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Highlights

Food Research International xxx (2013) xxx–xxx

Low-molecular procyanidin rich grape seed extract exert antihypertensive effect in males spontaneously hypertensive rats (SHR)

M. Quiñones [a], L. Guerrero [a,b], M. Suarez [a,c], Z. Pons [a], A. Aleixandre [d], L. Arola [a,c], B. Muguerza [a,c,]*

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► GSPE decreases SBP and DBP on hypertensive rats, but not in normotensive rats. ► Maximum decrease of SBP at 6 h post-administration of 375 mg/kg GSPE. ► GSPE increases activity of reduced glutathione, an endogenous antioxidant system. ► GSPE is rich in monomers, dimers and trimers in free form or linked to gallate. ► ACE activity and malondialdehyde, biomarker of oxidative stress, was not modified.

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ABSTRACT

Grapes are a good source of flavonoids, which have been previously demonstrated to exert beneficial healthy effects on cardiovascular diseases. The aims of this study were to extensively characterise a grape seed procyanidin extract (GSPE) (total phenolic content, antioxidant capacity and HPLC–MS phenolic profile) and, to assess its antihypertensive effect in spontaneously hypertensive rats (SHR) which is a model of genetically hypertensive rat analogue to the essential hypertension in humans. The hypotensive effect of GSPE was also proved in normotensive Wistar–Kyoto rats. Chromatographic analysis of the extract showed that the most abundant polyphenols are monomers and dimers, in their free forms and linked to a gallate. GSPE produced a significant decrease in systolic and diastolic blood pressure of SHR dose-dependently up to 375 mg/kg (maximum decrease 6 h post-administration) and did not affect blood pressure of Wistar–Kyoto rats. GSPE increased the activity of an antioxidant endogen system, but did not affect plasma ACE activity in these animals.

1. Introduction

Hypertension (HTN) is a major risk factor for stroke and is the most common disease found in primary care (Chobanian et al., 2003). It is estimated that by 2025, the incidence of hypertension will increase to 24% in developed countries and to 80% in developing countries (Messerli, Williams, & Ritz, 2007). The current and common method for controlling hypertension is the use of long-term drug therapy. However, it is well known that drugs have many side effects, which may complicate the patient's medical condition. New strategies for treating hypertension based on natural products could greatly benefit hypertensive patients. In this context, there is evidence that a diet rich in vegetables and fruits, which are rich in flavonoids and phenolic compounds, helps to control arterial blood pressure. In fact, increased fruit and vegetable intake has been included recently in the guidelines for the management of arterial hypertension (Mancia et al., 2007).

Grapes and wine are well known as significant sources of flavonoids (Aherne & O'Brien, 2002), which exhibit several pharmacological properties, including vasodilator (Andriambeloson et al., 1997; Diebolt, Bucher, & Andriantsitohaina, 2001; Moura et al., 2002; Zenebe, Pechanova, & Andriantsitohaina, 2003), antihypertensive (Diebolt et al., 2001; Jang & Lee, 2011) and antioxidant (Frankel, German, Kinsella, Parks, & Kanner, 1993; Jang & Lee, 2011; Moura et al., 2002) activities. These activities have led to grapes and wine being considered as functional food candidates (Barreiro-Hurlé; Colombo, & Cantos-Villar, 2008; Gollucke, 2010; Schieber, Stintzing, & Carle, 2001; Shrikhande, 2000). Nevertheless, different grape products often widely vary in both the type and content of flavonoids, and the characterisation of the types of polyphenols present in a grape variety or grape-derived product is important for understanding the possible health-promoting effects associated with its consumption. In fact, grape botanical variety/species, cultivation area, harvesting season, cultural practice, sun exposure, environmental factors, grape maturity, and manufacturing factors may affect the flavonoid content of grapes, grape extracts or wine (Aherne & O'Brien, 2002; Downey, Dokoozlian, & Krstic, 2006; Yang, Martinson, & Liu, 2009). In addition, the phenolic distribution in the juice, pulp, skins and seeds is very different; the phenolic contents of these components are approximately 5%, 1%, 30% and 64%, respectively (Singleton, 1981; Singleton & Esau, 1969). Moreover, in the grape fruit, flavonoids, such as anthocyanins and resveratrol, are mainly localised in the skins, whereas the procyanidins or flavanols are principally located in the seeds (Yang et al., 2009).

Grape seeds are a by-product of the grape/wine industry, but they are one of the richest sources of procyanidins (Nakamura, Tsuji, & Tonogai, 2003), and their beneficial effects have been extensively investigated. Our research group has demonstrated that a grape seed

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procyanidin-rich extract (GSPE) exhibits antioxidant capacity (Puiggròs et al., 2005), improves lipid metabolism (Del Bas et al., 2005), limits adipogenesis (Pinent et al., 2005), acts as an insulin-mimetic agent (Pinent et al., 2004) and reduces inflammation (Terra et al., 2011).

Procyanidin-rich foods, such as cocoa, have demonstrated antihypertensive properties (Buijsse, Feskens, Kok, & Kromhout, 2006; Taubert, Roesen, Lehmann, Jung, & Schömig, 2007). The antihypertensive properties of procyanidins are associated with different biological activities, such as nitric oxide-mediated vasodilation (Duffy et al., 2001; Fisher, Hughes, Gerhard-Herman, & Hollenberg, 2003; Mukai & Sato, 2009; Schroeter et al., 2006; Stein, Keevil, Wiebe, Aeschlimann, & Folts, 1999; Yamamoto, Suzuki, & Hase, 2008), angiotensin-converting enzyme (ACE) inhibition (Actis-Goretta, Ottaviani, & Fraga, 2006; Actis-Goretta, Ottaviani, Keen, & Fraga, 2003; Dong, Xu, Liang, Head, & Bennett, 2011; Ottaviani, Actis-Goretta, Villordo, & Fraga, 2006) and reduction of oxidative stress (Mane, Loonis, Juhel, Dufour, & Malien-Aubert, 2011; Ramiro-Puig et al., 2007). Although there are evidences in human studies that the whole grape fruit improves blood pressure and other factors related to vascular function in men with metabolic syndrome (Barona, Aristizabal, Blesso, Volek, & Fernandez, 2012) no investigation has yet been performed on the antihypertensive effects of GSPE in male hypertensive rats. As has been explained above, the phenolic composition of the grape seed differs from the composition of the whole grape. Therefore, it is necessary to extensively characterise the grape seed extract to better relate the resulting effects with the specific combination and concentration of molecules present in the extract.

The aims of the present study were to characterise and quantify both the flavonoid content present in GSPE and the total antioxidant capacity of this extract. We also evaluated the short-term effects of GSPE in an experimental model of hypertension. The underlying mechanisms involved in the antihypertensive effects of procyanidins have not been clarified in detail, but a better understanding of these mechanisms will allow a rational development of functional foods rich in polyphenols for blood pressure control. Therefore, in this study, we also investigated the possible mechanisms involved in the antihypertensive effects of GSPE.

2. Material and methods

2.1. Grape seed procyanidin-rich extract

The grape seed procyanidin-rich extract (GSPE) was obtained from white grape seeds and was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France). According to the manufacturer, the procyanidin profile of the extract was composed of monomers or flavan-3-ols (21.3%), dimers (17.4%), trimers (16.3%), tetramers (13.3%) and oligomers (5–13 units; 31.7%) of procyanidins.

2.2. Characterisation of GSPE

2.2.1. Solvents and phenolic standards

The following commercial standards were used for quantitative determination by HPLC: protocatechuic acid, eriodictyol-7-*O*-glucoside, chlorogenic acid, quercetin-3-*O*-galactoside, quercetin-4-*O*-glucoside, kaempferol-3-*O*-rutinoside, naringenin-7-*O*-glucoside, isorhamnetin-3-*O*-rutinoside, kaempferol-3-*O*-glucoside, isorhamnetin-3-*O*-glucoside, eriodictyol, isorhamnetin and procyanidin B2, which were purchased from Extrasynthese (Genay, France). (+)-Catechin and (−)-epicatechin were purchased from Fluka Co. (Buchs, Switzerland), and naringenin, kaempferol, vanillic acid, *p*-coumaric acid, 3-hydroxybenzoic acid, gallic acid, rutin, and (−)-epigallocatechin gallate were purchased from Sigma Aldrich (St. Louis, MO). 2,2′-Azo-bis(2-methylpropionamidine) dihydrochloride (AAPH) was purchased from Acros Organics. Fluorescein was purchased from Fluka/Sigma-Aldrich (Madrid, Spain), and Folin–Ciocalteu's reagent and Trolox were purchased from Sigma (Barcelona, Spain). Organic solvents (high performance liquid chromatography [HPLC]-grade) were obtained from Scharlab (Barcelona, Spain) and Merck (Darmstadt, Germany).

2.2.2. Quantification of the total phenolic content of GSPE

The total phenolic content of GSPE was estimated spectrophotometrically using a Hitachi U-1900 Spectrophotometer by means of the Folin–Ciocalteu assay at 725 nm (Singleton & Esau, 1969). The assay was performed in triplicate, and the samples were dissolved in ethanol:water (1:1). The results were expressed as mg of gallic acid per g of fresh GSPE extract.

2.2.3. Analysis of individual phenolic compounds of GSPE by reverse phase chromatography coupled to mass spectrometry

To study the extract in greater detail, individual phenolic compounds of the GSPE (both flavan-3-ols and phenolic acids) were characterised by an HPLC coupled to a UV detector (Agilent 1200 Series) and a time-of-flight mass spectrometer (TOF 6210, Agilent). The HPLC–MS system consisted of an Agilent 1200 Series instrument (Agilent Technologies) with a Zorbax SB-Aq column (3.5 μm, 150 mm × 2.1 mm internal diameter [i.d.]) equipped with a Pre-Column Zorbax SB-C18 (3.5 μm, 15 mm × 2.1 mm i.d.), which was also from Agilent, and Masshunter software. During the analysis, the column was kept at 30 °C and the flow rate was 0.21 mL/min. The solvent composition of solvent A was Milli-Q water/acetic acid (99.8:0.2 v/v), and that of solvent B was acetonitrile/acetic acid (99.8:0.2 v/v). Initially, 2% solvent B was used. The proportion of solvent B was gradually increased, reaching 20% at 33 min, 22.5% at 34.2 min, 23.2% at 40 min, 25% at 63 min and 100% at 72 min. Then, solvent B was reduced to the initial proportion at 75 min and maintained at this level until 90 min to re-equilibrate the column at these initial conditions. The injection volume was 9.4 μL, and all the freeze-dried samples were re-dissolved in water:acetone: acetic acid (27.5:70:0.5 v/v/v).

The wavelength of the UV detector was set at 280 nm. Ionisation in the mass spectrometer was performed by electrospray (ESI) in the negative mode, and the source parameters were as follows: capillary voltage, 4 kV; fragmentor, 125 V, source temperature, 150 °C; desolvation gas temperature, 350 °C, with a flow rate of 9 L/min and a drying gas flow rate of 12 L/min. Nitrogen was used as the cone gas.

Individual phenols were quantified with a six-point regression curve by using standards obtained from commercial suppliers.

2.2.4. Oxygen radical absorbance capacity assay

The characterisation of the GSPE was completed with the evaluation of its antioxidant activity in terms of its hydrophilic oxygen radical absorbance capacity (ORAC assay). The ORAC assay was performed according to the methodology reported previously (Huang, Ou, Hampsch-Woodill, Flanagan, & Prior, 2002) with some modifications (Suárez, Romero, Ramo, Maciá, & Motilva, 2009). This method analyses the peroxyl radical-scavenging activity of the samples. The assay was performed in 96-well microplates with an FLx800 Fluorescence Microplate Reader (Bio-Tek-IZASA, Barcelona, Spain) with an excitation filter set at 485 nm and an emission filter set at 520 nm. The Gen5™ Data Analysis Software controlled the fluorescence plate reader. The experiment was performed at 37 °C in phosphate buffer at pH 7.4. The reaction mixture consisted of 150 μL of 68 nM fluorescein solution (substrate), 25 μL of 74 mM initiator solution (2,2′-Azobis(2-methylpropionamidine) dihydrochloride [AAPH]; prepared immediately before use in the assay buffer at 37 º C), and 25 μL of either GSPE or Trolox at different concentrations (ranging from 0.25 to 5 μg/mL of the phenolic extract and from 12.5 to 100 μM Trolox). The assay buffer was used as a blank. The ORAC values were calculated by using the area-under-curve (AUC) results for Trolox, expressed as micromoles (μmol) of Trolox equivalents per gram of the phenolic extract, and the sample calibration curves obtained in each analysis.

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2.3. Experimental procedure in rats

2.3.1. General protocol

In this study, we used 17–22-week-old male, spontaneously hypertensive rats (SHR) weighing 307.18 ± 1.64 g and 17–20-week-old male, normotensive Wistar–Kyoto (WKY) rats weighing 343.75 ± 2.75 g. All of these animals were obtained from Charles River Laboratories (Barcelona, Spain). The animals were maintained at 23 °C with 12-h light/dark cycles and were given tap water and a standard diet (A04 Panlab, Barcelona, Spain) *ad libitum* during the experiments. GSPE was dissolved in water and orally administered by gastric intubation, between 9 and 10 a.m. Water was used as a negative control, and Captopril (Sigma, USA) (50 mg/kg), a known antihypertensive drug, was given as a positive control. Different doses of GSPE (250, 375 and 500 mg/kg) were administered to the SHR, and 375 mg/kg GSPE was also evaluated in the Wistar–Kyoto rats. The time-length of both studies was 48 h. The animals were always orally administered either 1 mL of water or 1 mL of the appropriate solution of GSPE or Captopril. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were recorded in the rats by the tail-cuff method (Buñag, 1973) before administration and at 2, 4, 6, 8, 24 and 48 h post-administration. Before the measurements, the rats were maintained at 38 °C for 10 min to detect the pulsations of the tail artery. Five measurements of SBP and DBP were taken, and their averages were calculated. To minimise stress-induced variations in blood pressure, all measurements were taken by the same person in the same peaceful environment. Moreover, to guarantee the reliability of the measurements, we established a training period of two weeks prior to the actual trial to allow the rats to become acclimated to this procedure.

Additionally, fifteen 22-week-old SHR were sacrificed by decapitation after overnight fasting. Eight of these animals were administered 375 mg/kg GSPE 6 h before being sacrificed, and the remaining animals (seven) were administered water 6 h before being sacrificed. The GSPE and water were orally administered by gastric intubation, between 9 and 10 a.m. Blood samples were obtained from the sacrificed rats to analyse plasma ACE activity. Liver samples were obtained from these rats to assess malondialdehyde (MDA) and reduced glutathione (GSH) levels. The procedures used to evaluate all of these parameters are described below.

All the above-mentioned experiments were performed as authorised for scientific research (European Directive 86/609/CEE and Royal Decree 223/1988 of the Spanish Ministry of Agriculture, Fisheries and Food).

2.3.2. Biochemical assays

2.3.2.1. Plasma and liver preparations for biochemical assays. Blood samples from the sacrificed animals were collected in tubes containing sodium heparin as an anticoagulant. These samples were centrifuged at 2500 *g* for 20 min at 4 °C to obtain the plasma, which was divided into aliquots and stored at −80 °C until analysis of ACE activity.

The liver tissue was homogenised at 4 °C in phosphate-buffered saline (PBS, 0.27 mM KCl, 0.15 M NaCl, 1.5 mM KH_2PO_4, 8 mM $Na_2PO_4 \cdot H_2O$, pH 7.4) with Teflon beats (Lysing Matrix D, MP biomedicals, Barcelona, Spain) in a Fast-Prep instrument (MP Biomedicals, Barcelona, Spain). The homogenates were centrifuged at 5000 ×*g* for 15 min at 4 °C, and the supernatants of the centrifuged samples were stored at −80 °C until use. The protein content of the homogenates was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin as the standard.

2.3.2.2. Malondialdehyde analysis. The MDA levels in the liver were measured by a thiobarbituric acid assay based on one proposed in the literature (Rodriguez-Martinez & Ruiz-Torres, 1992), which was modified as previously described (Manso et al., 2008). The liver homogenate was mixed with 20% trichloroacetic acid in 0.6 M HCl (1:1, v/v), and the sample tubes were kept on ice for 20 min to precipitate the plasma components and thus avoid any interference. The samples were centrifuged at 1500 ×*g* for 15 min before adding thiobarbituric acid (120 mM in Tris 260 mM, pH 7) to the supernatant in a ratio of 1:5 (v/v). The mixture was subsequently boiled at 97 °C for 30 min. Spectrophotometric measurements at 540 nm were made at 20 °C. The plasma MDA values were expressed as nmol MDA/g tissue protein.

2.3.2.3. Reduced glutathione assay. The GSH level in the liver was measured by the monochlorobimane fluorimetric method (Kamencic, Lyon, Paterson, & Juurlink, 2000). For this analysis, 90 μL of homogenised supernatant from the liver was mixed with monochlorobimane (100 mM; Sigma, Barcelona, Spain) and 10 μL of the catalyst (glutathione S-transferase) solution (1 U/mL), which was obtained from horse liver (Sigma, Barcelona, Spain). The levels of GSH were quantified using a fluorimeterFLx800 Fluorescence Microplate Reader (Bio-Tek-IZASA, Barcelona, Spain) and were expressed as μmol/g tissue protein.

2.3.2.4. Determination of ACE activity in plasma. The ACE activity in plasma was measured using a fluorimetric method, as previously reported (Miguel, Alonso, Salaices, Aleixandre, & López-Fandiño, 2007). Briefly, plasma aliquots were incubated in triplicate for 15 min at 37 °C with 40 μL of assay buffer containing the ACE substrate (5 mM of Hip-His-Leu; 0.1 M sodium tetraborate decahydrate, 300 mM NaCl; pH 8.3; Sigma, Barcelona, Spain). The reaction was quenched by the addition of 190 μL of 0.35 M NaOH. The concentration of the product, His-Leu, was measured fluorimetrically after 30 min of incubation with 17 μL of 2% *O*-phthaldialdehyde in methanol. The fluorescence measurements were performed at 37 °C in a FLx800 Fluorescence Microplate Reader (Bio-Tek-IZASA, Barcelona, Spain) with 350-nm excitation and 520-nm emission filters and Gen5™ Data Analysis Software. Black 96-well polystyrene microplates (Thermo Scientific, MERCK, Barcelona, Spain) were used. A calibration curve was constructed by adding different concentration of rabbit lung ACE (Sigma, Barcelona, Spain) to each plate. The ACE activity was expressed as mU ACE/mL of plasma.

2.4. Statistical analysis

The results are expressed as the mean ± standard error of the mean (SEM) and were analysed by one-way and two-way analyses of variance (ANOVA) by using the GraphPad Prism software. Differences between the groups were assessed with the Bonferroni test. Differences between the means were considered to be significant when $p < 0.05$.

3. Results

3.1. Characterisation of GSPE

The Folin–Ciocalteu assay revealed a total phenolic content of 516.8 ± 12.1 mg gallic acid equivalents/g of fresh GSPE. Fig. 1 shows the results of the analysis of the individual phenolic compounds (both flavan-3-ols and phenolic acids) by means of reversed phase HPLC–UV–MS (TOF). Table 1 presents the amounts of the identified compounds quantified using calibration curves constructed with commercial standards.

GSPE had an activity of 16,935 ± 651 μmol Trolox equivalents/g of fresh GSPE as determined by the ORAC assay.

3.2. Effects of GSPE on arterial blood pressure

Before administration of the different treatments, the SHR had a SBP of 210.60 ± 4.63 mm Hg and a DBP of 182.22 ± 4.78 mm Hg. Fig. 2 shows the changes in SBP (Fig. 2A) and DBP (Fig. 2B) observed

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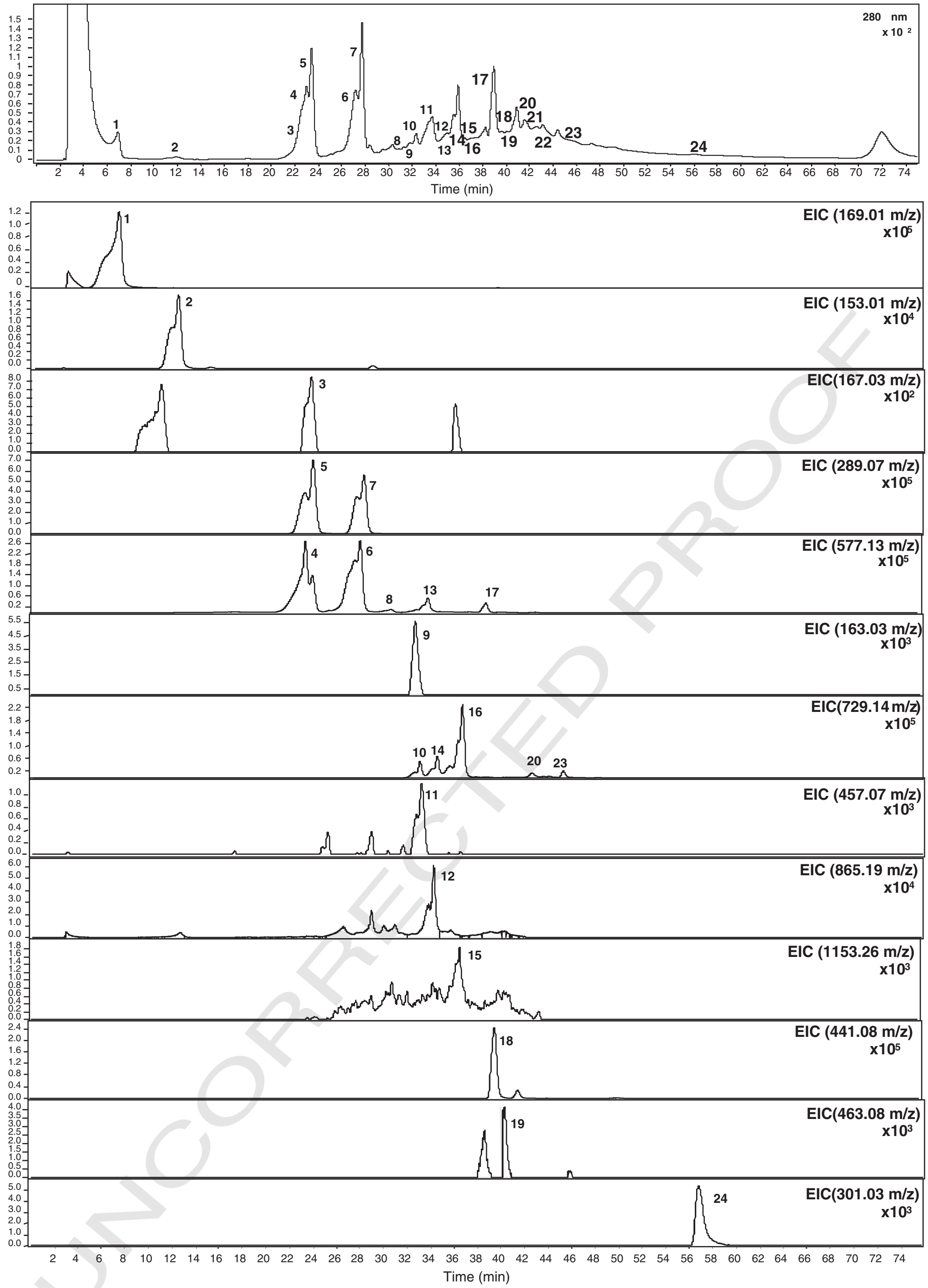

Fig. 1. UV-detected (280 nm) and extracted ion chromatograms (LC-TOF) of phenolic compounds from GSPE extract: (A) the HPLC–UV chromatogram of the GSPE extract at a wavelength of 280 nm and (B) the extracted ion chromatogram of the compounds included in Table 1. The results are presented as the means of three replicates, and the values in Table 1 are expressed as mg compound/g of fresh extract using the calibration curves of commercial standards. See Table 1 for codification of the peak numbers.

in SHR before and after the administration of water, Captopril or different doses of GSPE. The values of SBP and DBP obtained before and after oral administration of water were very similar. Captopril (50 mg/kg) caused an obvious decrease in arterial blood pressure in SHR, and the maximum decreases in SBP and DBP were observed 4 h post-administration. These variables returned to baseline 48 h after the administration of this drug. The oral administration of GSPE also resulted in significant decreases in SBP and DBP in the SHR. The decrease in SBP caused by GSPE was dose-dependent up to 375 mg/kg, and the decrease corresponding to this dose (−48.20 ± 6.28 mm Hg), which was observed 6 h post-administration, was the maximum observed for this extract. Nevertheless, as for Captopril, SBP returned to baseline 48 h after the administration of 375 mg/kg GSPE. In contrast to the results in SHR, the 375 mg/kg dose of the extract did not affect the arterial blood pressure in normotensive WKY rats (Fig. 3). The maximum decrease in SBP caused by 250 mg/kg GSPE

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Table 1
Individual phenolic compounds of GSPE (flavanols and phenolic acids) determined by reverse-phase HPLC–MS. Values are expressed as mg compound/g extract and are the means of 3 samples.

Number	Phenolic compound	$[M-H]^-$	Calibration curve	Total amount (mg/g)
1	Gallic acid	169.0136	y = 501,094x	17.7 ± 2.0
2	Protocatechuic acid	153.0187	y = 1,370,971.97x	1.0 ± 0.1
3	Vanillic acid	167.0342	y = 553,787x	0.1 ± 0.0
4, 6, 8, 13, 17	Procyanidin dimer[a]	577.1346	y = 250,456x	144.2 ± 32.2
5	Catechin	289.0712	y = 494,478x	90.7 ± 7.6
7	Epicatechin	289.0712	y = 556,794x	55.0 ± 0.8
9	*p*-Coumaric acid	163.0395	y = 1,943,720.52x	0.1 ± 0.0
10. 14, 16, 20, 23	Dimer gallate[a]	729.1455	y = 250,456x	39.7 ± 7.1
11	Epigallocatechin gallate	457.0770	y = 136,996x	0.4 ± 0.1
12	Procyanidin trimer[a]	865.1979	y = 250,456x	28.4 ± 2.0
15	Procyanidin tetramer [a]	1153.2613	y = 250,456x	2.0 ± 0.2
18	Epicatechin gallate [b]	441.0821	y = 556,794x	55.3 ± 1.5
19	Quercetin-3-*O*-galactoside	463.0877	y = 453,509x	0.2 ± 0.0
21	Naringenin-7-glucoside	433.1135	y = 188,637x	0.1 ± 0.0
22	Kaempferol-3-glucoside	447.0927	y = 489454x	0.1 ± 0.0
24	Quercetin	301.0348	y = 704,090x	0.3 ± 0.0

[a] Quantified using the calibration curve of procyanidin B2.
[b] Quantified using the calibration curve of epigallocatechin gallate.

(−31.77 ± 8.78 mm Hg) occurred 2 h post-administration. The decreases in DBP caused by the 375 and 250 mg/kg GSPE doses were even greater than the decrease in DBP caused by 50 mg/kg Captopril. The maximum decrease in DBP (−57.39 ± 7.98 mm Hg) caused by 375 mg/kg GSPE was observed 6 h post-administration in SHR. Paradoxically, 500 mg/kg GSPE exerted the lowest antihypertensive effect in SHR, as the largest changes in SBP (−19.85 ± 6.35 mm Hg) and DBP (−12.78 ± 6.25 mm Hg) caused by this dose of GSPE were less than the changes in these variables caused by the lower doses of the extract.

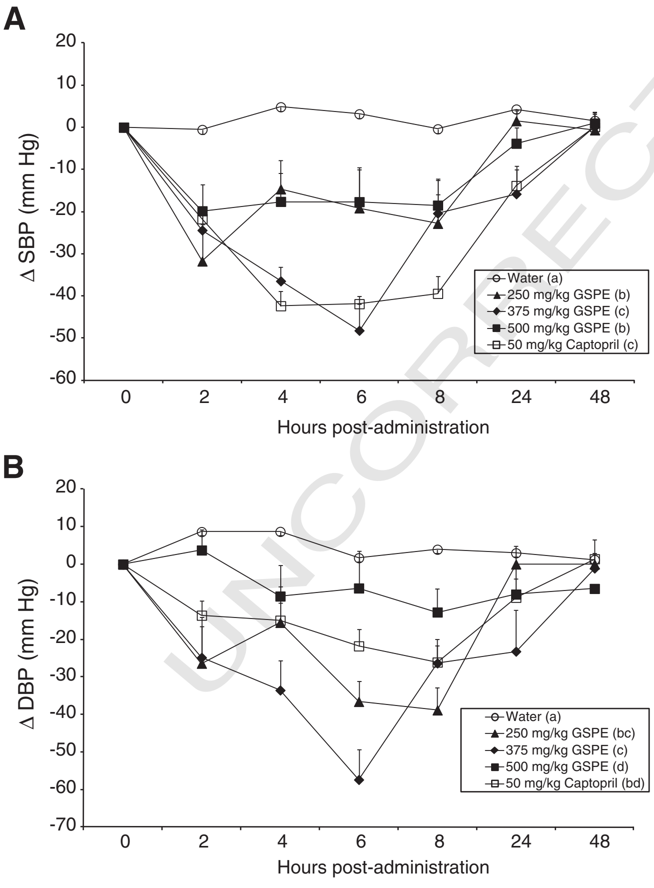

Fig. 2. Decrease in systolic blood pressure (SBP) (A) and diastolic blood pressure (DBP) (B) caused in spontaneously hypertensive rats after the administration of water (○), Captopril (50 mg/kg) (□) or different doses of GSPE: 250 mg/kg (▲), 375 mg/kg (◆), 500 mg/kg (■). Data are expressed as the mean ± SEM. All of the experimental groups include a minimum of 8 animals. Different letters represent significant differences ($p < 0.05$). *p* was estimated by two-way ANOVA.

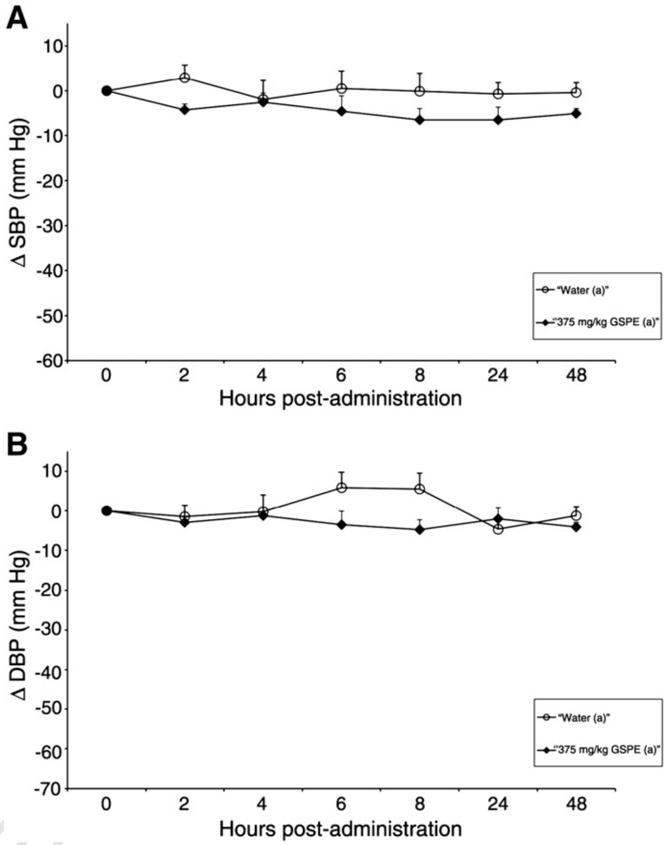

Fig. 3. Decrease in systolic blood pressure (SBP) and diastolic blood pressure (DBP) in Wistar–Kyoto rats after administration of water (○) or 375 mg/kg GSPE (◆). Data are expressed as the mean ± SEM. Both experimental groups have a minimum of 6 animals. No significant differences were observed.

3.3. MDA, GSH and ACE assays

The MDA and reduced GSH levels as well as plasma ACE activity were measured in both untreated SHR and in SHR treated with 375 mg/kg GSPE 6 h after administration. The livers of GSPE-treated and untreated SHR had very similar MDA levels (Fig. 4A). The levels of reduced GSH were increased in the livers of treated SHR (Fig. 4B), whereas plasma ACE activity was similar in untreated and GSPE-treated rats 6 h after administration of 375 mg/kg GSPE (Fig. 4C).

4. Discussion

Grapes, wine and other products obtained from grapes have demonstrated many beneficial effects (Dell'Agli, Buscialà, & Bosisio, 2004; Xia, Deng, Guo, & Li, 2010), including antihypertensive activity in rats (Diebolt et al., 2001; Moura et al., 2002; Peng et al., 2005; Sarr et al., 2006) and humans (Flechtner-Mors, Biesalski, Jenkinson, Adler, & Ditschuneit, 2004; Hansen et al., 2005; Park, Kim, & Kang, 2004). Polyphenols are considered to be major contributors to the health benefits of grapes (Diebolt et al., 2001; Jang & Lee, 2011). However, it is important to note that not all polyphenols exhibit the same

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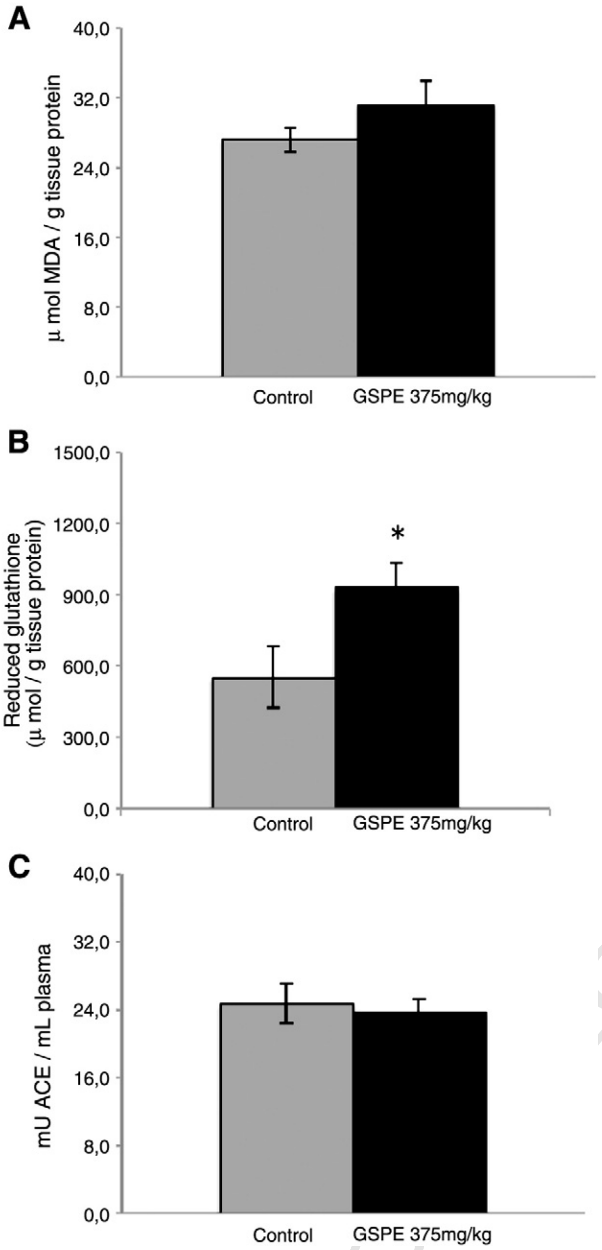

Fig. 4. Histograms of (A) liver malondialdehyde (MDA), (B) liver reduced glutathione and (C) plasma angiotensin-converting enzyme activity (ACE) in spontaneously hypertensive rats 6 h after administration of 375 mg/kg GSPE (■) or water (■). Data are expressed as the mean ± SEM. The experimental groups include a minimum of 7 animals. Different letters represent significant differences ($p<0.05$). *p* was estimated by one-way ANOVA.

bioactivity (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004; Williamson & Manach, 2005). In fact, as different grape products do not contain the same amounts or types of polyphenols (Bunea et al., 2012; Liang, Yang, Cheng, & Zhong, 2012; Liang et al., 2011), the beneficial properties of these different grape products will also not be equivalent. This fact necessitates the characterisation of these grape products to identify their bioactive constituents.

In this study, the results of the total phenolic content assay showed that phenolic compounds constituted 52% of GSPE, which supports to the substantial potential of this extract. In fact, the total polyphenol content of GSPE was 2.3 times higher than that reported for grape seeds (Pastrana-Bonilla, Akoh, Sellappan, & Krewer, 2003). The reverse-phase LC–MS analysis of the individual phenolic compounds of GSPE revealed that the most abundant phenols in GSPE are monomers, namely catechin (90.7 mg/g GSPE) and epicatechin (55.0 mg/g GSPE), and dimers, both in their free forms and linked to gallate (144.1 and 39.7 mg/g, respectively). Reverse-phase chromatographic analysis is suitable for small compounds (up to trimers), whereas the degree of polymerisation can be determined by normal-phase chromatography (Yang & Chien, 2000). Therefore, the information obtained from our analysis is a very useful complement to the characterisation provided by the manufacturer, which indicated the extent of polymerisation.

The most abundant phenolic acid in GSPE was gallic acid (17.7 mg/g), followed by protocatechuic acid (1 mg/g). In addition, the large amounts of epicatechin and its derivatives (dimers and gallates) found in the extract are noteworthy due to the beneficial effects of these compounds, which have been demonstrated in previous studies. In fact, any concentration of plasma epicatechin is accompanied by a dose-dependent increase in the plasma antioxidant capacity (Rein et al., 2000; Serafini et al., 2003). Also, a decrease in the plasma lipid oxidation (Rein et al., 2000; Serafini et al., 2003), a beneficial effect on vascular function (Schroeter et al., 2006) and a significant reduction in the serum oxidative stress (Flammer et al., 2007) is proportionally related with epicatechin plasma concentration. In addition, recent studies have shown the antihypertensive properties of epicatechin in different rat models of hypertension (Gómez-Guzmán, Jiménez, Sánchez, Romero, et al., 2011; Gómez-Guzmán, Jiménez, Sánchez, Zarzuelo, et al., 2011).

With regard to the antioxidant activity of GSPE, it is important to state that our result of 16,935 ± 651 µmol Trolox equivalents/g of fresh GSPE from the ORAC assay is in agreement with an earlier result (Corrales, García, Butz, & Tauscher, 2009) of 17,180 ± 129 µmol Trolox equivalents/g of extract obtained from the seeds of the Riesling grape in an antioxidant assay using 2,2′-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS). Moreover, the antioxidant activity of GSPE extract was higher than the antioxidant activity of an antihypertensive cocoa extract used in a previous study by our research group (12,134 ± 379 µmol Trolox equivalents/g of dry extract) (Quiñones, Miguel, Muguerza, & Aleixandre, 2011).

Antioxidant therapy has been extensively studied in hypertensive patients (Kitiyakara & Wilcox, 1998), and polyphenols are well-known antioxidants (Dudonné, Vitrac, Coutière, Woillez, & Mérillon, 2009; Scalbert, Johnson, & Saltmarsh, 2005). In this study, GSPE was evaluated in SHR, the experimental animal model that best mimics essential hypertension in humans (FitzGerald, Murray, & Walsh, 2004; Okamoto & Aoki, 1963). These *in vivo* trials revealed an obvious decrease in arterial blood pressure 6 h post-administration of 375 mg/kg GSPE in SHR. This observed decrease (approximately 50 mm Hg) was a promising result because small reductions in blood pressure may have an important impact on cardiovascular events in the hypertensive population (2–3% reduction in risk for each mm Hg) (Turnbull et al., 2003). The effect of 375 mg/kg GSPE in SHR was quite similar to that of 50 mg/kg of Captopril, which is considered a very effective antihypertensive treatment in clinical practice. Therefore, we believe that GSPE could be used as an ingredient in functional foods, with potential therapeutic benefits regarding the prevention and treatment of hypertension. Nevertheless, the quantity of GSPE necessary to decrease arterial blood pressure in humans should be definitively established when clinical trials were conducted.

Paradoxically, 500 mg/kg GSPE, the highest dose of GSPE used in this study, demonstrated poorer antihypertensive effects in SHR than did the lower doses of this extract. A similar paradox could be observed with different doses of a cocoa powder and of a cocoa extract, both of which are rich in procyanidins, in SHR (Cienfuegos-Jovellanos et al., 2009; Quiñones, Miguel, Muguerza, & Aleixandre, 2011). Some researchers have proposed that a high quantity of polyphenols could exhibit pro-oxidant properties instead of antioxidant properties (Azam, Hadi, Khan, & Hadi, 2004; Cotelle, 2001; Lahouel et al., 2006). The poorer antihypertensive effect observed with increased doses of the GSPE could be attributed to this proposed effect.

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The association between free radical production, lipid peroxidation, oxidative stress and hypertension is well known (Corrales et al., 2009; Martínez-Revelles et al., 2012; Quiñones, Sánchez, Muguerza, Miguel, & Aleixandre, 2011; Rodrigo, González, & Paoletto, 2011; Schulz, Gori, & Münzel, 2011). Nevertheless, it is controversial whether the antioxidant properties of polyphenols could explain their health benefits. Although monomeric flavan-3-ols are among those polyphenols showing higher bioavailability (Tomas-Barberán et al., 2007), they appear in the plasma not as the parent compounds, but as phase II metabolites. Moreover, considerable quantities of the ingested procyanidins reach the large intestine where they are degraded by colonic microbiota, giving origin to other smaller molecules that are also absorbed into the body (Del Rio et al., 2012). Therefore, all these metabolites will be responsible of the biological effects of procyanidins, and they physiological properties are different than the original compounds ingested, including their antioxidant activities.

Many different molecular targets and mechanisms have been proposed to explain the cardiovascular effects of polyphenols. However, experimental data indicate that polyphenols present in fruits and vegetables affect endothelial function, and hence, blood pressure, by regulating the bioavailability of nitric oxide, which is known to destroy reactive oxygen species (Galleano, Pechanova, & Fraga, 2010; Quiñones, Muguerza, Miguel, & Aleixandre, 2011). In addition, activation of the deacetylase sirtuin 1 (SIRT1) and up-regulation of endothelial nitric oxide synthase (Li, Xia, & Förstermann, 2012; Mattagajasingh et al., 2007; Wallerath et al., 2002; Zhang et al., 2008) have also been proposed to explain the cardiovascular effects of polyphenols. The effects of polyphenols could also be attributed to the induction of antioxidant enzymes in cardiovascular tissues and the increased expression of γ-glutamylcysteine synthetase, the rate-limiting enzyme for glutathione synthesis (Cao & Li, 2004; Li, Cao, & Zhu, 2006; Li et al., 2012). In this study, the administration of 375 mg/kg GSPE increased liver GSH, although the levels of MDA, a biomarker of lipid peroxidation, were similar in both the GSPE-treated and untreated (water-treated) groups of SHR. Nevertheless, the rapid increase in hepatic GSH, observed only 6 h post-administration in the GSPE group of SHR, is an important result as this antioxidant molecule plays a protective role against oxidative stress and free radical damage.

Other mechanisms, such as the inhibition of ACE activity, could also explain the antihypertensive effect of GSPE. ACE plays a key role in the regulation of arterial blood pressure and procyanidins can decrease both *in vitro* (Actis-Goretta et al., 2003; Ottaviani et al., 2006) and *in vivo* ACE activity (Quiñones, Miguel, Muguerza, & Aleixandre, 2011). Although our research group has previously shown that GSPE inhibited *in vitro* ACE activity (Guerrero et al., in press), plasma ACE activity in the GSPE-treated SHR in the present study was very similar to the corresponding value in untreated SHR. These results could be explained by the short period of time that had elapsed after GSPE administration, which was potentially insufficient to allow the observation of changes in the activity of this enzyme.

This study demonstrates the antihypertensive effect of GSPE in SHR but not in normotensive Wistar–Kyoto rats, establishing the specific effects of GSPE on the hypertensive condition.

5. Conclusion

In conclusion, GSPE could be a potential antihypertensive ingredient for functional foods, and the improvement of oxidative stress may be one of the mechanisms involved in its beneficial effects on arterial blood pressure. Nevertheless, additional research is needed to determine the real clinical value of GSPE. Being hypertension a chronic pathology that needs chronic treatment, future studies are in particular needed to study the long-term effect of this extract, but we believe also recommendable to better clarify the mechanisms involved in its antihypertensive effect.

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References

Actis-Goretta, L., Ottaviani, J. I., & Fraga, C. G. (2006). Inhibition of angiotensin converting enzyme activity by flavanol-rich foods. *Journal of Agricultural and Food Chemistry*, *54*(1), 229–234.

Actis-Goretta, L., Ottaviani, J. I., Keen, C. L., & Fraga, C. G. (2003). Inhibition of angiotensin converting enzyme (ACE) activity by flavan-3-ols and procyanidins. *FEBS Letters*, *555*(3), 597–600.

Aherne, S. A., & O'Brien, N. M. (2002). Dietary flavonols: Chemistry, food content, and metabolism. *Nutrition*, *18*(1), 75–81.

Andriambeloson, E., Kleschyov, A. L., Muller, B., Beretz, A., Stoclet, J. C., & Andriantsitohaina, R. (1997). Nitric oxide production and endothelium-dependent vasorelaxation induced by wine polyphenols in rat aorta. *British Journal of Pharmacology*, *120*(6), 1053–1058.

Azam, S., Hadi, N., Khan, N. U., & Hadi, S. M. (2004). Prooxidant property of green tea polyphenols epicatechin and epigallocatechin-3-gallate: Implications for anticancer properties. *Toxicology in Vitro*, *18*(5), 555–561.

Barona, J., Aristizabal, J. C., Blesso, C. N., Volek, J. S., & Fernandez, M. L. (2012). Grape polyphenols reduce blood pressure and increase flow-mediated vasodilation in men with metabolic syndrome. *Journal of Nutrition*, *142*(9), 1626–1632.

Barreiro-Hurlé, J., Colombo, S., & Cantos-Villar, E. (2008). Is there a market for functional wines? Consumer preferences and willingness to pay for resveratrol-enriched red wine. *Food Quality and Preference*, *19*(4), 360–371.

Buijsse, B., Feskens, E. J. M., Kok, F. J., & Kromhout, D. (2006). Cocoa intake, blood pressure, and cardiovascular mortality: The Zutphen Elderly Study. *Archives of Internal Medicine*, *166*(4), 411.

Buñag, R. D. (1973). Validation in awake rats of a tail-cuff method for measuring systolic pressure. *Journal of Applied Physiology*, *34*(2), 279–282.

Bunea, C. I., Pop, N., Babeş, A. C., Matea, C., Dulf, F. V., & Bunea, A. (2012). Carotenoids, total polyphenols and antioxidant activity of grapes (*Vitis vinifera*) cultivated in organic and conventional systems. *Chemistry Central Journal*, *6*(1), 66.

Cao, Z., & Li, Y. (2004). Protecting against peroxynitrite-mediated cytotoxicity in vascular smooth muscle cells via upregulating endogenous glutathione biosynthesis by 3H-1, 2-dithiole-3-thione. *Cardiovascular Toxicology*, *4*(4), 339–353.

Chobanian, A. V., Bakris, G. L., Black, H. R., Cushman, W. C., Green, L. A., Izzo, J. L., et al. (2003). Seventh report of the joint national committee on prevention, detection, evaluation, and treatment of high blood pressure. *Hypertension*, *42*(6), 1206–1252.

Cienfuegos-Jovellanos, E., Quiñones, M. M., Muguerza, B., Moulay, L., Miguel, M., & Aleixandre, A. (2009). Antihypertensive effect of a polyphenol-rich cocoa powder industrially processed to preserve the original flavonoids of the cocoa beans. *Journal of Agricultural and Food Chemistry*, *57*(14), 6156–6162.

Corrales, M., García, A. F., Butz, P., & Tauscher, B. (2009). Extraction of anthocyanins from grape skins assisted by high hydrostatic pressure. *Journal of Food Engineering*, *90*(4), 415–421.

Cotelle, N. (2001). Role of flavonoids in oxidative stress. *Current Topics in Medicinal Chemistry*, *1*(6), 569–590.

Del Bas, J. M., Fernández-Larrea, J., Blay, M., Ardèvol, A., Salvadó, M. J., Arola, L., et al. (2005). Grape seed procyanidins improve atherosclerotic risk index and induce liver CYP7A1 and SHP expression in healthy rats. *The FASEB Journal*, *19*(3), 479–481.

Del Rio, D., Mateos, A. M. R., Spencer, J. P. E., Massimiliano, T., Borges, G., & Crozier, A. (2012). Dietary (poly) phenolics in human health: structures, bioavailability and evidence of protective effects against chronic diseases. *Antioxidants & Redox Signaling*. **Q2**

Dell'Agli, M., Busciaià, A., & Bosisio, E. (2004). Vascular effects of wine polyphenols. *Cardiovascular Research*, *63*(4), 593–602.

Diebolt, M., Bucher, B., & Andriantsitohaina, R. (2001). Wine polyphenols decrease blood pressure, improve NO vasodilatation, and induce gene expression. *Hypertension*, *38*(2), 159–165.

Dong, J., Xu, X., Liang, Y., Head, R., & Bennett, L. (2011). Inhibition of angiotensin converting enzyme (ACE) activity by polyphenols from tea (*Camellia sinensis*) and links to processing method. *Food and Function*, *2*(6), 310–319.

Downey, M. O., Dokoozlian, N. K., & Krstic, M. P. (2006). Cultural practice and environmental impacts on the flavonoid composition of grapes and wine: A review of recent research. *American Journal of Enology and Viticulture*, *57*(3), 257–268.

Dudonné, S., Vitrac, X., Coutière, P., Woillez, M., & Mérillon, J. M. (2009). Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC assays. *Journal of Agricultural and Food Chemistry*, *57*(5), 1768–1774.

Duffy, S. J., Keaney, J. F., Jr., Holbrook, M., Gokce, N., Swerdloff, P. L., Frei, B., et al. (2001). Short-and long-term black tea consumption reverses endothelial dysfunction in patients with coronary artery disease. *Circulation*, *104*(2), 151–156.

Fisher, N. D. L., Hughes, M., Gerhard-Herman, M., & Hollenberg, N. K. (2003). Flavanol-rich cocoa induces nitric-oxide-dependent vasodilation in healthy humans. *Journal of Hypertension*, *21*(12), 2281.

FitzGerald, R. J., Murray, B. A., & Walsh, D. J. (2004). Hypotensive peptides from milk proteins. *The Journal of Nutrition*, *134*(4), 980S–988S.

Flammer, A. J., Hermann, F., Sudano, I., Spieker, L., Hermann, M., Cooper, K. A., et al. (2007). Dark chocolate improves coronary vasomotion and reduces platelet reactivity. *Circulation*, *116*(21), 2376–2382.

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Flechtner-Mors, M., Biesalski, H., Jenkinson, C., Adler, G., & Ditschuneit, H. (2004). Effects of moderate consumption of white wine on weight loss in overweight and obese subjects. *International Journal of Obesity*, *28*(11), 1420–1426.

Frankel, E., German, J., Kinsella, J., Parks, E., & Kanner, J. (1993). Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. *The Lancet*, *341*(8843), 454–457.

Galleano, M., Pechanova, O., & Fraga, C. G. (2010). Hypertension, nitric oxide, oxidants, and dietary plant polyphenols. *Current Pharmaceutical Biotechnology*, *11*(8), 837–848.

Gollucke, A. P. (2010). Recent applications of grape polyphenols in foods, beverages and supplements. *Recent Patents on Food, Nutrition & Agriculture*, *2*(2), 105–109.

Gómez-Guzmán, M., Jiménez, R., Sánchez, M., Romero, M., O'Valle, F., Lopez-Sepulveda, R., et al. (2011). Chronic (J)-epicatechin improves vascular oxidative and inflammatory status but not hypertension in chronic nitric oxide deficient rats. *British Journal of Nutrition*, *106*, 1337–1348.

Q3 Gómez-Guzmán, M., Jiménez, R., Sánchez, M., Zarzuelo, M. J., Galindo, P., Quintela, A. M., et al. (2011). Epicatechin lowers blood pressure, restores endothelial function and decreases oxidative stress, endothelin-1 and NADPH oxidase activity in DOCA-salt hypertension. *Free Radical Biology & Medicine*.

Guerrero, L., Castillo, J., Quiñones, M., Garcia-Vallvé, S., Arola, L., Pujadas, G. et al., (in press). Inhibition of antiotensin-converting enzyme activity by flavonoids: Structure-activity relationship studies. *Plos ONE*. Q4

Hansen, A. S., Marckmann, P., Dragsted, L., Nielsen, I. L. F., Nielsen, S., & Grønbaek, M. (2005). Effect of red wine and red grape extract on blood lipids, haemostatic factors, and other risk factors for cardiovascular disease. *European Journal of Clinical Nutrition*, *59*(3), 449–455.

Huang, D., Ou, B., Hampsch-Woodill, M., Flanagan, J. A., & Prior, R. L. (2002). High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. *Journal of Agricultural and Food Chemistry*, *50*(16), 4437–4444.

Jang, J. H., & Lee, J. S. (2011). Antihypertensive angiotensin I-converting enzyme inhibitory activity and antioxidant activity of *Vitis* hybrid — *Vitis coignetiae* red wine made with *Saccharomyces cerevisiae*. *Mycobiology*, *39*(2), 137–139.

Kamencic, H., Lyon, A., Paterson, P. G., & Juurlink, B. H. J. (2000). Monochlorobimane fluorometric method to measure tissue glutathione. *Analytical Biochemistry*, *286*(1), 35–37.

Kitiyakara, C., & Wilcox, C. S. (1998). Antioxidants for hypertension. *Current Opinion in Nephrology and Hypertension*, *7*(5), 531.

Lahouel, M., Amedah, S., Zellagui, A., Touil, A., Rhouati, S., Benyache, F., et al. (2006). The interaction of new plant flavonoids with rat liver mitochondria: Relation between the anti- and pro-oxidant effect and flavonoids concentration. *Therapie*, *61*(4), 347–355.

Li, Y., Cao, Z., & Zhu, H. (2006). Upregulation of endogenous antioxidants and phase 2 enzymes by the red wine polyphenol, resveratrol in cultured aortic smooth muscle cells leads to cytoprotection against oxidative and electrophilic stress. *Pharmacological Research*, *53*(1), 6–15.

Q5 Li, H., Xia, N., & Förstermann, U. (2012). Cardiovascular effects and molecular targets of resveratrol. *Nitric Oxide*.

Liang, Z., Sang, M., Fan, P., Wu, B., Wang, L., Duan, W., et al. (2011). Changes of polyphenols, sugars, and organic acid in 5 *Vitis* genotypes during berry ripening. *Journal of Food Science*, *76*(9), C1231–C1238.

Q6 Liang, Z., Yang, Y., Cheng, L., & Zhong, G. Y. (2012). Characterization of polyphenolic metabolites in the seeds of *Vitis* germplasm. *Journal of Agricultural and Food Chemistry*.

Manach, C., Scalbert, A., Morand, C., Rémésy, C., & Jiménez, L. (2004). Polyphenols: Food sources and bioavailability. *The American Journal of Clinical Nutrition*, *79*(5), 727–747.

Mancia, G., De Backer, G., Dominiczak, A., Cifkova, R., Fagard, R., Germano, G., et al. (2007). 2007 Guidelines for the management of arterial hypertension: The Task Force for the Management of Arterial Hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). *Journal of Hypertension*, *25*(6), 1105.

Mane, C., Loonis, M., Juhel, C., Dufour, C., & Malien-Aubert, C. (2011). Food grade lingonberry extract: Polyphenolic composition and in vivo protective effect against oxidative stress. *Journal of Agricultural and Food Chemistry*, *59*(7), 3330–3339.

Manso, M. A., Miguel, M., Even, J., Hernández, R., Aleixandre, A., & López-Fandiño, R. (2008). Effect of the long-term intake of an egg white hydrolysate on the oxidative status and blood lipid profile of spontaneously hypertensive rats. *Food Chemistry*, *109*(2), 361–367.

Q7 Martínez-Revelles, S., Avendaño, M. S., Garcia-Redondo, A.B., Álvarez, Y., Aguado, A., Pérez-Girón, J.V., et al. (2012). Reciprocal relationship between reactive oxygen species and cyclooxygenase-2 and vascular dysfunction in hypertension. *Antioxidants & Redox Signaling* (ja).

Mattagajasingh, I., Kim, C. S., Naqvi, A., Yamamori, T., Hoffman, T. A., Jung, S. B., et al. (2007). SIRT1 promotes endothelium-dependent vascular relaxation by activating endothelial nitric oxide synthase. *Proceedings of the National Academy of Sciences*, *104*(37), 14855.

Messerli, F. H., Williams, B., & Ritz, E. (2007). Essential hypertension. *The Lancet*, *370*(9587), 591–603.

Miguel, M., Alonso, M. J., Salaices, M., Aleixandre, A., & López-Fandiño, R. (2007). Antihypertensive, ACE-inhibitory and vasodilator properties of an egg white hydrolysate: Effect of a simulated intestinal digestion. *Food Chemistry*, *104*(1), 163–168.

Moura, R. S., Viana, F., Souza, M., Kovary, K., Guedes, D., Oliveira, E., et al. (2002). Antihypertensive, vasodilator and antioxidant effects of a vinifera grape skin extract. *Journal of Pharmacy and Pharmacology*, *54*(11), 1515–1520.

Mukai, Y., & Sato, S. (2009). Polyphenol-containing azuki bean (*Vigna angularis*) extract attenuates blood pressure elevation and modulates nitric oxide synthase and caveolin-1 expressions in rats with hypertension. *Nutrition, Metabolism, and Cardiovascular Diseases*, *19*(7), 491–497.

Nakamura, Y., Tsuji, S., & Tonogai, Y. (2003). Analysis of proanthocyanidins in grape seed extracts, health foods and grape seed oils. *Journal of Health Science*, *49*(1), 45–54.

Okamoto, K., & Aoki, K. (1963). Development of a strain of spontaneously hypertensive rats. *Japanese Circulation Journal*, *27*(3), 282–293.

Ottaviani, J. I., Actis-Goretta, L., Villordo, J. J., & Fraga, C. G. (2006). Procyanidin structure defines the extent and specificity of angiotensin I converting enzyme inhibition. *Biochimie*, *88*(3), 359–365.

Park, Y. K., Kim, J. S., & Kang, M. H. (2004). Concord grape juice supplementation reduces blood pressure in Korean hypertensive men: Double blind, placebo controlled intervention trial. *Biofactors*, *22*(1–4), 145–147.

Pastrana-Bonilla, E., Akoh, C. C., Sellappan, S., & Krewer, G. (2003). Phenolic content and antioxidant capacity of muscadine grapes. *Journal of Agricultural and Food Chemistry*, *51*(18), 5497–5503.

Peng, N., Clark, J. T., Prasain, J., Kim, H., White, C. R., & Wyss, J. M. (2005). Antihypertensive and cognitive effects of grape polyphenols in estrogen-depleted, female, spontaneously hypertensive rats. *American Journal of Physiology — Regulatory, Integrative and Comparative Physiology*, *289*(3), R771–R775.

Pinent, M., Blade, M., Salvado, M., Arola, L., Hackl, H., Quackenbush, J., et al. (2005). Grape-seed derived procyanidins interfere with adipogenesis of 3T3-L1 cells at the onset of differentiation. *International Journal of Obesity*, *29*(8), 934–941.

Pinent, M., Blay, M., Blade, M., Salvado, M., Arola, L., & Ardevol, A. (2004). Grape seed-derived procyanidins have an antihyperglycemic effect in streptozotocin-induced diabetic rats and insulinomimetic activity in insulin-sensitive cell lines. *Endocrinology*, *145*(11), 4985–4990.

Puiggròs, F., Llópiz, N., Ardévol, A., Bladé, C., Arola, L., & Salvadó, M. J. (2005). Grape seed procyanidins prevent oxidative injury by modulating the expression of antioxidant enzyme systems. *Journal of Agricultural and Food Chemistry*, *53*(15), 6080–6086.

Quiñones, M., Miguel, M., Muguerza, B., & Aleixandre, A. (2011). Effect of a cocoa polyphenol extract in spontaneously hypertensive rats. *Food and Function*, *2*(11), 649–653.

Quiñones, M., Muguerza, B., Miguel, M., & Aleixandre, A. (2011). Evidence that nitric oxide mediates the blood pressure lowering effect of a polyphenol-rich cocoa powder in spontaneously hypertensive rats. *Pharmacological Research*. Q8

Quiñones, M., Sánchez, D., Muguerza, B., Miguel, M., & Aleixandre, A. (2011). Mechanisms for antihypertensive effect of CocoanOX, a polyphenol-rich cocoa powder, in spontaneously hypertensive rats. *Food Research International*, *44*(5), 1203–1208.

Ramiro-Puig, E., Urpí-Sardà, M., Pérez-Cano, F. J., Franch, À., Castellote, C., Andrés-Lacueva, C., et al. (2007). Cocoa-enriched diet enhances antioxidant enzyme activity and modulates lymphocyte composition in thymus from young rats. *Journal of Agricultural and Food Chemistry*, *55*(16), 6431–6438.

Rein, D., Lotito, S., Holt, R. R., Keen, C. L., Schmitz, H. H., & Fraga, C. G. (2000). Epicatechin in human plasma: in vivo determination and effect of chocolate consumption on plasma oxidation status. *The Journal of Nutrition*, *130*(8), 2109S–2114S.

Rodrigo, R., González, J., & Paoletto, F. (2011). The role of oxidative stress in the pathophysiology of hypertension. *Hypertension Research*, *34*(4), 431–440.

Rodriguez-Martinez, M., & Ruiz-Torres, A. (1992). Homeostasis between lipid peroxidation and antioxidant enzyme activities in healthy human aging. *Mechanisms of Ageing and Development*, *66*(2), 213–222.

Sarr, M., Chataigneau, M., Martins, S., Schott, C., El Bedoui, J., Oak, M. H., et al. (2006). Red wine polyphenols prevent angiotensin II-induced hypertension and endothelial dysfunction in rats: role of NADPH oxidase. *Cardiovascular Research*, *71*(4), 794–802.

Scalbert, A., Johnson, I. T., & Saltmarsh, M. (2005). Polyphenols: Antioxidants and beyond. *The American Journal of Clinical Nutrition*, *81*(1), 215S–217S.

Schieber, A., Stintzing, F., & Carle, R. (2001). By-products of plant food processing as a source of functional compounds — Recent developments. *Trends in Food Science & Technology*, *12*(11), 401–413.

Schroeter, H., Heiss, C., Balzer, J., Kleinbongard, P., Keen, C. L., Hollenberg, N. K., et al. (2006). (−)-Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(4), 1024–1029.

Schulz, E., Gori, T., & Münzel, T. (2011). Oxidative stress and endothelial dysfunction in hypertension. *Hypertension Research*, *34*(6), 665–673.

Serafini, M., Bugianesi, R., Maiani, G., Valtuena, S., De Santis, S., & Crozier, A. (2003). Plasma antioxidants from chocolate. *Nature*, *424*(6952), 1013.

Shrikhande, A. J. (2000). Wine by-products with health benefits. *Food Research International*, *33*(6), 469–474.

Singleton, V. L. (1981). Naturally occurring food toxicants: Phenolic substances of plant origin common in foods. *Advances in Food Research*, *27*, 149–242.

Singleton, V. L., & Esau, P. (1969). Phenolic substances in grapes and wine, and their significance. *Advances in Food Research. Supplement*, *1*, 1–261.

Stein, J. H., Keevil, J. G., Wiebe, D. A., Aeschlimann, S., & Folts, J. D. (1999). Purple grape juice improves endothelial function and reduces the susceptibility of LDL cholesterol to oxidation in patients with coronary artery disease. *Circulation*, *100*(10), 1050–1055.

Suárez, M., Romero, M. P., Ramo, T., Macià, A., & Motilva, M. J. (2009). Methods for preparing phenolic extracts from olive cake for potential application as food antioxidants. *Journal of Agricultural and Food Chemistry*, *57*(4), 1463–1472.

Taubert, D., Roesen, R., Lehmann, C., Jung, N., & Schömig, E. (2007). Effects of low habitual cocoa intake on blood pressure and bioactive nitric oxide. *JAMA: The Journal of the American Medical Association*, *298*(1), 49–60.

Terra, X., Pallarés, V., Ardèvol, A., Bladé, C., Fernández-Larrea, J., Pujadas, G., et al. (2011). Modulatory effect of grape-seed procyanidins on local and systemic inflammation in diet-induced obesity rats. *The Journal of Nutritional Biochemistry*, *22*(4), 380–387.

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Tomas-Barberán, F. A., Cienfuegos-Jovellanos, E., Marín, A., Muguerza, B., Gil-Izquierdo, Á., Cerdá, B., et al. (2007). A new process to develop a cocoa powder with higher flavonoid monomer content and enhanced bioavailability in healthy humans. *Journal of Agricultural and Food Chemistry*, *55*(10), 3926–3935.

Turnbull, F., Neal, B., Algert, C., Chalmers, J., Woodward, M., MacMahon, S., et al. (2003). Effects of different blood-pressure-lowering regimens on major cardiovascular events: Results of prospectively-designed overviews of randomised trials. *Lancet*, *362*(9395), 1527–1535.

Wallerath, T., Deckert, G., Ternes, T., Anderson, H., Li, H., Witte, K., et al. (2002). Resveratrol, a polyphenolic phytoalexin present in red wine, enhances expression and activity of endothelial nitric oxide synthase. *Circulation*, *106*(13), 1652–1658.

Williamson, G., & Manach, C. (2005). Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. *The American Journal of Clinical Nutrition*, *81*(1), 243S–255S.

Xia, E. Q., Deng, G. F., Guo, Y. J., & Li, H. B. (2010). Biological activities of polyphenols from grapes. *International Journal of Molecular Sciences*, *11*(2), 622–646.

Yamamoto, M., Suzuki, A., & Hase, T. (2008). Short-term effects of glucosyl hesperidin and hesperetin on blood pressure and vascular endothelial function in spontaneously hypertensive rats. *Journal of Nutritional Science and Vitaminology*, *54*(1), 95–98.

Yang, Y., & Chien, M. (2000). Characterization of grape procyanidins using high-performance liquid chromatography/mass spectrometry and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Journal of Agricultural and Food Chemistry*, *48*(9), 3990–3996.

Yang, J., Martinson, T. E., & Liu, R. H. (2009). Phytochemical profiles and antioxidant activities of wine grapes. *Food Chemistry*, *116*(1), 332–339.

Zenebe, W., Pechanova, O., & Andriantsitohaina, R. (2003). Red wine polyphenols induce vasorelaxation by increased nitric oxide bioactivity. *Physiological Research*, *52*(4), 425–432.

Zhang, Q., Wang, Z., Chen, H., Zhou, S., Zheng, W., Liu, G., et al. (2008). Endothelium-specific overexpression of class III deacetylase SIRT1 decreases atherosclerosis in apolipoprotein E-deficient mice. *Cardiovascular Research*, *80*(2), 191–199.

Please cite this article as: Quiñones, M., et al., Low-molecular procyanidin rich grape seed extract exert antihypertensive effect in males spontaneously hypertensive rats (SHR), *Food Research International* (2013), http://dx.doi.org/10.1016/j.foodres.2013.01.023