichlorofluorescein diacetate (DCFH-DA), DMEM containing either 5.6 mM (Low GL) and 25 mM D-Glucose (High GL), foetal bovine serum (FBS), gallic acid, 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), L-glutamine solution (200 mM), 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), magnesium chloride hexahydrate (MgCl$_2$.6H$_2$O), N-hippuryl-L-histidyl-L-leucine (HHL) tetrahydrate, β-nicotinamide adenine dinucleotide reduced (NADH) disodium salt hydrate, porcine pancreas pancreatin, 10,000 U/mL penicillin and 100 mg/mL streptomycin solution (P/S), 85% (v/v) phosphoric acid solution (H$_3$PO$_4$), sodium pyruvate, 1,1,3,3-tetramethoxypropane (TMP), 2-thiobarbituric acid (TBA), thiazolyl blue tetrazolium bromide (MTT), 2-vinylpyridine, cellulose membrane dialysis tubing (12,000 Da molecular weight cut-off), and the enzymes used in the simulated gastrointestinal digestion (α-amylase (EC 3.2.1.1), amyloglucosidase (EC 3.2.1.3), lipase (EC 3.1.1.3), and pepsin (E.C 3.4.23.1)) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Folin-Ciocalteu (FC) reagent, 70% (v/v) and perchloric acid (PCA) were purchased from Panreac Quimica S.L.U. (Barcelona, Spain). DMSO and PBS tablets were purchased from Merck Millipore Co. (Darmstadt, Germany).

2.2. Seasoning obtained from wine pomace

The powdered seasoning examined in this study was made at the University of Burgos according to a previously described method (González-Sanjosé, García-Lomillo, Del Pino-García, Muñiz-Rodriguez, &
Rivero-Pérez, 2013). This product was obtained from seed-free red wine pomace using a heat treatment as a stabilization process, and its main characteristics and composition (dietary fibre, fat, protein, minerals and phenolic classes) have previously been reported (García-Lomillo et al., 2014).

2.3. *In vitro* gastrointestinal digestion and colonic fermentation of the seasoning

The wine pomace seasoning underwent *in vitro* digestion following the method described by Saura-Calixto et al. (2010). This procedure mainly comprised two sequential steps that simulate conditions along the gut: a) enzymatic gastrointestinal digestion; and b) colonic microbial fermentation. Both soluble fractions obtained after these steps were further subjected to a controlled dialysis, chosen to model the mechanical aspects of the epithelial barrier (Fernández & Labra, 2013). Briefly, 7.5 mL of each fraction were transferred into cellulose membrane dialysis tubing and dialyzed against a total of 50 mL of Milli-Q water for 4 h (changing the water twice). The solutions extracted from the dialysis tubing were collected, lyophilized, weighed, and stored at -20 ºC. These samples containing possibly bioavailable compounds present in each fraction were labelled as "BGID" (potentially bioavailable samples after gastrointestinal digestion) and "BCF" (potentially bioavailable samples after colonic fermentation). Three replicates were carried out for each sample. Negative digested controls (without seasoning) for both types of samples were also prepared.

2.4. Analysis of BGID and BCF treatments

2.4.1. Total antioxidant capacity

The *in vitro* antioxidant activity of each treatment was directly evaluated in the lyophilized powdered samples using QUENCHER (Q-) methods (Del Pino-García et al., 2015). The antioxidant capacities assessed by Q-Folin-Ciocalteu (Q-FC) assay and by Q-ABTS assay were expressed as μmol of gallic acid equivalents per gram of product (GAE/g product), and as μmol of Trolox equivalents per gram of product (TE/g product), respectively.

2.4.2. *In vitro* determination of angiotensin I-converting enzyme (ACE) activity

ACE activity was measured using the method described by Cinq-Mars & Li-Chan (2007). In this assay, ACE activity is quantified by spectrophotometric absorbance (abs) at 228 nm produced by the liberation of
hippuric acid from the synthetic substrate HHL. As ACE activity is depressed in the presence of ACE inhibitors, percentage (%) ACE inhibition was calculated, as described in the following equation [1]:

\[
\% \text{ ACE inhibition} = 1 - \frac{(\text{abs} \text{sample} - \text{abs} \text{ negative control})}{(\text{abs} \text{ positive control} - \text{abs} \text{ negative control})} \times 100
\]  

[1]

where, the positive control (treatments replaced by the buffer) was taken as 100% ACE activity, and the negative control (ACE added only after reaction termination) was taken as 0% ACE activity.

2.5. Cell culture and treatment

The immortalized cell line HUVEC EA.hy926 was kindly provided by Dr. Diana Hernández-Romero from the research group "Arterial thrombosis and interstitial, vascular and myocardial remodelling" (IMIB-Arrixaca/University of Murcia, Murcia, Spain). Cells were maintained in DMEM Low GL supplemented with 10% FBS, 1% P/S, and 1% L-glutamine solution at 37 \(^\circ\)C in a humidified incubator with 5% CO\(_2\). The medium during the treatment incubation period was DMEM Low or High GL supplemented with 1% FBS, 1% P/S, 1% L-glutamine solution, and 0.1% DMSO. HUVECs EA.hy926 for experiments were taken from exponential phase cultures at passages 4–8 and seeded at a 2 \times 10^4 cells/cm\(^2\) density in all assays. Cells were grown for 24 h under basal conditions. Then, the treated-cells were exposed for 24 h to either the BGID or the BCF treatments (0.5 mg of product/mL as a final concentration) or the respective negative digested control treatments. Non-treated control cells were only incubated with Low GL (normoglycaemic control) and High GL (hyperglycaemic control) mediums. All experiments were carried out as three independent assays. Those assays to determine cell viability, intracellular ROS, and NO production were performed in 96-well cell culture multiplates and the remaining experiments in 25 cm\(^2\) cell culture flasks. After the treatment period, the cells were scraped and centrifuged (1,500 g, 5 min, 25 \(^\circ\)C). Supernatant was collected and frozen at -80 \(^\circ\)C for further analyses. The cell pellets were resuspended in 1 mL of PBS and used in the analyses, as explained below.

2.6. Cell viability assessment

Cell viability was analysed using the MTT method (Twentyman & Luscombe, 1987). Absorbance at 550 nm was measured in a PowerWave XS2 microplate spectrophotometer (BioTek Instruments Inc., Vermont, USA). The results were expressed as % cell viability with respect to control cells grown in the Low GL medium.
2.7. Glutathione reduced/oxidized (GSH/GSSG) ratio analysis

Aliquots of the HUVECs EA.hy926 suspensions collected after the treatment period were immediately acidified with PCA (2% final concentration), centrifuged (6,500 g, 5 min, 4 ºC), and the supernatants were frozen at -80 ºC until their use as samples. GSH and GSSG levels in these samples were determined using the Cayman's GSH assay kit. This kinetic spectrophotometric assay measures total GSH (reduced+oxidized). Quantification of GSSG was performed following GSH derivatisation with 2-vinylpyridine, and GSH was estimated by subtracting GSSG from total GSH. All these assays followed the manufacturer's instructions. The results were finally expressed as a GSH/GSSG ratio.

2.8. Malondialdehyde (MDA) determination

Lipid peroxidation was estimated from the MDA levels according to the method described by Grotto et al. (2007), with slight modifications to perform the assay in cell homogenates obtained by sonication of the HUVEC EA.hy926 suspensions collected after the treatment period. Briefly, a volume of 75 μL of cell homogenate was added to 25 μL of Milli-Q water and 25 μL of 3 M NaOH and incubated at 60 ºC for 30 min in a shaking water bath system. After this, 125 μL of 6 % (v/v) H₃PO₄ and 125 μL of 0.8% (w/v) TBA were added and the mixture was heated at 90 ºC for 45 min. Then, the mixture was cooled and extracted with 300 μL of n-butanol by vortex-mixing for 1 min and centrifugation at 3,000 g for 10 min. The butanol layer was collected and a volume of 20 μL was injected into an Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA) equipped with a diode array detector. The column was a Spherisorb3® ODS2 reversed phase C18 (250 mm x 4.6 mm, 3 μm particle size; Waters Cromatografia S.A., Barcelona, Spain). The mobile phase was a mixture of Milli-Q water:methanol (1:1, v/v). The flow rate was maintained isocratically at 0.6 ml/min, the absorbance at 532 nm of the eluent was monitored, and the total run time was 8 min. Concentrations of MDA were calculated from calibration curves obtained using TMP as the standard. MDA levels were normalized using each cell homogenate total protein concentration, which was determined according to Bradford (1976). The results were finally expressed as nmol MDA/mg of protein.

2.9. Carbonyl groups (CGs) determination

Changes in cell proteins due to oxidative stress were determined in sonicated cell lysates using the spectrophotometric method described by Levine et al. (1990) to quantify protein CGs. The CG levels were
normalized by the protein content of each cell homogenate, expressing the final results as nmol CGs/mg of protein.

2.10. Lactate dehydrogenase (LDH) leakage assessment

The LDH activity was measured in the extra- and intracellular medium using the method reported by Vassault (1983), with some modifications. Briefly, a reaction mixture containing 100 mM potassium phosphate buffer, 0.7 mM sodium pyruvate, and 0.48 mM NADH was freshly prepared. A volume of 1.25 mL of the reaction mixture was transferred to a quartz cuvette and mixed with 50 µL of the extra- or intracellular medium. The absorbance decay at 340 nm was monitored for 2 min every 15 s. The units of extra- and intracellular LDH activity (1 unit = 1 µmol NADH/min) were determined considering a molar absorption coefficient for NADH of 6,220 M⁻¹ cm⁻¹. Finally, cell membrane integrity was estimated from LDH activity, in terms of % LDH release to the extracellular medium with respect to total LDH (intracellular + extracellular).

2.11. Intracellular reactive oxygen species (ROS) measurement

Overall intracellular ROS production in HUVECs EA.hy926 was measured by the 2,7-dichlorofluorescein (DCF) assay (Wang & Joseph, 1999), with certain modifications. Following their treatment and incubation, the cells were washed with a colourless external medium containing 145 mM NaCl, 5 mM KCl, 1 mM MgCl₂.6H₂O, and 10 mM HEPES. They were then added 100 µL of 20 µM DHCF-DA solution in external medium and placed in the chamber reader of a Cary Eclipse Fluorescence Spectrophotometer (Varian Australia Pty. Ltd., Victoria, Australia) with a microplate reader accessory to take the initial fluorescence reading (t₀) using 480 nm excitation/530 nm emission filters. Fluorescence emission was also recorded after incubation in the dark at 37 °C for 30 min (t₃₀) and the variations in relative fluorescence units (t₃₀ - t₀) were calculated for each sample. The MTT assay plates were run in parallel and the % cell viability results were used to normalize the recorded increase in fluorescence intensity, thereby taking into account the amount of viable cells per well. ROS levels were then estimated as DCF fluorescence increases and the final results were expressed as folds of the control cells grown in the Low GL medium.

2.12. Extracellular nitric oxide (NO) determination
The quantification of NO breakdown products, nitrite (NO$_2^{-}$) and nitrate (NO$_3^{-}$), in the culture medium was used as an indirect method to assess the cellular production of NO. Total nitrite and nitrate (NO$_2^{-}$+NO$_3^{-}$) was determined using the Nitrate/Nitrite Colorimetric Assay kit (Cayman Chemical, Michigan, USA) according to the supplier's instructions. The % viability determined by the MTT assays run in parallel was used to normalize cellular production of NO, which was finally expressed as μM NO$_2^{-}$+NO$_3^{-}$.

2.13. Quantitative real-time PCR (qPCR) analysis

Total RNA was isolated from HUVECs Ea.hy926 suspensions using TRI Reagent solution (Applied Biosystems). After treatment with DNase I (Thermo Scientific™), 1 μg of total RNA was reverse-transcribed using a First Strand cDNA Synthesis kit (Thermo Scientific™), and finally amplified using iQ™ SYBR®Green Supermix (Bio-Rad). All the procedures were performed according to the manufacturers' protocols. Primers were designed using Primer3 v.0.4.0 software (Untergasser et al., 2012) and synthesized by Metabion (Metabion International AG, Steinkirchen, Germany). The sequences of primer sets (forward and reverse) were: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-

GCTCTCCAGAACATCATCCC-3' and 5'-GTCCACCACGTGACACGT-3'; NOX4, 5'-

GGAAGAGCCCAGATTCCAG-3' and 5'-AGTCTTTCCGACAGAATG-3'; eNOS, 5'-

GAAGCTGCCAGGTGGCCCTTT-3' and 5'-CGGTTGGTGACATGCT-3'; SOD2, 5'-

GGAACGGGGAGACTTACAAA-3' and 5'-ACTGAAGGTAGTAAGGAGC-3'; HO-1, 5'-

GCCAGCAACAAAGTGCAAG-3' and 5'-AAAGCTGAGTGTAAGGACC-3'. qPCR was carried out with an iCycler iQ Real-Time PCR Detection System (Bio-Rad) under the following conditions: 1 cycle of initial denaturing and enzyme activation at 95 ºC for 3 min; 45 cycles of denaturing at 95 °C for 15 s and annealing/extension at 60 ºC for 30 s. The proper product amplification was verified by melting curve analysis. Amplification efficiencies were calculated for each pair of primers and quantification was performed using the efficiency-corrected ΔΔC$_t$ method, with GAPDH as the reference gene. Relative gene expression was finally expressed as folds of change compared to control cells grown in the Low GL medium.

2.14. Ex vivo determination of angiotensin I-converting enzyme (ACE) activity

After the treatment and incubation of HUVECs EA.hy926, the ACE activity in sonicated cell lysates was measured using the ACE substrate HHL following the spectrofluorometric method reported by Hermanns,
Müller, Tsokos, & Kirkpatrick (2014). ACE activity was considered proportional to fluorescence intensity and was normalized to the protein concentration of cell homogenates. The final results were expressed as % ACE activity compared to control cells grown in the Low GL medium.

2.15. Data presentation and statistical analysis

Data were expressed as means ± standard deviation of independent experiments (n=3). Statistical analysis was performed using Statgraphics® Centurion XVI, version 16.2.04 (Statpoint Technologies, Inc., Warranton, VA). One-way analysis of variance (ANOVA), using Fisher's least significant difference (LSD) test, was used to determine significant differences ($p < 0.05$) between data from cells incubated with the different treatments (control, BGID, and BCF) in the same medium (represented with Roman or Greek letters), and between the results of cells cultured in different mediums (Low and High GL) for each of the treatments (represented as % of change of hyperglycaemic with respect to normoglycaemic cells).

3. Results and discussion

Powdered seasonings obtained from winery by-products could be used by the food industry as innovative condiments and ingredients with antioxidant functional effects. Thus, several experiments were conducted to evaluate the properties of a red wine pomace-derived seasoning and its potential protective effects against oxidative stress and endothelial dysfunction induced by high glucose conditions in vascular cells. Considering the transformations that would take place in bioactive compounds following ingestion of the seasoning, a simulated gastrointestinal digestion and colonic fermentation of the seasoning was performed, obtaining two fractions that were dialyzed to emulate trans-epithelial passage and then used as cell treatments. These digestion products (BGID and BCF, respectively) were first characterized in vitro and then used ex vivo for treating HUVECs EA.hy926.

3.1. Analysis of the seasoning-derived digestion products

The digestion products evaluated in this study derived from a seed-free wine pomace seasoning that has been characterized in previous works. This product has a high content of dietary fibre (48.6 ± 0.7 %), and is a rich source of (poly)phenols (24.4 ± 0.1 mg GAE/g), especially proanthocyanidins and anthocyanins (García-Lomillo et al., 2014). More detailed data about its individual (poly)phenolic contents are provided as
Supplementary Table S1. In addition, the undigested seasoning presents high *in vitro* total antioxidant capacities (Q-FC index: 59.9 ± 1.5 µmol GAE/g; Q-ABTS values: 103 ± 2.7 µmol TE/g), which was determined directly in the powdered product, as previously described (Del Pino-García et al., 2015). In the present study, the antioxidant and ACE inhibitory activities the digestion products derived from such seasoning (BGID and BCF) were firstly assessed *in vitro*. The Q-FC index and Q-ABTS values for the BGID product were 48.0 ± 1.3 µmol GAE/g product and 153 ± 21 µmol TE/g product, respectively, whereas the results for the BCF product were 30.8 ± 8.7 µmol GAE/g product and 435 ± 48 µmol TE/g product. Therefore, both fractions showed an interesting but different *in vitro* total antioxidant capacity profile. Q-FC values were around 35% higher before the action of colonic microbiota than after it, but the ABTS$^+$ antiradical capacity of the metabolites that could be absorbed in the colon was around 3-fold higher than those antioxidants present in the BGID fraction. This antioxidant capacity shown by the BGID and the BCF products are certainly due to their (poly)phenolic composition. In this regard, it is well-known that proanthocyanidins from wine pomace are partly depolymerized into their flavan-3-ols monomers during transit along the digestive tract, and further transformed into free or conjugated microbial-derived phenolic metabolites in the colon (Touriño et al., 2011). Similarly, the extensive microbial catabolism of main flavonoids (anthocyanins and flavan-3-ols) and phenolic acids in red wine extracts appears to result in the formation of metabolites, such as phenylvaleric, phenylpropionic, phenylacetic and benzoic acid derivatives (Sánchez-Patán et al., 2012). Many of these metabolites still contain free hydroxyl groups and could contribute to enhance antioxidant capacity *in vivo* (Fernández-Panchón, Villano, Troncoso, & García-Parrilla, 2008; Pérez-Jiménez & Saura-Calixto, 2008; Scalbert & Williamson, 2000b).

Regarding the ability of the seasoning-derived compounds to act as ACE inhibitors *in vitro*, higher capacity was shown by the BGID (32.9 ± 0.9%) than the BCF (11.2 ± 4.5%) treatment. Several studies have confirmed the inhibitory power of certain polyphenols against ACE activity by competing with the substrate (Ang I) for the active sites of the enzyme (Actis-Gorettta, Ottaviani, Keen, & Fraga, 2003; Guerrero et al., 2012; Hidalgo et al., 2012). The ACE inhibitory capacity of flavonoid-rich foods appears to be especially related to their procyanidin and total flavan-3-ol content, and may depend on the mean degree of polymerization (mDP) of proanthocyanidins. An IC$_{50}$ in the 100 µM range for dimer and trimer fractions, and within the mM range for monomeric flavan-3-ols and other phenolic compounds have been described (Actis-Gorettta, Ottaviani, & Fraga, 2006). Recently, the effect of *in vitro* gastrointestinal digestion on the ACE inhibitory activity of a grape skin extract was studied (Fernández & Labra, 2013). These authors noted
that 80% of polymeric proanthocyanidins (mDP \( \sim 19 \)) were degraded during digestion in the small intestine to low-molecular-weight (mDP \( \sim 2 \)) oligomers, which crossed the dialysis membrane and efficiently inhibited around 60% of ACE activity. However, to the best of our knowledge, the present study has for the first time evaluated the ACE inhibitory power of fractions obtained after simulating the action of colonic microbiota. Structural changes of flavonoids can have an important impact on their potential as ACE inhibitors (Guerrero et al., 2012). Accordingly, the lower in vitro ACE inhibitory ability observed for the BCF rather than the BGID products may be due to the presence of simpler molecules in the BCF samples following depolymerisation and transformation of phenolic precursors by colonic microbiota (Aura, 2008). In fact, previous investigations using chemically synthesized anthocyanidin-3-O-glucosides and several of their putative gut breakdown metabolites have shown that standard compounds of phenolic acids exhibited lower ACE inhibitory capacity than their supposed parent molecules (Hidalgo et al., 2012).

3.2. Cell viability and biomarkers of oxidative stress and endothelial dysfunction

Reduced cell proliferation and viability have been reported after short-term (McGinn, Saad, Poronnik, & Pollock, 2003) and long-term (Varma et al., 2005) exposure of HUVECs to high glucose concentrations, independently of osmotic changes. Likewise, around 15% lower cell viability was observed in the current 24-hour study for hyperglycaemic than normoglycaemic cells (Fig. 1). After the incubation of HUVECs EA.hy926 with the seasoning-derived digestion products, in both Low and High GL mediums, the number of viable cells in comparison with control cells was about 85% (BGID) and 90% (BCF). Therefore, non-cytotoxic doses of the treatments were used, as previously considered for concentrations of phenolic compounds resulting in higher than 80% cell viability (Margina, Gradinaru, Manda, Neagoe, & Ilie, 2013; Müller et al., 2009; Stagos et al., 2014).

The state of oxidative stress in vivo is governed by the intricate interplay between enzymatic pathways accountable for ROS production and the various endogenous or exogenous anti-oxidant mechanisms responsible for ROS elimination (Lee et al., 2012). Intracellular GSH was selected as representative of the endogenous non-enzymatic mechanisms protecting against oxidative damage, so the GSH/GSSG ratio served as a marker of the overall cell redox environment (Moskaug, Carlsen, Myhrstad, & Blomhoff, 2005). The results showed a decrease higher than 40% in the GSH/GSSG ratio of control cells in response to high glucose conditions (Fig. 2). This finding indicates an enhanced use of GSH to scavenge free radicals, either directly or acting as a substrate for GSH-dependent enzymes (GSH peroxidases and transferases) to protect
against hydrogen peroxides, lipid hydroperoxides and electrophilic toxic compounds (Masella, Di Benedetto, Vari, Filesi, & Giovannini, 2005). Furthermore, the reduced GSH/GSSG ratio may be due to the decreased availability of NAD(P)H under oxidative stress induced by hyperglycaemia, with NAD(P)H being required for the activity of glutathione reductase to transform GSSG into GSH (Srivastava et al., 1989). Under hyperglycaemic conditions, the incubation of HUVECs EA.hy926 with the BCF compounds was able to improve the intracellular redox environment significantly with respect to control cells. These results suggest that bioavailable colonic fermented metabolites derived from the seasoning may have a great capacity to restore a balanced redox state in hyperglycaemic endothelial cells. Phenolic metabolites might exert direct protection in biological systems by scavenging ROS, thereby decreasing the need for endogenous GSH. Nonetheless, several lines of research support an indirect action as a more probable mechanism, as exogenous antioxidants may activate the endogenous defence systems through antioxidant responsive elements (AREs), which are known to stimulate the transcription of the rate-limiting enzyme for GSH synthesis in a nuclear factor erythroid 2-related factor 2 (Nrf2)-dependent manner (Martinez, Mitjans, & Vinardell, 2014; Masella et al., 2005; Moskaug et al., 2005).

The oxidation products of biological molecules are also widely used as biomarkers of oxidative damage and provide specific evidence of cellular oxidative stress status and changes induced by exposure to antioxidant nutrients (Ho, Karimi Galougahi, Liu, Bhindi, & Figtree, 2013). Oxidative stress and subsequent lipid peroxidation are known to disturb the integrity of cellular membranes, leading to the release of cytoplasmic enzymes and pro-inflammatory factors to the extracellular medium (Margina et al., 2013). These aspects were assessed using MDA and CG levels (Fig. 3A and B) as biomarkers of oxidative damage to lipids and proteins, respectively, and LDH leakage (Fig. 3C) as an indicator of cell membrane damage (L. Li & Lau, 1993). In the control cells, hyperglycaemic conditions caused a significant increase in the levels of MDA and protein CGs, as well as in the plasma membrane permeability (LDH leakage). The BGID and the BCF treatments tended to ameliorate high glucose-induced protein oxidation and, more markedly, lipid peroxidation and LDH leakage. Concretely, a significant protection of lipids in the BCF-treated cells, and of the cell membrane in both the BGID- and the BCF-treated cells, was observed with respect to the control HUVECs EA.hy926 incubated in the High GL medium. The results suggested that the BGID and especially the BCF compounds helped to preserve endothelial cell membrane integrity under hyperglycaemia-induced oxidative conditions. This finding agrees with previous studies reporting the ability of certain polyphenols to
restore the cell membrane fluidity and transmembrane potential in hyperglycaemic HUVECs (Margina et al., 2013).

Oxidative stress implicated in the cell membrane and biomolecules damage is also linked to functional alterations in vascular cells. Endothelial function is controlled by a complex network that regulates ROS production and clearance, concurrently with NO generation and inactivation, but this network is impaired under hyperglycaemic conditions (Hadi & Suwaidi, 2007; Inoguchi et al., 2000). In the current study, the exposure of control cells to the High GL medium caused a marked 76.4% increase in ROS levels and, simultaneously, a significant 34.8% decrease in the concentration of NO breakdown products (Fig. 4). This endothelial dysfunction was in line with previous studies reporting that the exposure of HUVECs to high glucose concentrations more or less caused ROS generation to double (Chao, Hou, Lee Chao, Weng, & Ho, 2009; Koziel et al., 2012) and led to a significant decrease in NO bioavailability (Juhong Yang, Wang, Li, Zhang, & Feng, 2010; Zhou et al., 2012). It is widely recognized that NOXs represent the primary source of ROS (O$_2^·$ and H$_2$O$_2$) in the vascular wall, production of which is triggered by stimulation of vasoconstrictor agents such as Ang II and endothelin-1 (Virdis et al., 2011). A number of additional sources of ROS have been identified under conditions of oxidative stress and hyperglycaemia (Münzel et al., 2010; Pennathur & Heinecke, 2007; Zhou et al., 2012), including uncoupled eNOS, which implies that the physiological activity of the enzyme for NO production is decreased and switched to the deleterious O$_2^·$ generation (Roe & Ren, 2012; Virdis et al., 2011). NO bioavailability also depends on its rapid inactivation by reaction with O$_2^·$ to form ONOO$^·$ (W. Chen et al., 2010), which can further explain the decreased NO levels under the hyperglycaemic conditions observed in this study. Interestingly, the BGID and the BCF treatments were able to partly restore the ROS levels detected in hyperglycaemic cells to levels found in normoglycaemic cells. Furthermore, incubation with these treatments prevented hyperglycaemia-impaired NO bioavailability in HUVECs EA.hy926, with significantly higher NO concentrations found in the medium of the treated rather than the control cells. The seasoning-derived BCF compounds showed a higher potential than the BGID compounds to improve endothelial function. Similar trends in these beneficial treatment effects were observed in cells exposed to the Low and the High GL mediums, but more markedly under hyperglycaemic conditions. According to previous studies (Edwards, Czank, Woodward, Cassidy, & Kay, 2015; Ruijters, Weseler, Kicken, Haenen, & Bast, 2013; Steffen, Gruber, Schewe, & Sies, 2008), the results could be due to the anti-ROS effects of those phenolic compounds and metabolites present in the seasoning-derived treatments. Although only a limited number of individual phenolic metabolites were able to enhance the
endothelial NO levels (Appeldoorn et al., 2009), the NO-preserving actions of these compounds have been
suggested to rely mainly on NOX inhibition and perhaps partially in combination with their $O_2^\cdot$ scavenging
capacity (Steffen et al., 2008). In fact, the lower ROS levels and reduced NO inactivation detected in the
BCF-treated cells were in accordance with the higher antiradical activity (ABTS values) for the BCF than the
BGID products observed in vitro. In addition, phenolic compounds may increase NO production through
eNOS protein activation mediated by the phosphatidylinositol 3-kinase/Akt pathway (Schini-Kerth, Auger,
Kim, Étienne-Selloum, & Chataigneau, 2010; Schmitt & Dirsch, 2009).

3.3. Possible mechanisms of action implicated in the protection against oxidative stress and endothelial
dysfunction under hyperglycaemia

3.3.1. Changes in NOX4, eNOS, SOD2 and HO-1 gene expression

In physiological terms, controlled ROS production plays an important role in cell signalling and maintains
normal vascular contraction-relaxation and cell growth. The enhanced ROS production under pathological
conditions is also known to stimulate several redox-sensitive signalling pathways (Virdis et al., 2011;
Zinkevich & Gutterman, 2011). In the present study, changes were therefore assessed in the mRNA levels of
four genes involved in the endothelial cells ROS and NO balance (NOX4 and eNOS) and the endogenous
antioxidant system (SOD2 and HO-1) (Fig. 5). This assessment was done to elucidate some of the
mechanisms underlying the observed protective effects of the potentially bioavailable compounds derived
from the seasoning.

In the hyperglycaemic control cells, gene expression of the endothelial ROS generating enzyme NOX4
increased more than 2-fold, whereas no changes in the eNOS mRNA levels were detected (Fig. 5A and B,
respectively). The observed NOX4 up-regulation agrees with previously published works and has been
described as mediated by the activation of phosphatidylinositol 3-kinase/Akt (Zhou et al., 2012) and protein
kinase C pathways (Chai et al., 2008), and by the stimulation of Ang II (Selemidis, Sobey, Wingler, Schmidt,
& Drummond, 2008). However, it is unclear how hyperglycaemia affects eNOS gene expression in
HUVECs, which might be up-regulated (Ding et al., 2004; H. Li et al., 1998), down-regulated (Sun & Liao,
2004) or, in accordance with our observations, not significantly affected (Patel et al., 2013). The results of
the present study suggest that the decreased NO levels observed in the hyperglycaemic control cells were
unlikely to be due to down-regulation of eNOS gene expression. It might therefore involve other previously cited mechanisms, such as ROS-mediated NO inactivation and eNOS uncoupling.

Interestingly, the treatment of hyperglycaemic HUVECs EA.hy926 with the BGID and the BCF compounds both resulted in a significant decrease in the levels of NOX4 and eNOS mRNA. In line with different authors working with grape pomace extracts and standard phenolic compounds (Álvarez et al., 2012; Spanier et al., 2009), the current results could be associated with the ability of the seasoning-derived phenolic compounds to down-regulate NOX4 gene expression, with a higher potential observed for the potential colonic fermented metabolites. This capacity may be a key mechanism to explain the lower ROS levels found in the BGID and the BCF-treated cells. In addition, our results appear to suggest a compensatory decrease in the eNOS gene expression in response to the enhanced NO levels described above for hyperglycaemic cells treated with the BGID and especially the BCF products.

In parallel to the mRNA level changes observed for the ROS and NO generating enzymes, the gene expression of the mitochondrial antioxidant enzyme SOD2 was 6-fold up-regulated, whereas the HO-1 was 0.6-fold down-regulated, in the hyperglycaemic control cells (Fig. 5C and D). These results are in agreement with other authors (Y. Y. Chen et al., 2012; Koziel et al., 2012) and they may reflect the endothelial cell response under hyperglycaemic conditions in an attempt to counteract over-production of \( \text{O}_2^- \) (Patel et al., 2013). The vascular up-regulation of SOD2 is attributed to the presence of response elements in its promoter for the redox-sensitive transcription factors activator protein-1 (AP-1) and nuclear factor-kappa B (NF-κB) (Faraci & Didion, 2004), which are known to be activated under hyperglycaemia (S. Chen, Mukherjee, Chakraborty, & Chakrabarti, 2003). However, controversial results can be found in the literature on the modulation of HO-1 gene expression by glucose in the vasculature, as this modulation has been suggested to depend upon levels of glucose (Iori et al., 2008).

SOD2 mRNA levels were considerably decreased in the hyperglycaemic BCF-treated cells, and also significantly reduced in normoglycaemic cells after incubation with both treatments. In contrast, the HO-1 gene was over-expressed to a similar extent by the BGID and the BCF treatments, but the effects were more marked for hyperglycaemic (3.7-fold) than normoglycaemic cells (2.6-fold). These results are consistent with several lines of evidence suggesting that some phenolic compounds and their metabolites have the capacity to improve endothelial function by HO-1 gene over-expression, mainly through the Nrf2/ARE pathway activation (Edwards et al., 2015; Ungvari et al., 2010). The observed HO-1 up-regulation almost certainly contributed to the decreased ROS levels observed in the treated cells, a fact that may further influence other
redox-sensitive signalling pathways and enzymes that were over-expressed due to the increased oxidative environment in cells, such as might be the case of SOD2. The direct ROS scavenging capacity of the seasoning-derived phenolic compounds and their ability to inhibit the high glucose-induced NF-κB translocation to the nucleus, as previously reported for certain flavan-3-ols (Jian Yang et al., 2013), might also explain the decreased requirement for SOD2 gene expression in the treated cells.

3.3.2. Changes in *ex vivo* ACE activity

Renin-angiotensin aldosterone system (RAAS) and ACE activation are associated with alterations in endothelial function observed in diabetes and hypertension (Hadi & Suwaidi, 2007; Hsueh & Wyne, 2011). In this regard, the Ang II over-production observed in hyperglycaemic cells is known to contribute to NOXs over-expression and activation, increased ROS generation, and subsequent endothelial dysfunction (McFarlane et al., 2003; Selemidis et al., 2008; Virdis et al., 2011). In addition, Ang II significantly up-regulates mRNA expression of renin, ACE, and angiotensinogen (the Ang II precursor), thus resulting in a vicious circle of RAAS activation in endothelial cells (Ide, Hirase, Nishimoto-Hazuku, Ikeda, & Node, 2008). Interestingly, there is accumulating evidence that flavonoids and polyphenol-enriched plant extracts are effective ACE inhibitors, although most of them have been shown to be more effective *in vitro* than *ex vivo* or *in vivo* (Balasuriya & Rupasinghe, 2011). Consequently, the *ex vivo* capacity of the BGID and the BCF products to down-regulate ACE activity in HUVECs EA.hy926 was also evaluated in this study. The results showed that ACE activity was around 21% higher in lysates obtained from hyperglycaemic rather than from normoglycaemic cells (*Fig. 6*). This significant increase in ACE activity was in accordance with the enhanced ACE-1 expression previously reported in HUVECs under hyperglycaemia (F. Chen et al., 2014).

However, *ex vivo* ACE activity was significantly decreased in the hyperglycaemic treated cells by about 13.6% (BGID) and 9.7% (BCF). These findings reveal that the BGID and the BCF treatments interfered in high glucose-induced RAAS activation. This effect may be partly explained by the direct capacity of the phenolic compounds released from the seasoning along digestion to inhibit ACE activity, which is supported by our *in vitro* ACE results, and by previous experiments *in vitro* and in HUVECs that demonstrated the ACE inhibitory capacity of certain flavonol metabolites (Balasuriya & Rupasinghe, 2012). In addition, the decreased Ang II production resulting from this direct ACE inhibition might lead to an indirect effect of the
BGID and the BCF compounds down-regulating ACE expression in hyperglycaemic cells during the incubation period with these treatments (Ide et al., 2008).

4. Conclusions

The findings of this study have provided novel and compelling evidence of the potential protective effects of pre- and post-colonic digested compounds derived from a seasoning obtained from wine pomace against hyperglycaemia-induced endothelial dysfunction and oxidative damage.

Probable mechanisms of action by which these compounds restore the redox environment and endothelial function in HUVECs EA.hy926 are: direct ROS scavenging; modulation of NOX4, SOD2 and HO-1 gene expression by inducing different redox-sensitive signalling pathways; and, their capacity to decrease ACE activity.

The metabolism of bioactive compounds by colonic microbiota was found to play a key role along the digestive process in the beneficial vascular effects observed for the seasoning that has been assessed.

Altogether, this ex vivo study encourages the incorporation of seasonings derived from wine pomace in functional foods and sets the stage for future animal and clinical trials in order to confirm in vivo the promising healthy properties of such seasonings in cases of diabetes, hypertension, and other cardiovascular diseases.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

The authors thank the financial support of the Autonomous Government of Castilla y León (Research project BU282U13). The PhD grants of RDPG and JGL (FPU grants) are funded by the Spanish Ministry of Education, Culture and Sports. The authors also thank Jover E. and Hernández-Romero D. ("Arterial thrombosis and interstitial, vascular and myocardial remodelling" research group, IMIB-Arrixaca, Murcia, Spain) for their advice in the HUVECs EA.hy926 culture.

Appendix: Supplementary material

Supplementary data to this article (Table S1) can be found online at
References


Fatehi-Hassanabad, Z., Chan, C. B., & Furman, B. L. (2010). Reactive oxygen species and endothelial...


Ho, E., Karimi Galougahi, K., Liu, C.-C., Bhindi, R., & Figtree, G. a. (2013). Biological markers of


Spanier, G., Xu, H., Xia, N., Tobias, S., Deng, S., Wojnowski, L., … Li, H. (2009). Resveratrol reduces endothelial oxidative stress by modulating the gene expression of superoxide dismutase 1 (SOD1), glutathione peroxidase 1 (GPx1) and NADPH oxidase subunit (NOX4). *Journal of Physiology and...


Figure captions

**Fig. 1.** Cell viability of normoglycaemic (Low GL) and hyperglycaemic (High GL) HUVECs EA.hy926 treated with the digested products obtained from the seasoning. BGID: potentially bioavailable samples after simulated gastrointestinal digestion; BCF: potentially bioavailable samples after simulated colonic fermentation. Data expressed in % as mean values ± standard deviation (n = 3) with respect to the control normoglycaemic cells (100%). Significant differences (p<0.05) between hyperglycaemic and normoglycaemic cells are also indicated in % values. Significant changes (p<0.05) between the control, the BGID-, and the BCF-treated cells are expressed in Roman (Low GL) and Greek letters (High GL).

**Fig. 2.** Intracellular redox status (GSH/GSSG ratio) of normoglycaemic (Low GL) and hyperglycaemic (High GL) HUVECs EA.hy926 treated with the digested products obtained from the seasoning. BGID: potentially bioavailable samples after simulated gastrointestinal digestion; BCF: potentially bioavailable samples after simulated colonic fermentation. Data expressed as mean values ± standard deviation (n = 3). Percentage (%) values indicate significant differences (p<0.05) between hyperglycaemic and normoglycaemic cells. Significant differences (p<0.05) between the control, the BGID-, and the BCF-treated cells are expressed in Roman (Low GL) and Greek letters (High GL).

**Fig. 3.** Biomarkers of lipid, protein and cell membrane oxidative damage of normoglycaemic (Low GL) and hyperglycaemic (High GL) HUVECs EA.hy926 treated with the seasoning digested products. A) Malondialdehyde (MDA). B) Carbonyl groups (CGs). C) % lactate dehydrogenase (LDH) leakage. Cells for all assays were treated with the potentially bioavailable compounds obtained after the *in vitro* gastrointestinal digestion (BGID) or colonic fermented (BCF) of the seasoning under evaluation. Data expressed as mean values ± standard deviation (n = 3). Significant variations (p<0.05) between cells cultured in different mediums are indicated as % of hyperglycaemic with respect to normoglycaemic cells. Significant differences (p<0.05) between the control, the BGID-, and the BCF-treated cells are expressed in Roman (Low GL) and Greek letters (High GL).

**Fig. 4.** Intracellular ROS and extracellular NO levels of normoglycaemic (Low GL) and hyperglycaemic (High GL) HUVECs EA.hy926 treated with the seasoning digested products. A)
Overall intracellular ROS production by HUVECs EA.hy926 was estimated by folds of 2,7-dichlorofluorescein (DCF) fluorescence increases with respect to the control normoglycaemic cells. B) NO generation by HUVECs EA.hy926 was estimated as the concentration (µM) in the culture medium of the total sum of NO breakdown products (NO₂⁻ + NO₃⁻). Cells for both assays were treated with the potentially bioavailable compounds obtained after the in vitro gastrointestinal digestion (BGID) or colonic fermented (BCF) of the seasoning assessed. Data expressed as mean values ± standard deviation (n = 3). Significant differences (p<0.05) between hyperglycaemic and normoglycaemic cells are indicated in % values. Significant differences (p<0.05) between the control, the BGID-, and the BCF-treated cells are expressed in Roman (Low GL) and Greek letters (High GL).

**Fig. 5. Gene expression in normoglycaemic (Low GL) and hyperglycaemic (High GL) HUVECs**

EA.hy926 treated with the seasoning digested products. Genes analysed: A) NOX4, B) eNOS, C) SOD2, and D) HO-1. Cells were treated with the potentially bioavailable compounds obtained after the in vitro gastrointestinal digestion (BGID) or colonic fermented (BCF) of the seasoning under study. mRNA levels of the genes under assessment were determined by quantitative real-time PCR, normalized to the GAPDH gene expression. The results are presented as relative fold changes with respect to the control normoglycaemic cells. Data expressed as mean values ± standard deviation (n = 3). Significant changes (p<0.05) between hyperglycaemic and normoglycaemic cells are indicated with an asterisk (*). Significant differences (p<0.05) between the control, the BGID-, and the BCF-treated cells are expressed in Roman (Low GL) and Greek letters (High GL).

**Fig. 6. ACE activity determined ex vivo in normoglycaemic (Low GL) and hyperglycaemic (High GL) HUVECs**

HUVECs EA.hy926 treated with the digested products obtained from the seasoning evaluated. BGID: potentially bioavailable samples after simulated gastrointestinal digestion; BCF: potentially bioavailable samples after simulated colonic fermentation. Data expressed in % as mean values ± standard deviation (n = 3) with respect to the control normoglycaemic cells (100%). Significant differences (p<0.05) between hyperglycaemic and normoglycaemic cells are indicated in % values. Significant variations (p<0.05) between the control, the BGID-, and the BCF-treated cells are expressed in Roman (Low GL) and Greek letters (High GL).
Fig. 1 (One-column Figure)
Fig. 2 (One-column Figure)
Fig. 3 (One-column Figure)
Fig. 4 (Two-column Figure)
Fig. 5 (Two-column Figure)
Fig. 6 (One-column Figure)

![Bar chart showing ACE activity (%) for Control, BGID, and BCF groups.](image-url)
Table S1. Detailed HPLC-DAD characterization of the wine pomace seasoning (poly)phenolic composition.

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<th>(Poly)phenolic compounds</th>
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<td>Hydroxybenzoic acids</td>
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<td>p-OH-benzoic acid</td>
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<td>Salicylic acid</td>
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*Results expressed as mean value ± standard deviation (n=3).