1 **Wine pomace seasoning attenuates hyperglycaemia-induced endothelial dysfunction and oxidative**

2 **damage in endothelial cells.**

3

4 Short title:

5 **Wine pomace seasoning attenuates endothelial cell dysfunction and oxidative damage.**

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19 **Abstract**

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

32

33 **Keywords:**

34 Colonic metabolites; Dietary antioxidants; Endothelial function; Hyperglycaemia; Redox signalling; Wine
35 pomace

36

37 **Abbreviations:**

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

48

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 ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_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 $O_2^{\cdot -}$ 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).

79 Living organisms have developed several effective mechanisms to protect themselves from the detrimental
80 effects of ROS. The endogenous antioxidant-defence system includes enzymes such as superoxide
81 dismutases (SOD), catalase, glutathione peroxidases, heme oxygenases (HO), and others, as well as non-
82 enzymatic antioxidants such as glutathione (GSH) (Lee, Margaritis, Channon, & Antoniades, 2012).
83 In addition, several exogenous interventions using antioxidant dietary compounds have been proposed to
84 prevent endothelial dysfunction and to restore the redox status in the vascular microenvironment, with
85 certain phenolic compounds being highlighted among the most promising natural antioxidants with
86 beneficial cardiovascular effects (Stoclet et al., 2004). In this regard, a number of studies have been initiated
87 dealing with the antioxidant, hypotensive, and vascular health-promoting effects of (poly)phenols in grapes,
88 wine and wine pomace. The antioxidant capacity of these phytochemicals is not simply related to their direct
89 ROS scavenging ability, but also to the inhibition of enzymatic sources of oxidative stress, such as NOX and
90 ACE, as well as the stimulation of endogenous antioxidant enzymes (Eriz, Sanhueza, Roeckel, & Fernández,
91 2011; Münzel et al., 2010; Nijveldt et al., 2001).
92 However, one of the principal topics concerning the beneficial effects of phenolic compounds is their
93 bioavailability and metabolic fate, which are dependent upon their digestive stability, their bioaccessibility
94 (release from the food matrix), the efficiency of their trans-epithelial passage, and their further metabolism in
95 enteric and hepatic tissue, as well as by gut microbiota (Manach, Scalbert, Morand, Rémésy, & Jiménez,
96 2004; Scalbert & Williamson, 2000a; Tagliazucchi, Verzelloni, Bertolini, & Conte, 2010). Therefore, despite
97 the limitations of simulated digestion systems (Alminger et al., 2014), the combination of *in vitro* digestion
98 protocols with cell assays has recently been proposed as a suitable approach to determine the antioxidant
99 activity of (poly)phenols once they have been ingested (Huang, Sun, Lou, & Ye, 2014).
100 Hyperglycaemia-induced oxidative stress is one cause of the vascular complications that characterize
101 diabetes (Fatehi-Hassanabad, Chan, & Furman, 2010; Hadi & Suwaidi, 2007). As such, several studies have
102 investigated endothelial function impairment and associated pro-oxidative environment *ex vivo,* by using
103 vascular cells such as human umbilical vein endothelial cells (HUVECs) grown under high glucose
104 concentrations (Inoguchi et al., 2000; Koziel, Woyda-Ploszczyca, Kicinska, & Jarmuszkiewicz, 2012; Patel,
105 Chen, Das, & Kavdia, 2013; Zhou, Xie, Zhou, & Li, 2012).
106 Recently, new powdered seasonings obtained from wine pomace have been developed (García-Lomillo,
107 González-Sanjosé, Del Pino-García, Rivero-Pérez, & Muñiz, 2014). These (poly)phenol-rich products have
108 demonstrated significant antioxidant properties *in vitro* (Del Pino-García, García-Lomillo, Rivero-Pérez,

109 González-Sanjosé, & Muñiz, 2015), so their use as additives or ingredients in functional foods could enhance
110 exogenous antioxidant dietary pool and contribute to maintaining/restoring a healthy internal redox status.
111 This paper is focused on the potential protective effects against endothelial dysfunction and oxidative
112 damage in the vasculature of vegetal products proposed as functional food ingredients. Its major aim was to
113 evaluate the specific effects of a red wine pomace-derived seasoning under hyperglycaemia-induced
114 oxidative stress in the endothelial cell line HUVEC EA.hy926. The cell treatments used to achieve this
115 objective consisted of those compounds released after a simulated gastrointestinal digestion and colonic
116 fermentation of the powdered seasoning under study.
117
118 **2. Materials and methods**
119
120 **2.1. Chemicals**
121 2,2'-Azinobis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS), porcine bile extract, 2',7'-dichlorofluorescein
122 diacetate (DCFH-DA), DMEM containing either 5.6 mM (Low GL) and 25 mM D-Glucose (High GL),
123 foetal bovine serum (FBS), gallic acid, 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), L-
124 glutamine solution (200 mM), 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), magnesium
125 chloride hexahydrate ($MgCl_2.6H_2O$), N-hippuryl-L-histidyl-L-leucine (HHL) tetrahydrate, β-nicotinamide
126 adenine dinucleotide reduced (NADH) disodium salt hydrate, porcine pancreas pancreatin, 10,000 U/mL
127 penicillin and 100 mg/mL streptomycin solution (P/S), 85% (v/v) phosphoric acid solution (H_3PO_4), sodium
128 pyruvate, 1,1,3,3-tetramethoxypropane (TMP), 2-thiobarbituric acid (TBA), thiazolyl blue tetrazolium
129 bromide (MTT), 2-vinylpyridine, cellulose membrane dialysis tubing (12,000 Da molecular weight cut-off),
130 and the enzymes used in the simulated gastrointestinal digestion (α-amylase (EC 3.2.1.1), amyloglucosidase
131 (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.
132 Louis, MO, USA). Folin-Ciocalteu (FC) reagent, 70% (v/v) and perchloric acid (PCA) were purchased from
133 Panreac Quimica S.L.U. (Barcelona, Spain). DMSO and PBS tablets were purchased from Merck Millipore
134 Co. (Darmstadt, Germany).
135
136 **2.2. Seasoning obtained from wine pomace**
137 The powdered seasoning examined in this study was made at the University of Burgos according to a
138 previously described method (González-Sanjosé, García-Lomillo, Del Pino-García, Muñiz-Rodríguez, &

139 Rivero-Pérez, 2013). This product was obtained from seed-free red wine pomace using a heat treatment as
140 stabilization process, and its main characteristics and composition (dietary fibre, fat, protein, minerals and
141 phenolic classes) have previously been reported (García-Lomillo et al., 2014).
142

143 **2.3. *In vitro* gastrointestinal digestion and colonic fermentation of the seasoning**

144 The wine pomace seasoning underwent *in vitro* digestion following the method described by Saura-Calixto
145 et al. (2010). This procedure mainly comprised two sequential steps that simulate conditions along the gut: a)
146 enzymatic gastrointestinal digestion; and b) colonic microbial fermentation. Both soluble fractions obtained
147 after these steps were further subjected to a controlled dialysis, chosen to model the mechanical aspects of
148 the epithelial barrier (Fernández & Labra, 2013). Briefly, 7.5 mL of each fraction were transferred into
149 cellulose membrane dialysis tubing and dialyzed against a total of 50 mL of Milli-Q water for 4 h (changing
150 the water twice). The solutions extracted from the dialysis tubing were collected, lyophilized, weighed, and
151 stored at -20 ºC. These samples containing possibly bioavailable compounds present in each fraction were
152 labelled as "BGID" (potentially bioavailable samples after gastrointestinal digestion) and "BCF" (potentially
153 bioavailable samples after colonic fermentation). Three replicates were carried out for each sample. Negative
154 digested controls (without seasoning) for both types of samples were also prepared.
155

156 **2.4. Analysis of BGID and BCF treatments**

157

158 **2.4.1. Total antioxidant capacity**

159 The *in vitro* antioxidant activity of each treatment was directly evaluated in the lyophilized powdered
160 samples using QUENCHER (Q-) methods (Del Pino-García et al., 2015). The antioxidant capacities assessed
161 by Q-Folin-Ciocalteu (Q-FC) assay and by Q-ABTS assay were expressed as µmol of gallic acid equivalents
162 per gram of product (GAE/g product), and as µmol of Trolox equivalents per gram of product (TE/g
163 product), respectively.
164

165 **2.4.2. *In vitro* determination of angiotensin I-converting enzyme (ACE) activity**

166 ACE activity was measured using the method described by Cinq-Mars & Li-Chan (2007). In this assay, ACE
167 activity is quantified by spectrophotometric absorbance (abs) at 228 nm produced by the liberation of

168 hippuric acid from the synthetic substrate HHL. As ACE activity is depressed in the presence of ACE
169 inhibitors, percentage (%) ACE inhibition was calculated, as described in the following equation [1]:

170 $$\% \text{ ACE inhibition} = 1 - [(abs_{sample} - abs_{negative\ control})/(abs_{positive\ control} - abs_{negative\ control})] \times 100 \quad [1]$$

171 where, the positive control (treatments replaced by the buffer) was taken as 100% ACE activity, and the
172 negative control (ACE added only after reaction termination) was taken as 0% ACE activity.
173

174 **2.5. Cell culture and treatment**

175 The immortalized cell line HUVEC EA.hy926 was kindly provided by Dr. Diana Hernández-Romero from
176 the research group "Arterial thrombosis and interstitial, vascular and myocardial remodelling"(IMIB-
177 Arrixaca/University of Murcia, Murcia, Spain). Cells were maintained in DMEM Low GL supplemented
178 with 10% FBS, 1% P/S, and 1% L-glutamine solution at 37 ºC in a humidified incubator with 5% CO_2. The
179 medium during the treatment incubation period was DMEM Low or High GL supplemented with 1% FBS,
180 1% P/S, 1% L-glutamine solution, and 0.1% DMSO. HUVECs EA.hy926 for experiments were taken from
181 exponential phase cultures at passages 4–8 and seeded at a 2×10^4 cells/cm^2 density in all assays. Cells were
182 grown for 24 h under basal conditions. Then, the treated-cells were exposed for 24 h to either the BGID or
183 the BCF treatments (0.5 mg of product/mL as a final concentration) or the respective negative digested
184 control treatments. Non-treated control cells were only incubated with Low GL (normoglycaemic control)
185 and High GL (hyperglycaemic control) mediums. All experiments were carried out as three independent
186 assays. Those assays to determine cell viability, intracellular ROS, and NO production were performed in
187 96-well cell culture multiplates and the remaining experiments in 25 cm^2 cell culture flasks. After the
188 treatment period, the cells were scraped and centrifuged (1,500 g, 5 min, 25 ºC). Supernatant was collected
189 and frozen at -80 ºC for further analyses. The cell pellets were resuspended in 1 mL of PBS and used in the
190 analyses, as explained below.
191

192 **2.6. Cell viability assessment**

193 Cell viability was analysed using the MTT method (Twentyman & Luscombe, 1987). Absorbance at 550 nm
194 was measured in a PowerWave XS2 microplate spectrophotometer (BioTek Instruments Inc., Vermont,
195 USA). The results were expressed as % cell viability with respect to control cells grown in the Low GL
196 medium.
197

198 **2.7. Glutathione reduced/oxidized (GSH/GSSG) ratio analysis**

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

206

207 **2.8. Malondialdehyde (MDA) determination**

208 Lipid peroxidation was estimated from the MDA levels according to the method described by Grotto et al.
209 (2007), with slight modifications to perform the assay in cell homogenates obtained by sonication of the
210 HUVEC EA.hy926 suspensions collected after the treatment period. Briefly, a volume of 75 µL of cell
211 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
212 in a shaking water bath system. After this, 125 µL of 6 % (v/v) H_3PO_4 and 125 µL of 0.8% (w/v) TBA were
213 added and the mixture was heated at 90 ºC for 45 min. Then, the mixture was cooled and extracted with 300
214 µL of *n*-butanol by vortex-mixing for 1 min and centrifugation at 3,000 *g* for 10 min. The butanol layer was
215 collected and a volume of 20 µL was injected into an Agilent 1100 series HPLC system (Agilent
216 Technologies, Palo Alto, CA) equipped with a diode array detector. The column was a Spherisorb3® ODS2
217 reversed phase C18 (250 mm x 4.6 mm, 3 µm particle size; Waters Cromatografia S.A., Barcelona, Spain).
218 The mobile phase was a mixture of Milli-Q water:methanol (1:1, v/v). The flow rate was maintained
219 isocratically at 0.6 ml/min, the absorbance at 532 nm of the eluent was monitored, and the total run time was
220 8 min. Concentrations of MDA were calculated from calibration curves obtained using TMP as the standard.
221 MDA levels were normalized using each cell homogenate total protein concentration, which was determined
222 according to Bradford (1976). The results were finally expressed as nmol MDA/mg of protein.

223

224 **2.9. Carbonyl groups (CGs) determination**

225 Changes in cell proteins due to oxidative stress were determined in sonicated cell lysates using the
226 spectrophotometric method described by Levine et al. (1990) to quantify protein CGs. The CG levels were

227 normalized by the protein content of each cell homogenate, expressing the final results as nmol CGs/mg of
228 protein.
229
230 **2.10. Lactate dehydrogenase (LDH) leakage assessment**
231 The LDH activity was measured in the extra- and intracellular medium using the method reported by
232 Vassault (1983), with some modifications. Briefly, a reaction mixture containing 100 mM potassium
233 phosphate buffer, 0.7 mM sodium pyruvate, and 0.48 mM NADH was freshly prepared. A volume of 1.25
234 mL of the reaction mixture was transferred to a quartz cuvette and mixed with 50 µL of the extra- or
235 intracellular medium. The absorbance decay at 340 nm was monitored for 2 min every 15 s. The units of
236 extra- and intracellular LDL activity (1 unit = 1 µmol NADH/min) were determined considering a molar
237 absorption coefficient for NADH of 6,220 $M^{-1}cm^{-1}$. Finally, cell membrane integrity was estimated from
238 LDH activity, in terms of % LDH release to the extracellular medium with respect to total LDH (intracellular
239 + extracellular).
240
241 **2.11. Intracellular reactive oxygen species (ROS) measurement**
242 Overall intracellular ROS production in HUVECs EA.hy926 was measured by the 2,7-dichlorofluorescein
243 (DCF) assay (Wang & Joseph, 1999), with certain modifications. Following their treatment and incubation,
244 the cells were washed with a colourless external medium containing 145 mM NaCl, 5 mM KCl, 1 mM
245 $MgCl_2.6H_2O$, and 10 mM HEPES. They were then added 100 µL of 20 µM DHCF-DA solution in external
246 medium and placed in the chamber reader of a Cary Eclipse Fluorescence Spectrophotometer (Varian
247 Australia Pty. Ltd., Victoria, Australia) with a microplate reader accessory to take the initial fluorescence
248 reading (t_0) using 480 nm excitation/530 nm emission filters. Fluorescence emission was also recorded after
249 incubation in the dark at 37 ºC for 30 min (t_{30}) and the variations in relative fluorescence units (t_{30} - t_0) were
250 calculated for each sample. The MTT assay plates were run in parallel and the % cell viability results were
251 used to normalize the recorded increase in fluorescence intensity, thereby taking into account the amount of
252 viable cells per well. ROS levels were then estimated as DCF fluorescence increases and the final results
253 were expressed as folds of the control cells grown in the Low GL medium.
254
255 **2.12. Extracellular nitric oxide (NO) determination**

256 The quantification of NO breakdown products, nitrite (NO_2^-) and nitrate (NO_3^-), in the culture medium was
257 used as an indirect method to assess the cellular production of NO. Total nitrite and nitrate (NO_2^-+NO_3^-) was
258 determined using the Nitrate/Nitrite Colorimetric Assay kit (Cayman Chemical, Michigan, USA) according
259 to the supplier's instructions. The % viability determined by the MTT assays run in parallel was used to
260 normalize cellular production of NO, which was finally expressed as µM NO_2^-+NO_3^-.
261

262 **2.13. Quantitative real-time PCR (qPCR) analysis**

263 Total RNA was isolated from HUVECs Ea.hy926 suspensions using TRI Reagent solution (Applied
264 Biosystems). After treatment with DNase I (Thermo Scientific ™), 1 µg of total RNA was reverse-
265 transcribed using a First Strand cDNA Synthesis kit (Thermo Scientific™), and finally amplified using iQ™
266 SYBR®Green Supermix (Bio-Rad). All the procedures were performed according to the manufacturers'
267 protocols. Primers were designed using Primer3 v.0.4.0 software (Untergasser et al., 2012) and synthesized
268 by Metabion (Metabion International AG, Steinkirchen, Germany). The sequences of primer sets (forward
269 and reverse) were: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-
270 GCTCTCCAGAACATCATCCC-3' and 5'-GTCCACCACTGACACGTTG-3'; NOX4, 5'-
271 GGAAGAGCCCAGATTCCAAG-3' and 5'-AGTCTTTCGGCACAGTACAG-3'; eNOS, 5'-
272 GAAGCTGCAGGTGTTCGAT-3' and 5'-CGGTTGGTGGCATACTTGAT-3'; SOD2, 5'-
273 GGAACGGGGACACTTACAAA-3' and 5'-ACTGAAGGTAGTAAGCGTGC-3'; HO-1, 5'-
274 GCCAGCAACAAAGTGCAAG-3' and 5'-AAAGCTGAGTGTAAGGACCC-3'. qPCR was carried
275 out with an iCycler iQ Real-Time PCR Detection System (Bio-Rad) under the following conditions: 1 cycle
276 of initial denaturing and enzyme activation at 95 ºC for 3 min; 45 cycles of denaturing at 95 °C for 15 s and
277 annealing/extension at 60 °C for 30 s. The proper product amplification was verified by melting curve
278 analysis. Amplification efficiencies were calculated for each pair of primers and quantification was
279 performed using the efficiency-corrected $\Delta\Delta C_t$ method, with GAPDH as the reference gene. Relative gene
280 expression was finally expressed as folds of change compared to control cells grown in the Low GL medium.
281

282 **2.14. *Ex vivo* determination of angiotensin I-converting enzyme (ACE) activity**

283 After the treatment and incubation of HUVECs EA.hy926, the ACE activity in sonicated cell lysates was
284 measured using the ACE substrate HHL following the spectrofluorometric method reported by Hermanns,

285 Müller, Tsokos, & Kirkpatrick (2014). ACE activity was considered proportional to fluorescence intensity
286 and was normalized to the protein concentration of cell homogenates. The final results were expressed as %
287 ACE activity compared to control cells grown in the Low GL medium.
288

289 **2.15. Data presentation and statistical analysis**

290 Data were expressed as means ± standard deviation of independent experiments (n=3). Statistical analysis
291 was performed using Statgraphics® Centurion XVI, version 16.2.04 (Statpoint Technologies, Inc.,
292 Warranton, VA). One-way analysis of variance (ANOVA), using Fisher's least significant difference (LSD)
293 test, was used to determine significant differences ($p < 0.05$) between data from cells incubated with the
294 different treatments (control, BGID, and BCF) in the same medium (represented with Roman or Greek
295 letters), and between the results of cells cultured in different mediums (Low and High GL) for each of the
296 treatments (represented as % of change of hyperglycaemic with respect to normoglycaemic cells).
297

298 **3. Results and discussion**

299

300 Powdered seasonings obtained from winery by-products could be used by the food industry as innovative
301 condiments and ingredients with antioxidant functional effects. Thus, several experiments were conducted to
302 evaluate the properties of a red wine pomace-derived seasoning and its potential protective effects against
303 oxidative stress and endothelial dysfunction induced by high glucose conditions in vascular cells.
304 Considering the transformations that would take place in bioactive compounds following ingestion of the
305 seasoning, a simulated gastrointestinal digestion and colonic fermentation of the seasoning was performed,
306 obtaining two fractions that were dialyzed to emulate trans-epithelial passage and then used as cell
307 treatments. These digestion products (BGID and BCF, respectively) were first characterized *in vitro* and then
308 used *ex vivo* for treating HUVECs EA.hy926.
309

310 **3.1. Analysis of the seasoning-derived digestion products**

311 The digestion products evaluated in this study derived from a seed-free wine pomace seasoning that has been
312 characterized in previous works. This product has a high content of dietary fibre (48.6 ± 0.7 %), and is a rich
313 source of (poly)phenols (24.4 ± 0.1 mg GAE/g), especially proanthocyanidins and anthocyanins (García-
314 Lomillo et al., 2014). More detailed data about its individual (poly)phenolic contents are provided as

315 **Supplementary Table S1**. In addition, the undigested seasoning presents high *in vitro* total antioxidant
316 capacities (Q-FC index: 59.9 ± 1.5 µmol GAE/g; Q-ABTS values: 103 ± 2.7 µmol TE/g), which was
317 determined directly in the powdered product, as previously described (Del Pino-García et al., 2015).
318 In the present study, the antioxidant and ACE inhibitory activities the digestion products derived from such
319 seasoning (BGID and BCF) were firstly assessed *in vitro*. The Q-FC index and Q-ABTS values for the BGID
320 product were 48.0 ± 1.3 µmol GAE/g product and 153 ± 21 µmol TE/g product, respectively, whereas the
321 results for the BCF product were 30.8 ± 8.7 µmol GAE/g product and 435 ± 48 µmol TE/g product.
322 Therefore, both fractions showed an interesting but different *in vitro* total antioxidant capacity profile. Q-FC
323 values were around 35% higher before the action of colonic microbiota than after it, but the $ABTS^{\cdot +}$
324 antiradical capacity of the metabolites that could be absorbed in the colon was around 3-fold higher than
325 those antioxidants present in the BGID fraction. This antioxidant capacity shown by the BGID and the BCF
326 products are certainly due to their (poly)phenolic composition. In this regard, it is well-known that
327 proanthocyanidins from wine pomace are partly depolymerized into their flavan-3-ols monomers during
328 transit along the digestive tract, and further transformed into free or conjugated microbial-derived phenolic
329 metabolites in the colon (Touriño et al., 2011). Similarly, the extensive microbial catabolism of main
330 flavonoids (anthocyanins and flavan-3-ols) and phenolic acids in red wine extracts appears to result in the
331 formation of metabolites, such as phenylvaleric, phenylpropionic, phenylacetic and benzoic acid derivatives
332 (Sánchez-Patán et al., 2012). Many of these metabolites still contain free hydroxyl groups and could
333 contribute to enhance antioxidant capacity *in vivo* (Fernández-Panchón, Villano, Troncoso, & García-
334 Parrilla, 2008; Pérez-Jiménez & Saura-Calixto, 2008; Scalbert & Williamson, 2000b).
335 Regarding the ability of the seasoning-derived compounds to act as ACE inhibitors *in vitro*, higher capacity
336 was shown by the BGID (32.9 ± 0.9%) than the BCF (11.2 ± 4.5%) treatment. Several studies have
337 confirmed the inhibitory power of certain polyphenols against ACE activity by competing with the substrate
338 (Ang I) for the active sites of the enzyme (Actis-Goretta, Ottaviani, Keen, & Fraga, 2003; Guerrero et al.,
339 2012; Hidalgo et al., 2012). The ACE inhibitory capacity of flavonoid-rich foods appears to be especially
340 related to their procyanidin and total flavan-3-ol content, and may depend on the mean degree of
341 polymerization (mDP) of proanthocyanidins. An IC_{50} in the 100 µM range for dimer and trimer fractions,
342 and within the mM range for monomeric flavan-3-ols and other phenolic compounds have been described
343 (Actis-Goretta, Ottaviani, & Fraga, 2006). Recently, the effect of *in vitro* gastrointestinal digestion on the
344 ACE inhibitory activity of a grape skin extract was studied (Fernández & Labra, 2013). These authors noted

345 that 80% of polymeric proanthocyanidins (mDP ~19) were degraded during digestion in the small intestine to
346 low-molecular-weight (mDP ~2) oligomers, which crossed the dialysis membrane and efficiently inhibited
347 around 60% of ACE activity. However, to the best of our knowledge, the present study has for the first time
348 evaluated the ACE inhibitory power of fractions obtained after simulating the action of colonic microbiota.
349 Structural changes of flavonoids can have an important impact on their potential as ACE inhibitors (Guerrero
350 et al., 2012). Accordingly, the lower *in vitro* ACE inhibitory ability observed for the BCF rather than the
351 BGID products may be due to the presence of simpler molecules in the BCF samples following
352 depolymerisation and transformation of phenolic precursors by colonic microbiota (Aura, 2008). In fact,
353 previous investigations using chemically synthesized anthocyanidin-3-*O*-glucosides and several of their
354 putative gut breakdown metabolites have shown that standard compounds of phenolic acids exhibited lower
355 ACE inhibitory capacity than their supposed parent molecules (Hidalgo et al., 2012).
356
357 **3.2. Cell viability and biomarkers of oxidative stress and endothelial dysfunction**
358 Reduced cell proliferation and viability have been reported after short-term (McGinn, Saad, Poronnik, &
359 Pollock, 2003) and long-term (Varma et al., 2005) exposure of HUVECs to high glucose concentrations,
360 independently of osmotic changes. Likewise, around 15% lower cell viability was observed in the current 24-
361 hour study for hyperglycaemic than normoglycaemic cells (**Fig. 1**). After the incubation of HUVECs
362 EA.hy926 with the seasoning-derived digestion products, in both Low and High GL mediums, the number of
363 viable cells in comparison with control cells was about 85% (BGID) and 90% (BCF). Therefore, non-
364 cytotoxic doses of the treatments were used, as previously considered for concentrations of phenolic
365 compounds resulting in higher than 80% cell viability (Margina, Gradinaru, Manda, Neagoe, & Ilie, 2013;
366 Müller et al., 2009; Stagos et al., 2014).
367 The state of oxidative stress *in vivo* is governed by the intricate interplay between enzymatic pathways
368 accountable for ROS production and the various endogenous or exogenous anti-oxidant mechanisms
369 responsible for ROS elimination (Lee et al., 2012). Intracellular GSH was selected as representative of the
370 endogenous non-enzymatic mechanisms protecting against oxidative damage, so the GSH/GSSG ratio served
371 as a marker of the overall cell redox environment (Moskaug, Carlsen, Myhrstad, & Blomhoff, 2005). The
372 results showed a decrease higher than 40% in the GSH/GSSG ratio of control cells in response to high
373 glucose conditions (**Fig. 2**). This finding indicates an enhanced use of GSH to scavenge free radicals, either
374 directly or acting as a substrate for GSH-dependent enzymes (GSH peroxidases and transferases) to protect

375 against hydrogen peroxides, lipid hydroperoxides and electrophilic toxic compounds (Masella, Di Benedetto,
376 Varì, Filesi, & Giovannini, 2005). Furthermore, the reduced GSH/GSSG ratio may be due to the decreased
377 availability of NAD(P)H under oxidative stress induced by hyperglycaemia, with NAD(P)H being required
378 for the activity of glutathione reductase to transform GSSG into GSH (Srivastava et al., 1989). Under
379 hyperglycaemic conditions, the incubation of HUVECs EA.hy926 with the BCF compounds was able to
380 improve the intracellular redox environment significantly with respect to control cells. These results suggest
381 that bioavailable colonic fermented metabolites derived from the seasoning may have a great capacity to
382 restore a balanced redox state in hyperglycaemic endothelial cells. Phenolic metabolites might exert direct
383 protection in biological systems by scavenging ROS, thereby decreasing the need for endogenous GSH.
384 Nonetheless, several lines of research support an indirect action as a more probable mechanism, as
385 exogenous antioxidants may activate the endogenous defence systems through antioxidant responsive
386 elements (AREs), which are known to stimulate the transcription of the rate-limiting enzyme for GSH
387 synthesis in a nuclear factor erythroid 2-related factor 2 (Nrf2)-dependent manner (Martinez, Mitjans, &
388 Vinardell, 2014; Masella et al., 2005; Moskaug et al., 2005).
389 The oxidation products of biological molecules are also widely used as biomarkers of oxidative damage and
390 provide specific evidence of cellular oxidative stress status and changes induced by exposure to antioxidant
391 nutrients (Ho, Karimi Galougahi, Liu, Bhindi, & Figtree, 2013). Oxidative stress and subsequent lipid
392 peroxidation are known to disturb the integrity of cellular membranes, leading to the release of cytoplasmic
393 enzymes and pro-inflammatory factors to the extracellular medium (Margina et al., 2013). These aspects
394 were assessed using MDA and CG levels (**Fig. 3**A and B) as biomarkers of oxidative damage to lipids and
395 proteins, respectively, and LDH leakage (**Fig. 3**C) as an indicator of cell membrane damage (L. Li & Lau,
396 1993). In the control cells, hyperglycaemic conditions caused a significant increase in the levels of MDA and
397 protein CGs, as well as in the plasma membrane permeability (LDH leakage). The BGID and the BCF
398 treatments tended to ameliorate high glucose-induced protein oxidation and, more markedly, lipid
399 peroxidation and LDH leakage. Concretely, a significant protection of lipids in the BCF-treated cells, and of
400 the cell membrane in both the BGID- and the BCF-treated cells, was observed with respect to the control
401 HUVECs EA.hy926 incubated in the High GL medium. The results suggested that the BGID and especially
402 the BCF compounds helped to preserve endothelial cell membrane integrity under hyperglycaemia-induced
403 oxidative conditions. This finding agrees with previous studies reporting the ability of certain polyphenols to

404 restore the cell membrane fluidity and transmembrane potential in hyperglycaemic HUVECs (Margina et al.,
405 2013).
406 Oxidative stress implicated in the cell membrane and biomolecules damage is also linked to functional
407 alterations in vascular cells. Endothelial function is controlled by a complex network that regulates ROS
408 production and clearance, concurrently with NO generation and inactivation, but this network is impaired
409 under hyperglycaemic conditions (Hadi & Suwaidi, 2007; Inoguchi et al., 2000). In the current study, the
410 exposure of control cells to the High GL medium caused a marked 76.4% increase in ROS levels and,
411 simultaneously, a significant 34.8% decrease in the concentration of NO breakdown products (**Fig. 4**). This
412 endothelial dysfunction was in line with previous studies reporting that the exposure of HUVECs to high
413 glucose concentrations more or less caused ROS generation to double (Chao, Hou, Lee Chao, Weng, & Ho,
414 2009; Koziel et al., 2012) and led to a significant decrease in NO bioavailability (Juhong Yang, Wang, Li,
415 Zhang, & Feng, 2010; Zhou et al., 2012). It is widely recognized that NOXs represent the primary source of
416 ROS ($O_2^{\cdot-}$ and H_2O_2) in the vascular wall, production of which is triggered by stimulation of vasoconstrictor
417 agents such as Ang II and endothelin-1 (Virdis et al., 2011). A number of additional sources of ROS have
418 been identified under conditions of oxidative stress and hyperglycaemia (Münzel et al., 2010; Pennathur &
419 Heinecke, 2007; Zhou et al., 2012), including uncoupled eNOS, which implies that the physiological activity
420 of the enzyme for NO production is decreased and switched to the deleterious $O_2^{\cdot-}$ generation (Roe & Ren,
421 2012; Virdis et al., 2011). NO bioavailability also depends on its rapid inactivation by reaction with $O_2^{\cdot-}$ to
422 form $ONOO^-$ (W. Chen et al., 2010), which can further explain the decreased NO levels under the
423 hyperglycaemic conditions observed in this study. Interestingly, the BGID and the BCF treatments were able
424 to partly restore the ROS levels detected in hyperglycaemic cells to levels found in normoglycaemic cells.
425 Furthermore, incubation with these treatments prevented hyperglycaemia-impaired NO bioavailability in
426 HUVECs EA.hy926, with significantly higher NO concentrations found in the medium of the treated rather
427 than the control cells. The seasoning-derived BCF compounds showed a higher potential than the BGID
428 compounds to improve endothelial function. Similar trends in these beneficial treatment effects were
429 observed in cells exposed to the Low and the High GL mediums, but more markedly under hyperglycaemic
430 conditions. According to previous studies (Edwards, Czank, Woodward, Cassidy, & Kay, 2015; Ruijters,
431 Weseler, Kicken, Haenen, & Bast, 2013; Steffen, Gruber, Schewe, & Sies, 2008), the results could be due to
432 the anti-ROS effects of those phenolic compounds and metabolites present in the seasoning-derived
433 treatments. Although only a limited number of individual phenolic metabolites were able to enhance the

434 endothelial NO levels (Appeldoorn et al., 2009), the NO-preserving actions of these compounds have been
435 suggested to rely mainly on NOX inhibition and perhaps partially in combination with their $O_2^{\cdot-}$ scavenging
436 capacity (Steffen et al., 2008). In fact, the lower ROS levels and reduced NO inactivation detected in the
437 BCF-treated cells were in accordance with the higher antiradical activity (ABTS values) for the BCF than the
438 BGID products observed *in vitro*. In addition, phenolic compounds may increase NO production through
439 eNOS protein activation mediated by the phosphatidylinositol 3-kinase/Akt pathway (Schini-Kerth, Auger,
440 Kim, Étienne-Selloum, & Chataigneau, 2010; Schmitt & Dirsch, 2009).
441
442 **3.3. Possible mechanisms of action implicated in the protection against oxidative stress and endothelial**
443 **dysfunction under hyperglycaemia**
444
445 **3.3.1. Changes in NOX4, eNOS, SOD2 and HO-1 gene expression**
446 In physiological terms, controlled ROS production plays an important role in cell signalling and maintains
447 normal vascular contraction-relaxation and cell growth. The enhanced ROS production under pathological
448 conditions is also known to stimulate several redox-sensitive signalling pathways (Virdis et al., 2011;
449 Zinkevich & Gutterman, 2011). In the present study, changes were therefore assessed in the mRNA levels of
450 four genes involved in the endothelial cells ROS and NO balance (NOX4 and eNOS) and the endogenous
451 antioxidant system (SOD2 and HO-1) (**Fig. 5**). This assessment was done to elucidate some of the
452 mechanisms underlying the observed protective effects of the potentially bioavailable compounds derived
453 from the seasoning.
454 In the hyperglycaemic control cells, gene expression of the endothelial ROS generating enzyme NOX4
455 increased more than 2-fold, whereas no changes in the eNOS mRNA levels were detected (**Fig. 5**A and B,
456 respectively). The observed NOX4 up-regulation agrees with previously published works and has been
457 described as mediated by the activation of phosphatidylinositol 3-kinase/Akt (Zhou et al., 2012) and protein
458 kinase C pathways (Chai et al., 2008), and by the stimulation of Ang II (Selemidis, Sobey, Wingler, Schmidt,
459 & Drummond, 2008). However, it is unclear how hyperglycaemia affects eNOS gene expression in
460 HUVECs, which might be up-regulated (Ding et al., 2004; H. Li et al., 1998), down-regulated (Sun & Liao,
461 2004) or, in accordance with our observations, not significantly affected (Patel et al., 2013). The results of
462 the present study suggest that the decreased NO levels observed in the hyperglycaemic control cells were

463 unlikely to be due to down-regulation of eNOS gene expression. It might therefore involve other previously
464 cited mechanisms, such as ROS-mediated NO inactivation and eNOS uncoupling.
465 Interestingly, the treatment of hyperglycaemic HUVECs EA.hy926 with the BGID and the BCF compounds
466 both resulted in a significant decrease in the levels of NOX4 and eNOS mRNA. In line with different authors
467 working with grape pomace extracts and standard phenolic compounds (Álvarez et al., 2012; Spanier et al.,
468 2009), the current results could be associated with the ability of the seasoning-derived phenolic compounds
469 to down-regulate NOX4 gene expression, with a higher potential observed for the potential colonic
470 fermented metabolites. This capacity may be a key mechanism to explain the lower ROS levels found in the
471 BGID and the BCF-treated cells. In addition, our results appear to suggest a compensatory decrease in the
472 eNOS gene expression in response to the enhanced NO levels described above for hyperglycaemic cells
473 treated with the BGID and especially the BCF products.
474 In parallel to the mRNA level changes observed for the ROS and NO generating enzymes, the gene
475 expression of the mitochondrial antioxidant enzyme SOD2 was 6-fold up-regulated, whereas the HO-1was
476 0.6-fold down-regulated, in the hyperglycaemic control cells (**Fig. 5**C and D). These results are in agreement
477 with other authors (Y. Y. Chen et al., 2012; Koziel et al., 2012) and they may reflect the endothelial cell
478 response under hyperglycaemic conditions in an attempt to counteract over-production of $O_2^{\cdot -}$ (Patel et al.,
479 2013). The vascular up-regulation of SOD2 is attributed to the presence of response elements in its promoter
480 for the redox-sensitive transcription factors activator protein-1 (AP-1) and nuclear factor-kappa B (NF-κB)
481 (Faraci & Didion, 2004), which are known to be activated under hyperglycaemia (S. Chen, Mukherjee,
482 Chakraborty, & Chakrabarti, 2003). However, controversial results can be found in the literature on the
483 modulation of HO-1 gene expression by glucose in the vasculature, as this modulation has been suggested to
484 depend upon levels of glucose (Iori et al., 2008).
485 SOD2 mRNA levels were considerably decreased in the hyperglycaemic BCF-treated cells, and also
486 significantly reduced in normoglycaemic cells after incubation with both treatments. In contrast, the HO-1
487 gene was over-expressed to a similar extent by the BGID and the BCF treatments, but the effects were more
488 marked for hyperglycaemic (3.7-fold) than normoglycaemic cells (2.6-fold). These results are consistent with
489 several lines of evidence suggesting that some phenolic compounds and their metabolites have the capacity
490 to improve endothelial function by HO-1 gene over-expression, mainly through the Nrf2/ARE pathway
491 activation (Edwards et al., 2015; Ungvari et al., 2010). The observed HO-1 up-regulation almost certainly
492 contributed to the decreased ROS levels observed in the treated cells, a fact that may further influence other

493 redox-sensitive signalling pathways and enzymes that were over-expressed due to the increased oxidative
494 environment in cells, such as might be the case of SOD2. The direct ROS scavenging capacity of the
495 seasoning-derived phenolic compounds and their ability to inhibit the high glucose-induced NF-kB
496 translocation to the nucleus, as previously reported for certain flavan-3-ols (Jian Yang et al., 2013), might
497 also explain the decreased requirement for SOD2 gene expression in the treated cells.
498

499 **3.3.2. Changes in *ex vivo* ACE activity**

500 Renin-angiotensin aldosterone system (RAAS) and ACE activation are associated with alterations in
501 endothelial function observed in diabetes and hypertension (Hadi & Suwaidi, 2007; Hsueh & Wyne, 2011).
502 In this regard, the Ang II over-production observed in hyperglycaemic cells is known to contribute to NOXs
503 over-expression and activation, increased ROS generation, and subsequent endothelial dysfunction
504 (McFarlane et al., 2003; Selemidis et al., 2008; Virdis et al., 2011). In addition, Ang II significantly up-
505 regulates mRNA expression of renin, ACE, and angiotensinogen (the Ang II precursor), thus resulting in a
506 vicious circle of RAAS activation in endothelial cells (Ide, Hirase, Nishimoto-Hazuku, Ikeda, & Node,
507 2008). Interestingly, there is accumulating evidence that flavonoids and polyphenol-enriched plant extracts
508 are effective ACE inhibitors, although most of them have been shown to be more effective *in vitro* than *ex*
509 *vivo* or *in vivo* (Balasuriya & Rupasinghe, 2011). Consequently, the *ex vivo* capacity of the BGID and the
510 BCF products to down-regulate ACE activity in HUVECs EA.hy926 was also evaluated in this study.
511 The results showed that ACE activity was around 21% higher in lysates obtained from hyperglycaemic rather
512 than from normoglycaemic cells (**Fig. 6**). This significant increase in ACE activity was in accordance with
513 the enhanced ACE-1 expression previously reported in HUVECs under hyperglycaemia (F. Chen et al.,
514 2014).
515 However, *ex vivo* ACE activity was significantly decreased in the hyperglycaemic treated cells by about
516 13.6% (BGID) and 9.7% (BCF). These findings reveal that the BGID and the BCF treatments interfered in
517 high glucose-induced RAAS activation. This effect may be partly explained by the direct capacity of the
518 phenolic compounds released from the seasoning along digestion to inhibit ACE activity, which is supported
519 by our *in vitro* ACE results, and by previous experiments *in vitro* and in HUVECs that demonstrated the
520 ACE inhibitory capacity of certain flavonol metabolites (Balasuriya & Rupasinghe, 2012). In addition, the
521 decreased Ang II production resulting from this direct ACE inhibition might lead to an indirect effect of the

522 BGID and the BCF compounds down-regulating ACE expression in hyperglycaemic cells during the
523 incubation period with these treatments (Ide et al., 2008).
524
525 **4. Conclusions**
526 The findings of this study have provided novel and compelling evidence of the potential protective effects of
527 pre- and post-colonic digested compounds derived from a seasoning obtained from wine pomace against
528 hyperglycaemia-induced endothelial dysfunction and oxidative damage.
529 Probable mechanisms of action by which these compounds restore the redox environment and endothelial
530 function in HUVECs EA.hy926 are: direct ROS scavenging; modulation of NOX4, SOD2 and HO-1 gene
531 expression by inducing different redox-sensitive signalling pathways; and, their capacity to decrease ACE
532 activity.
533 The metabolism of bioactive compounds by colonic microbiota was found to play a key role along the
534 digestive process in the beneficial vascular effects observed for the seasoning that has been assessed.
535 Altogether, this *ex vivo* study encourages the incorporation of seasonings derived from wine pomace in
536 functional foods and sets the stage for future animal and clinical trials in order to confirm *in vivo* the
537 promising healthy properties of such seasonings in cases of diabetes, hypertension, and other cardiovascular
538 diseases.
539
540 **Conflicts of interest**
541 The authors declare no conflict of interest.
542
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549
550 **Appendix: Supplementary material**
551 Supplementary data to this article (**Table S1**) can be found online at

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780

781

782 **Figure captions**

783

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

791

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

799

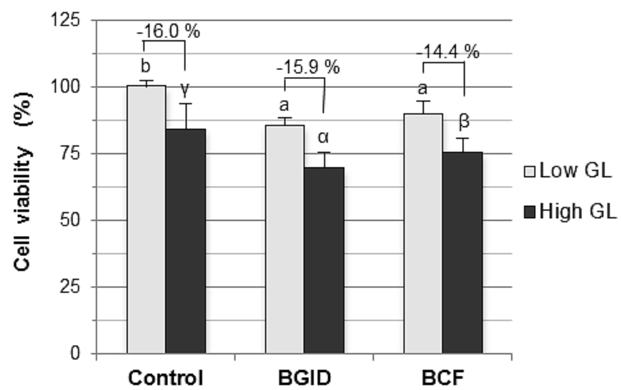
800 **Fig. 3. Biomarkers of lipid, protein and cell membrane oxidative damage of normoglycaemic (Low**
801 **GL) and hyperglycaemic (High GL) HUVECs EA.hy926 treated with the seasoning digested products.**
802 **A)** Malondialdehyde (MDA). **B)** Carbonyl groups (CGs). **C)** % lactate dehydrogenase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

809

810 **Fig. 4. Intracellular ROS and extracellular NO levels of normoglycaemic (Low GL) and**
811 **hyperglycaemic (High GL) HUVECs EA.hy926 treated with the seasoning digested products. A)**

812 Overall intracellular ROS production by HUVECs EA.hy926 was estimated by folds of 2,7-
813 dichlorofluorescein (DCF) fluorescence increases with respect to the control normoglycaemic cells. **B)** NO
814 generation by HUVECs EA.hy926 was estimated as the concentration (µM) in the culture medium of the
815 total sum of NO breakdown products ($NO_2^- + NO_3^-$). Cells for both assays were treated with the potentially
816 bioavailable compounds obtained after the *in vitro* gastrointestinal digestion (BGID) or colonic fermented
817 (BCF) of the seasoning assessed. Data expressed as mean values ± standard deviation (n = 3). Significant
818 differences (p<0.05) between hyperglycaemic and normoglycaemic cells are indicated in % values.
819 Significant differences (p<0.05) between the control, the BGID-, and the BCF-treated cells are expressed in
820 Roman (Low GL) and Greek letters (High GL).
821
822 **Fig. 5. Gene expression in normoglycaemic (Low GL) and hyperglycaemic (High GL) HUVECs**
823 **EA.hy926 treated with the seasoning digested products.** Genes analysed: **A**) NOX4, **B**) eNOS, **C**) SOD2,
824 and **D**) HO-1. Cells were treated with the potentially bioavailable compounds obtained after the *in vitro*
825 gastrointestinal digestion (BGID) or colonic fermented (BCF) of the seasoning under study. mRNA levels of
826 the genes under assessment were determined by quantitative real-time PCR, normalized to the GAPDH gene
827 expression. The results are presented as relative fold changes with respect to the control normoglycaemic
828 cells. Data expressed as mean values ± standard deviation (n = 3). Significant changes (p<0.05) between
829 hyperglycaemic and normoglycaemic cells are indicated with an asterisk (*). Significant differences (p<0.05)
830 between the control, the BGID-, and the BCF-treated cells are expressed in Roman (Low GL) and Greek
831 letters (High GL).
832
833 **Fig. 6. ACE activity determined *ex vivo* in normoglycaemic (Low GL) and hyperglycaemic (High GL)**
834 **HUVECs EA.hy926 treated with the digested products obtained from the seasoning evaluated.** BGID:
835 potentially bioavailable samples after simulated gastrointestinal digestion; BCF: potentially bioavailable
836 samples after simulated colonic fermentation. Data expressed in % as mean values ± standard deviation (n =
837 3) with respect to the control normoglycaemic cells (100%). Significant differences (p<0.05) between
838 hyperglycaemic and normoglycaemic cells are indicated in % values. Significant variations (p<0.05)
839 between the control, the BGID-, and the BCF-treated cells are expressed in Roman (Low GL) and Greek
840 letters (High GL).

Fig. 1 (One-column Figure)



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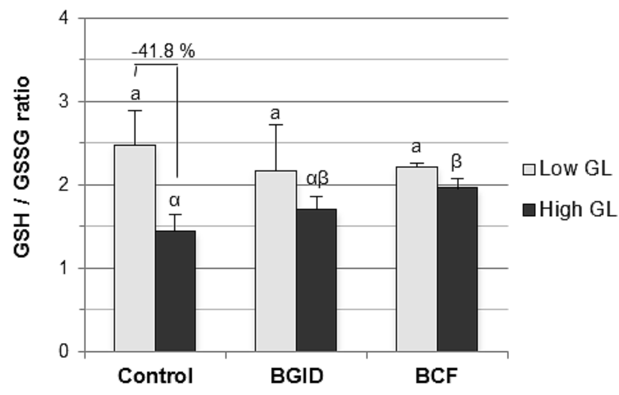

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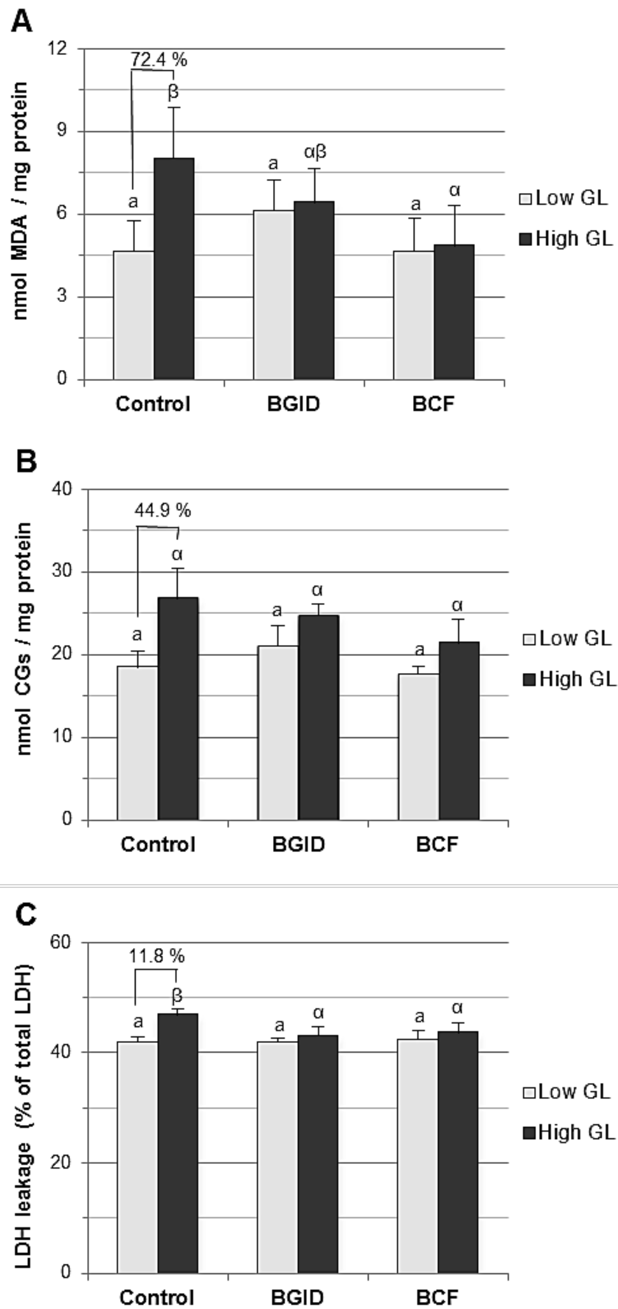

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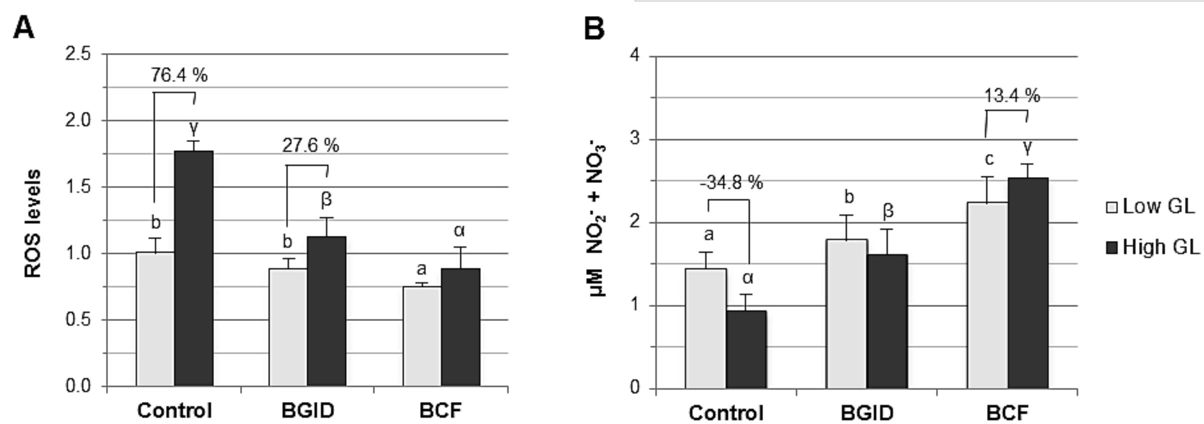

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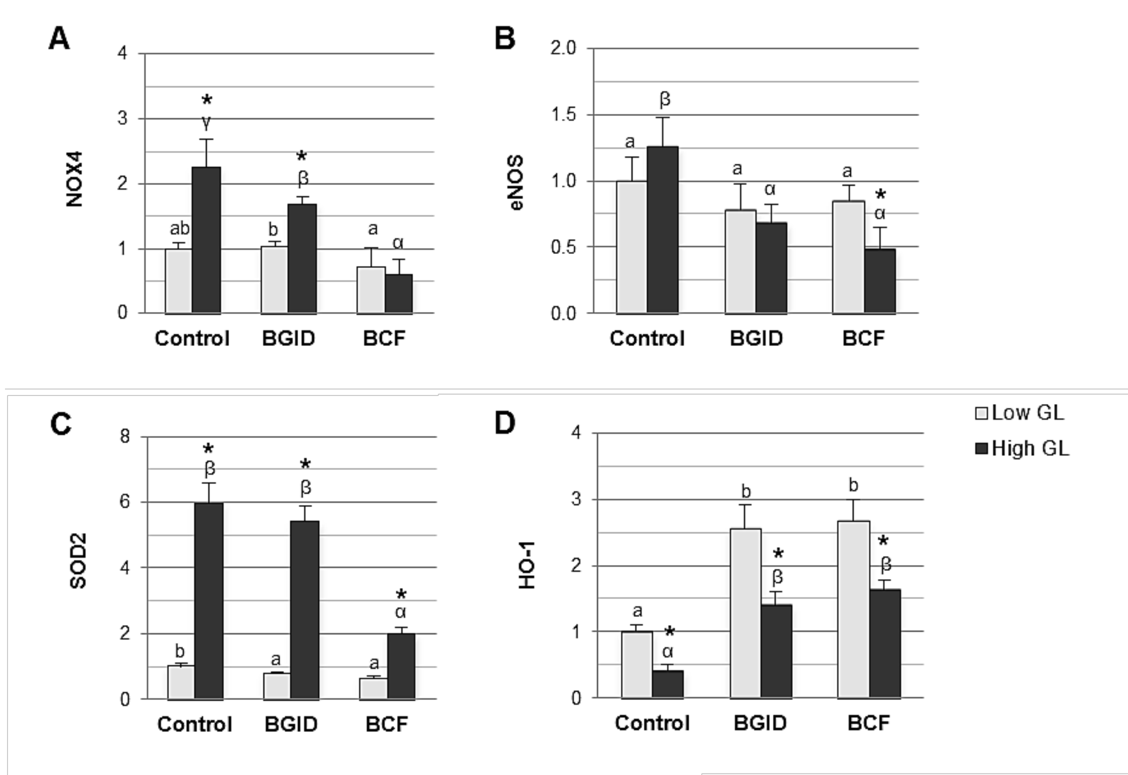

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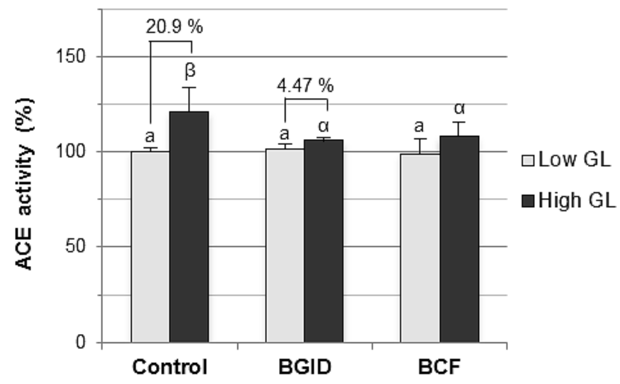

Table S1. **Detailed HPLC-DAD characterization of the wine pomace seasoning (poly)phenolic composition.**

(Poly)phenolic compounds	Concentration (µg/g)[a]
Phenolic acids	
Hydroxybenzoic acids	
p-OH-benzoic acid	6.27 ± 0.12
Salicylic acid	18.7 ± 0.1
Protocatechuic acid	21.3 ± 0.2
Gallic acid	73.1 ± 0.4
Vanillic acid	30.4 ± 0.3
Syringic acid	74.4 ± 3.5
Ellagic acid	21.4 ± 0.1
Ethyl gallate	17.1 ± 0.1
Hydroxycinnamic acids	
p-Coumaric acid	5.76 ± 0.07
Caffeic acid	4.04 ± 0.13
Ferulic acid	1.22 ± 0.02
Caftaric acid	11.4 ± 0.7
Stilbenes	
t-resveratrol	1.73 ± 0.13
t-piceid	1.42 ± 0.05
Flavan-3-ols	
Flavan-3-ols (monomers)	
Catechin	47.7 ± 2.0
Epicatechin	20.0 ± 1.7
Flavan-3-ols (dimers)	
Procyanidin B1	93.0 ± 4.3
Procyanidin B2	39.2 ± 1.6
Flavonols	
Flavonol aglycones	
Kaempferol	3.58 ± 0.09
Quercetin	19.0 ± 0.8
Myricetin	69.5 ± 2.7
Flavonol-3-*O*-glycosides	
Kaempferol-3-*O*-rutinoside	122 ± 2
Kaempferol-3-*O*-glucoside	51.6 ± 0.4
Quercetin-3-*O*-rutinoside	24.9 ± 0.4
Myricetin-3-*O*-rhamnoside	52.7 ± 2.5
Anthocyanidins	
Delphinidin	301 ± 8
Cyanidin	6.30 ± 1.06
Petunidin	118 ± 20
Peonidin	30.9 ± 2.8
Malvidin	1577 ± 109

a) Results expressed as mean value ± standard deviation (n=3).