WINE LEES AND THEIR DERIVED PRODUCTS TO MANAGE HYPERTENSION. IDENTIFICATION OF THEIR BIOACTIVE COMPOUNDS AND UNDERLYING MECHANISMS

Raúl López Fernández

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DOCTORAL THESIS
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Grupo de Investigación en Nutrigenómica
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FEM CONSTAR que aquest treball, titulat "**Wine lees and their derived products to manage hypertension. Identification of their bioactive compounds and underlying mechanisms**" que presenta *Raúl López Fernández* per a l'obtenció del títol de Doctor, ha estat realitzat sota la nostra direcció al Departament de Bioquímica i Biotecnologia de la Universitat Rovira i Virgili i que compleix els requisits per a l'obtenció de la Menció Internacional de Doctorat.

HACEMOS CONSTAR que el presente trabajo, titulado "**Wine lees and their derived products to manage hypertension. Identification of their bioactive compounds and underlying mechanisms**" que presenta *Raúl López Fernández* para la obtención del título de Doctor, ha sido realizado bajo nuestra dirección en el Departamento de Bioquímica i Biotecnologia de la Universitat Rovira i Virgili y cumple con los requisitos para la obtanción de la mención Internacional de Doctorado.

WE STATE that the present study, entitled "**Wine lees and their derived products to manage hypertension. Identification of their bioactive compounds and underlying mechanisms**" presented by *Raúl López Fernández* for the award of the degree of Doctor, has been carried out under our supervision at the Departament de Bioquímica i Biotecnologia from the Universitat Rovira i Virgili and that is eligible to apply for the International Doctoral Mention.

Tarragona, 2 Març 2021

El/s director/s de la tesi doctoral
El/los director/es de la tesis doctoral
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Esta tesis está enmarcada dentro del proyecto RETOS COLABORACIÓN: RTC-2017-6044-2 del Ministerio de Economía y Competitividad de España y Fundo Europeo de Desarrollo Regional y AEI-010300-2013-254, AEI-010500-2014-201, AEI-010500-2015-328 e IDI-20180101 del Centro Español para el Desarrollo de Tecnología Industrial (CDTI). Este proyecto tiene como principal objetivo el desarrollo y validación de una bebida funcional para el control de la presión arterial. Raúl López Fernández ha estado contratado con este proyecto en el grupo de Investigación en Nutrigenómica de la Universitat Rovira i Virgili durante la realización de la presente tesis doctoral (Octubre 2018-Marzo 2021).

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"The future depends on what you do today"

Mahatma Gandhi

LIST OF PUBLICATIONS

A) Published papers:

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B) Submitted papers:

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C) In preparation papers:

López-Fernández-Sobrino, R., Soliz-Rueda, J.R., Torres-Fuentes, C., Aragonès, G., Arola-Arnal, A., Bravo, F.I., Muguerza, B. Effect of dealcoholized wine lees on blood pressure, heart rate and locomotor activity in a long-term treatment in spontaneously hypertensive rats.

López-Fernández-Sobrino, R., Torres-Fuentes, C., Bravo, F.I., Muguerza, B. Grapes, Wine and Winery by-products: source of Phenolic compounds with antihypertensive properties.

PATENT

F. I. Bravo, **R. López-Fernández**, J.M Alcaide-Hidalgo, M. Margalef, A. Mas-Capdevila, J.M del Bas, M.E. Hérnandez, B. Muguerza. Wine lees, derivatives thereof and their uses. Aplication number: EP20382358.8. Priority date: 30/04/2020. PCT/EP2021/053051. Title-holder entity: Grandes Vinos y Viñedos. State: Under evaluation.

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López-Fernández Sobrino, R., Rodríguez, R.M., Mulero, M., Bravo, F.I., and Muguerza, B. A Phenol-enriched Grape-derived decreases Blood Pressure via Sirtuin-1 in spontaneously hypertensive Rats. From Foodomics to Nutrigenomics: Translating food composition data into healthy diets. NuGOweek 2019- 16th edition. Bern Switzerland.

López-Fernández Sobrino, R., Bravo, F.I. and Muguerza, B. Antihypertensive properties of low doses of a phenol-enriched grape-derived product in hypertensive rats. ICPH, the 9th International Conference on Polyphenols and Health. Kobe, Japon, 2019.

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SUMMARY

Hypertension (HTN) is currently one of the major risk factors for cardiovascular disease. Natural antihypertensive compounds are considered as a good strategy in decreasing blood pressure (BP). An interesting source of natural BP-lowering compounds are agri-food industries, which generate large amounts of by-products. This thesis aims to evaluate the potential of wine lees (WL), a winery by-product, as source of antihypertensive compounds. The BP-lowering properties of different WL were studied. WL from the Cabernet grape variety exhibited antihypertensive effect in spontaneously hypertensive rats (SHR) after acute administration. Their effects were associated to their high content in flavanols and anthocyanins. Additionally, these WL were submitted to enzyme-assisted extraction with Flavourzyme to release the phenolic compounds from WL non-soluble fraction and phenolic-enriched WL were obtained. Anthocyanins and flavanols were the largest families present in the hydrolyzed WL, which also exhibited enhanced antioxidant and antihypertensive activities. In addition to phenolic compounds, the hydrolysis also produced the release of the novel antihypertensive peptides FKTTDQQTRTTVA, NPKLVTIV, TVTNPARIA, LDSPSEGRAPG and LDSPSEGRAPGAD.

However, original WL also contain ethanol, which disappears in the drying process. Considering that the drying process of WL is essential for their commercialization, the antihypertensive activity of dried WL was investigated. WL powder showed enhanced antihypertensive properties after their acute administration at the most effective dose of 125 mg/kg bw. Their vasoprotective and antioxidant effects were confirmed as the mechanisms involved in their BP-lowering properties. However, since HTN is a chronic disease, the antihypertensive effect of dried WL was also confirmed in SHR. In addition, dried WL reduced heart rate and increased locomotor activity.

Therefore, the WL from the Cabernet grape variety, the obtained phenolic-enriched WL, the identified antihypertensive peptides and the dried WL could

be good candidates to be included in functional food, nutraceutical and food supplement for the management of HTN.

RESUMEN

Actualmente la hipertensión (HTN) es uno de los principales factores de riesgo de enfermedad cardiovascular. El uso de compuestos antihipertensivos naturales se consideran una buena estrategia para la reducción de la presión arterial (PA). La industria agroalimentaria genera grandes cantidades de subproductos ricos en compuestos naturales con propiedades antihipertensivas. Esta tesis tiene como objetivo evaluar el potencial de las lías del vino (WL), un subproducto de la industria vitivinícola, como fuente de compuestos antihipertensivos. Se estudiaron las propiedades antihipertensivas de diferentes WL, donde las WL de la variedad Cabernet demostraron tener efecto antihipertensivo tras una única administración en ratas espontáneamente hipertensas (SHR). Sus efectos se asociaron a su alto contenido en flavanoles y antocianinas. Adicionalmente se realizó una extracción de compuestos fenólicos a la fracción no soluble de éstas lías mediante hidrólisis enzimática con Flavourzime, obteniendo WL enriquecidas en compuestos fenólicos. Las actividades antioxidantes y antihipertensivas fueron mejoradas con el hidrolizado que presentaba principalmente antocianinas y flavanoles. La hidrólisis también generó la liberación de nuevos péptidos antihipertensivos FKTTDQQTRTTVA, NPKLVTIV, TVTNPARIA, LDSPSEGRAPG y LDSPSEGRAPGAD.

Sin embargo, las lías originales también contienen etanol, el cuál desaparece en el proceso de secado. De modo que el proceso de secado de las lías es esencial para su comercialización. Además, las lías en polvo fueron estudiadas mostrando una mejora de las propiedades antihipertensivas tras una única dosis de 125 mg/kg por peso del animal. También se confirmó su efecto vasoprotector y antioxidante mediante el estudio de los mecanismos implicados en la reducción de la PA. Sin embargo, la HTN es una enfermedad crónica, así, su efecto antihipertensivo a largo plazo también fue confirmado en

SHR. Además, las lías secas reducen el pulso e incrementan la actividad locomotora.

Por tanto, las lías de la variedad de Cabernet, las lías enriquecidas con compuestos fenólicos, los péptidos antihipertensivos identificados y las lías secas podrían ser buenos candidatos para ser incluidos como alimentos funcionales, nutracéuticos y suplementos alimenticios para el control de la HTN.

RESUM

Actualment la hipertensió (HTN) és un dels principals factors de risc de malaltia cardiovascular. L'ús de compostos antihipertensius naturals es consideren una bona estratègia per a la reducció de la pressió arterial (PA). La indústria agroalimentària genera grans quantitats de subproductes rics en compostos naturals amb propietats antihipertensives. Aquesta tesi té com a objectiu avaluar el potencial de les mares del vi (WL), un subproducte de la indústria vitivinícola, com a font de compostos antihipertensius. Es van estudiar les propietats antihipertensives de diferents WL, on les WL de la varietat Cabernet van demostrar tenir efecte antihipertensiu després d'una única administració en rates espontàniament hipertenses (SHR). Els seus efectes es van associar al seu alt contingut en flavanols i antocianines. Addicionalment es va realitzar una extracció de compostos fenòlics a la fracció no soluble d'aquestes mares mitjançant hidròlisi enzimàtica amb Flavourzime, obtenint WL enriquides en compostos fenòlics. Les activitats antioxidants i antihipertensives van ser millorades amb l'hidrolitzat que presentava principalment antocianines i flavanols. La hidròlisi també va generar l'alliberament de nous pèptids antihipertensius FKTTDQQTRTTVA, NPKLVTIV, TVTNPARIA, LDSPSEGRAPG i LDSPSEGRAPGAD.

No obstant això, les mares originals també contenen etanol, el cual desapareix en el procés d'assecat. De manera que el procés d'assecat de les mares és essencial per a la seva comercialització. A més, les mares en pols van ser estudiades mostrant una millora de les propietats antihipertensives després d'una única dosi de 125 mg/kg per pes de l'animal. També es va confirmar el seus efectes vasoprotector i antioxidant mitjançant l'estudi dels mecanismes implicats en la reducció de la PA. No obstant això, la HTN és una malaltia crònica, així, el seu efecte antihipertensiu a llarg termini també va ser confirmat en SHR. A més, les mares seques redueixen el pols i incrementen l'activitat locomotora.

Per tant, les mares de la varietat Cabernet, les mares enriquides amb compostos fenòlics, els pèptids antihipertensius identificats i les mares seques podrien ser bons candidats per a ser inclosos com a aliments funcionals, nutracèutics i suplements alimentaris per al control de la HTN.

LIST OF ABBREVIATIONS

ACE Angiotensin converting enzyme

ACE2 Angiotensin converting enzyme 2

ACEi Angiotensin converting enzyme inhibitory

ACh Acetilcoline

Ang Angiotensin

ARBs Ang II receptor antagonists

AT_1R Angiotensin type 1 receptor

AT_2R Angiotensin type 2 receptor

Akt Protein kinase B

BH_4 Tetrahydrobiopterin

BK Bradykinin

BP Blood pressure

BR_1 Bradykinin receptor 1

BR_2 Bradykinin receptor 2

BSO Buthionine sulfoximine

BW body weight

cAMP Cyclic adenosine monophosphate

cGMP Cyclic guanosine monophosphate

COX Cyclooxygenase

CVD Cardiovascular disease

DBP Diastolic blood pressure

DOCA Desoxycortisone acetate

ECE Endothelin converting enzyme

EDHF Endothelial-derived hyperpolarizing factors

eNOS Endothelial nitric synthase

ET Endothelin

ET_A Endothelin-1 receptor A

ET_B Endothelin-1 receptor B

FAD Flavin adenine dinucleotide

FMN Flavin mononucleotide

GA Gallic acid

GAE Gallic acid equivalents

GCS gamma-glutamylcysteine synthetase

GI Gastrointestinal

GP Grape pomace

GPx Glutathione peroxidase

GR Glutathione reductase

GSE Grape skin extract

GSH Reduced glutathione

GSPE Grape seed proanthocyanidins extract

GSSG oxided glutathione

GST Glutathione S-transferase

HDL High-density lipoprotein

HO-1 hemeoxygenase-1

HPLC High performance liquid chromatography

HTN Hypertension

H_2O_2 Hydrogen peroxide

ICAM Intercellular adhesion molecule-1

KKS Kallikrein-Kinin system

LDL Low-density lipoprotein

L-NAME N(ω)-monomethyl-L-arginine

LM-GSPE Low-molecular weight grape seed polyphenol extract

MDA Malondialdehyde

NADPH Nicotinamide adenine dinucleotide phosphate

NO nitric oxide

NOX NADPH oxidase

NOx Nitric oxide metabolites

O_2^- Superoxide

$ONOO^-$ Peroxide nitrite

PGF1α 6-keto-prostaglandin F1α

PGH_2 Prostaglandin H_2

PGI_2 Prostaglandin I_2 or prostacyclin

PWL Phenolic-enriched wine lees

RAAS Renin-angiotensin-aldosterone system

ROS Reactive oxygen species

SBP Systolic blood pressure

SD Sprague-Dawley

SHR Spontaneously hypertensive rats

Sirt Sirtuin

SOD Superoxide dismutase

TBARS Thiobarbituric acid reactive substances

TGF-β1 transforming growth factor-β1

TNFα Tumour necrosis factor alpha

UHPLC Ultra high performance liquid chromatography

VSMC Vascular smooth muscle cell

WKY Wistar Kyoto

WL Wine lees

WLPW Wine lees powder

INTRODUCTION

INTRODUCTION

Hypertension (HTN) is a common risk factor for cardiovascular disease (CVD) and a major global public health challenge. Treatments and prevention strategies for lowering blood pressure (BP) and slowing the progression of BP elevation are crucial for people with HTN. It has been suggested that the reduction of both diastolic blood pressure (DBP) and/or systolic blood pressure (SBP) by only 5 mmHg and10 mmHg respectively, reduce the risk of suffering cardiovascular events [1]–[3].

The increment of HTN prevalence is attributed to unhealthy diets (i.e. high sodium and low potassium intake) and lack of physical activity [4], [5]. Thus, the consumption of diets rich in fruits and vegetables have been associated with a lower risk of suffering CVD [6]. In this regard, several studies have shown different beneficial effects of grapes and wine on BP, attributed to their bioactive compound content, mainly phenolic compounds [7]–[9]. Grapes are one of the world's largest fruit crops and the 57 % of the production is destined to make wine [10]. Winery industry generates large amounts of by-products [11]. Food by-products have recently emerged as a source of bioactive compounds with a wide range of biological properties, including antihypertensive [12]. In addition, this new by-product use allows for a winemaking industry more sustainable and environmentally friendly [13], [14].

The objective of this review was to collect the reported evidences about the use of winery by-products as sources of antihypertensive compounds, identifying the potential bioactive compounds and highlighting the potential use of the less-studied by-products.

1. Blood pressure and Hypertension

BP is the force exerted by the blood upon the walls of vessels, especially arteries. It is represented by two events: 1) SBP, the pressure exerted in blood

vessels when the heart is contracted and 2) DBP, the pressure exerted in the vessels when the heart rests between beats [15].

HTN is defined as SBP ≥ 140 mmHg and/or DBP ≥ 90 mmHg and it is classified in 4 grades depending on the BP values (**Table 1**) [16]. HTN is the leading cause of death globally and produces an increased risk of developing brain, hear, kidney or other diseases [5], [17]. According to the World Health Organization (WHO), 1.13 billion of people worldwide suffer HTN. Furthermore, less than 20 % of hypertensive people are under control [15]. HTN is also associated with other diseases, where more than 50% of hypertensive people have other common cardiovascular risk factors such as diabetes, lipid disorders, overweight-obesity or metabolic syndrome [16].

Table 1. Blood pressure (BP) classification for adults.

Category	Systolic BP (mmHg)		Diastolic BP (mmHg)
Normal BP	<130	and	<85
High-normal BP or prehypertension	130-139	and/or	85-89
Grade 1 hypertension	140-159	and/or	90-99
Grade 2 hypertension	≥160	and/or	≥100

Source: Extracted from 2020 International Society of Hypertension Global Hypertension Practice Guidelines [16]

The development of HTN is due to different factors such as environmental, genetic, behavioural or a combination of these factors. The genetic predisposition to suffer HTN in combination with environmental factors such as smoking, unhealthy diet, obesity or physical inactivity, contribute to increase the pathogenesis of this disease [18]. In this regard, diet is the most important modifiable factor to prevent HTN and other cardiovascular disorders.

2. Blood pressure regulation

The regulation of BP is mediated by complex mechanisms that involve multiple organs and systems, including vasopressor and vasodepressor hormones,

autonomic nervous system, structure of the vascular system, renal function, oxidative stress and total body fluid volume [19]. Some of the principal mechanisms involved in the BP regulation are described below.

2.1. The renin-angiotensin-aldosterone system

One of the main mechanisms of BP regulation is the renin-angiotensin-aldosterone system (RAAS) (**Figure 1**), which is activated by a sudden decrease in blood pressure or Na^+ levels or increase K^+ levels. This system produces an increase in the reabsorption of water and Na^+ and excretion of K^+ in the nephrons [20]. The hormonal cascade system begins with the release of renin by juxtaglomerular cells in the kidney in response to various stimuli as changes in NaCl delivery, renal perfusion pressure, negative feedback by angiotensin II (Ang II) or sympathetic nerve stimulation [21]. In the circulation system, renin catalyses the conversion of angiotensinogen, released by liver, to the decapeptide angiotensin I (Ang I). Then, angiotensin-converting enzyme (ACE) cleavages the Ang I C-terminal end releasing the octapeptide Ang II. ACE is mainly expressed by capillary blood vessels in the lung, but it has also been described in kidneys, heart, adipose tissue, brain and some segments of the digestive tract [22], [23]. Ang II is a potent vasoconstrictor, exerting its action through Ang-II type 1 receptor (AT_1R). Recently, it has been evidenced that Ang II can also act as vasodilator when it binds to Ang-II type 2 receptor (AT_2R). However, this last action is less frequent as AT_2R is less expressed than AT_1R in the cells [24], [25]. Moreover, the binding of Ang II to AT_1R in adrenal glands, induce the production of aldosterone in zona glomerulosa, which produce the retention of Na^+ and water increasing the BP [26],[27].

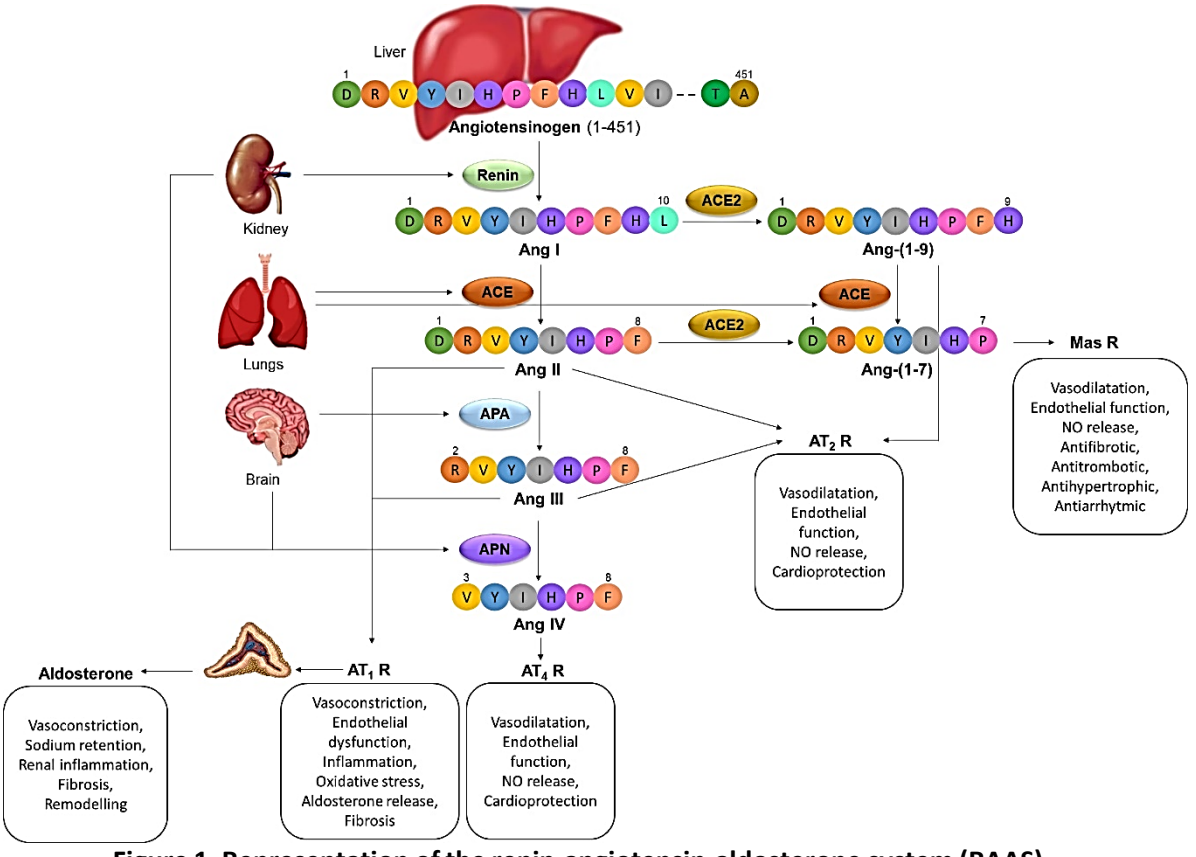

Figure 1. Representation of the renin-angiotensin-aldosterone system (RAAS). Hormonal cascade system beings with the release of renin by kidney. In the circulation system, renin catalyses' the conversion of angiotensinogen, released by liver, to the decapeptide angiotensin I (Ang I). Then, angiotensin-converting enzyme (ACE) cleavages the Ang I C-terminal end releasing the octapeptide Ang II. Ang II can act as vasoconstrictor, exerting its action through Ang-II type 1 receptor (AT_1R) or can also act as vasodilator when it binds to Ang-II type 2 receptor (AT_2R). Moreover, the binding of Ang II to AT_1R in adrenal glands, induce the production of aldosterone in zona glomerulosa, which produce the retention of Na^+ and water increasing the BP. Moreover, Ang II can also be broken down in its C-terminal region by ACE2, leading to the production of the nonapeptide Ang-(1-9), which binds to AT_2R exert a vasodilator effect. Ang-(1-9) is in turn hydrolysed by ACE to produce Ang-(1-7), which can be also generated by hydrolysing Ang II through ACE2. Ang-(1-7) exerts a vasoactive effect when it blinds to Mas receptor (MasR). In aditiion, Ang II also can

generate Ang III (also called Ang-(2-8)) by the action of the brain aminopeptidase A (APA) in the extreme N-terminal. Ang III has the same Ang II affinity for AT1R and AT_2R, leading to exhibit a vasoconstrictor effect. Ang III is degraded by aminopeptidase N (APN), produced by the brain, into Ang IV (also called Ang-(3–8)) that produces vasodilatation through Ang II type 4 receptor (AT_4R).

Moreover, Ang II can also be broken down at its C-terminal region by ACE2, leading to the production of the nonapeptide Ang-(1-9), which binds to AT_2R exert a vasodilator effect. Ang-(1-9) is in turn hydrolysed by ACE to produce Ang-(1-7), which can be also generated by hydrolysing Ang II through ACE2. Ang-(1-7) exerts a vasoactive effect when it blinds to Mas receptor (MasR) [28]. MasR is predominantly expressed in the brain and the testes, while moderate levels are found in the heart, kidney and vessels [29]. In addition, Ang II also can generate Ang III (also called Ang-(2-8)) by the action of the brain aminopeptidase A (APA) in the extreme N-terminal. Ang III has the same Ang II affinity for AT_1R and AT_2R, leading to exhibit a vasoconstrictor effect. Ang III is degraded by aminopeptidase N (APN), produced by the brain, into Ang IV (also called Ang-(3–8)) that produces vasodilatation through Ang II type 4 receptor (AT_4R) [26], [30].

As it has been shown, ACE plays an important role regulating BP as it mediates the passage from Ang I to the vasoconstrictor Ang II. However, this enzyme is also relevant because metabolizes other peptides, including the vasodilator bradykinin (BK) and kallidin to inactive metabolites [21]. Thus, an increase in ACE production results in the presence of high levels of Ang II and aldosterone, producing vasoconstriction and BP increase. The overactivation of RAAS contributes to the development of different diseases including HTN [31] and plays an important role in the development of endothelial dysfunction [32].

2.2. Endothelial function

The vascular endothelium has an important role in the regulation of vascular tone and BP. Specifically, it controls the fluidity and coagulation of the blood through the regulation of the clotting cascade and the fibrinolytic system [33], [34]. Moreover, vascular tone is regulated by the endothelium-derived factors by a balance between the production of vasodilators (nitric oxide (NO) or prostacyclin (PGI_2)) and vasoconstrictors (endothelin-1 (ET-1) or Ang II in the endothelium) **(Figure 2)** [35].

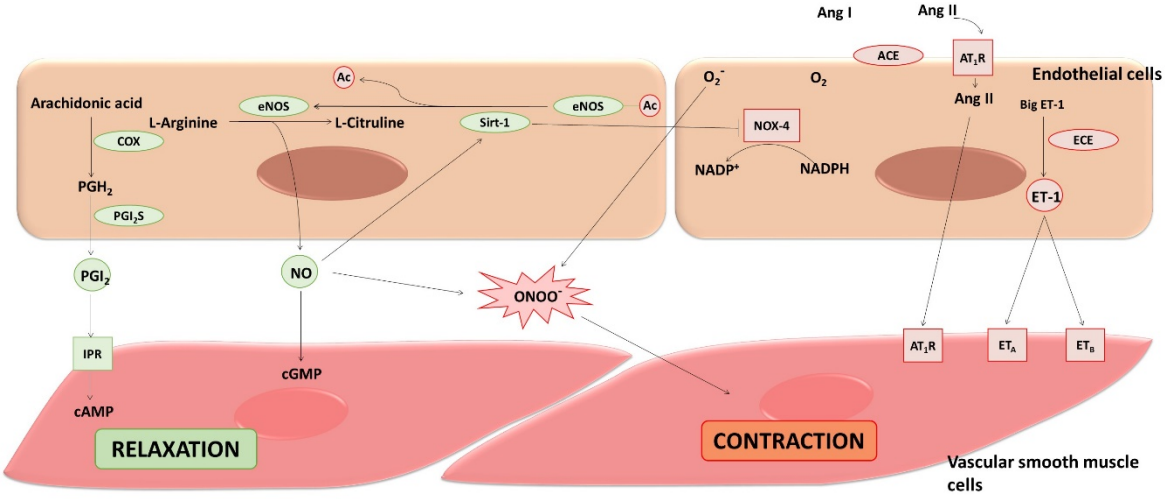

Figure 2. Endothelial-derived vasoactive molecules. Vasorelaxant enzymes and molecules are represented in green and vasoconstrictor enzymes and molecules in red. ACE: angiotensin converting enzyme; Ang: angiotensin; AT_1R: Angiotensin type 1 receptor; cAMP: cyclic adenosine monophosphate; cGMP: cyclic guanosine monophosphate; COX: cyclooxygenase; ECE: endothelin converting enzyme; ET: endothelin; ET_A: endothelin receptor A; ET_B: endothelin receptor B; eNOS: endothelial nitric oxide synthase; L-Arg: L-arginine; NADPH: nicotinamide adenine dinucleotide phosphate; NOX-4: nicotinamide adenine dinucleotide phosphate-oxidase 4; NO: nitric oxide; PGI_2: prostaglandin I_2 or prostacyclin; PGI_2S: Prostaglandin I_2 synthase; ROS: reactive oxygen species; Sirt-1: Sirtuin 1; IPR: prostaglandin receptor.

Endothelial dysfunction is characterized by an imbalance in endothelium-dependent vasodilators and vasoconstrictors [36]. In a healthy state, the endothelium promotes a vasodilator, antithrombotic, and anti-inflammatory state. However, endothelial dysfunction is associated with different risk factors such as hypercholesterolemia, hyperglycemia, HTN, established atherosclerosis

or other CVD, which alter endothelial phenotype promoting atherogenesis, lesion progression, and plaque vulnerability [37]. All risk factors are associated with an attenuation/loss of endothelium-dependent vasodilation [35], [36], [38].

2.2.1. Endothelial-derived vasodilator factors

NO is the main endothelium-derived vasodilator factor. It inhibits platelets, leukocyte adhesion to the endothelial surface, and proliferation and contraction of vascular smooth muscle cells (VSMC). NO is produced by the NO synthase (NOS), which can be found in different isoforms including neuronal (nNOS), inducible (iNOS) and endothelial (eNOS). In endothelial cells, eNOS is located in the plasmatic membrane as inactive form [37] [39]. Upon activation by different stimuli, eNOS uses L-arginine and O_2 to reduce nicotinamide-adenine-dinucleotide phosphate (NADPH) and produce NO [40]. eNOS activity is enhanced by agonists such as bradykinin, acetylcholine (ACh), and histamine, which increase intracellular calcium (Ca^{2+}). Hemodynamic shear stress and the hormones insulin or plasma albumin, increment activity of eNOS by inducing eNOS phosphorylation, independent of changes in intracellular Ca^{2+} [41], [42]. Sirtuin 1 (Sirt-1), also called deacetylase silent information regulator factor 2 related enzyme 1, is one of the most relevant factors that activate eNOS. The role of Sirt-1 is crucial in the regulation of NO production and in endothelium-dependent vascular tone. Sirt-1 deacetylates eNOS activating it and increasing NO production [43]. Moreover, many studies have demonstrated that Sirt-1 may inhibit endothelial apoptosis and improve vascular endothelial function through increasing *eNos* expression, exerting anti-atherosclerotic effects [44], [45]. Given the important role of Sirt-1 in maintaining vascular endothelial homeostasis, Sirt-1 and their activators have been investigated considering their potential as a target in pharmacological therapies for CVD treatment in animals and humans [46], [47]. In HTN, NO levels are reduced [48] and this can mainly occur by one of the following three methods: i) reduction of NO

production due to decreased eNOS levels [49], ii) decrease in the NO availability caused by reactive oxygen species (ROS) which convert NO to peroxynitrite [50] or iii) antagonism of NO by endothelium derived contracting factors [51].

Endothelial function is also mediated by other vasodilator, Prostacyclin I_2 (PGI_2) [52]. PGI_2 is a central cardioprotective hormone along with NO. Its main functions, besides vasodilatation, are the inhibition of platelet activation, reduction of the risk of thrombosis and reduction in VSMC remodelling and cholesterol uptake [53]. For PGI_2 synthesis, arachidonic acid must be released from membrane-bound lipids via the enzymatic actions of phospholipase A2 [54]. In endothelial cells, phospholipase A2 activation is a calcium-dependent step [55]. Once liberated, arachidonic acid is metabolized by cyclooxygenase (COX) enzymes to produce prostaglandin H_2 (PGH_2) and in turn, PGH_2 is transformed to PGI_2 by prostacyclin synthase (PGI_2S). Two COX isoforms are involved in the endothelial PGI_2 synthesis, the endothelial-constitutive COX-1, which generates the majority of prostanoids during physiological processes and COX-2, that is only expressed when the endothelium is damaged and exposed to inflammatory cytokines [52], [53], [56], [57].

2.2.2. Endothelial-derived vasoconstrictor factors

Vasoconstrictors factors also play an important role in the BP regulation [58]. Among these, Ang II is one of the most relevant since ACE acts on the endothelium converting Ang I to Ang II. Ang II binds to the AT_1R of endothelial cells promoting vasoconstriction. In addition, Ang II also promotes the endothelin-1 (ET-1) production, another potent endothelium-derived vasoconstrictor factor. An increase of these both factors induce endothelial dysfunction [38]. ET-1 is produced by the endothelin converting enzyme (ECE) from the inactive precursor big ET-1 [59]–[61]. ET-1 contributes to vascular tone and regulates cell proliferation through activation of ET_A and ET_B receptors. ET_A receptor is located exclusively on VSMC while ET_B receptors on VSMC and endothelial surfaces [62], [63]. When ET-1 binds to ET_A or ET_B receptors, smooth

muscle Ca^{2+} channels open, allowing the entrance of extracellular Ca^{2+} into the cell and producing vasoconstriction. However, when ET_B is activated at the endothelial surface, stimulates the release of NO and PGI_2 producing vasodilation. ET-1 effect is determined by the receptor localisation and the balance between ET_A and ET_B receptors [64]. Nevertheless, under physiological conditions, the main ET-1 effect is vasoconstriction mediated by the ET_A receptor, which is partly counteracted by ET_B receptor-mediated NO release. Moreover, in endothelial dysfunction, it has been described that individuals present an ET_B receptor upregulation in VSMC, whereas in endothelial cells ET_B receptors are downregulated. This dysregulation resulting in an enhance in ET-1-mediated vasoconstriction [65].

2.3. Oxidative stress and vascular homeostasis

Oxidative stress is defined as an imbalance between the generation of reactive oxygen species (ROS) and antioxidant defence systems [66]. Overproduction of ROS contributes to the development of CVD and endothelial dysfunction [67]. Furthermore, the increment of ROS levels in cardiovascular control organs elicit targeted immune response, which potentiates systemic HTN and its complications. Thus, oxidative stress induced by pro-inflammatory signals worsens the immunologic response in endothelium, which produces the progressive deterioration of vascular function [68].

High levels of oxidative stress in hypertensive states increase Ang II levels, which stimulate the production of ROS through NADPH oxidase. The main vascular sources of ROS are mitochondrial enzymes, superoxide-producing enzyme NADPH oxidase, xanthine oxidase and uncoupling of NOS (state in which this enzyme produce superoxide instead of NO) [19]. NADPH oxidases (NOX) is a complex with multiple subunits of NOX (NOX1, NOX2, NOX4 and NOX-5). NOX participates in diverse processes as cell growth, migration, inflammation, fibrosis and contraction [69]. The production of ROS by NOX is through its catalytic domain which allow the electrons transfer from cytosolic NADPH to

leads the reduction of the oxygen to O_2^- as well as to secondary ROS by-products [65]. From the five NOX isoforms described, NOX2 and NOX4 are those producing ROS in the endothelium, being NOX4 the most abundant isoform [70]. NOX1/NOX2 are involved in the development of endothelial dysfunction, HTN and inflammation. While NOX4 may have a protective role in vasculature under stress condition; however recent studies showed that suppression of NOX4 could prevent HTN [71], [72]. In fact, NOX4 contributes to increased ROS generation when is stimulated by Ang II, glucose, tumour necrosis factor alpha (TNFα) and growth factors [73]. NOX5 has been reported to be related to oxidative damage in human arthrosclerosis [72]. ROS activity and NOX expression and activity are increased in VSCM in endothelial dysfunction and are associated with oxidative stress and aberrant redox signalling [73], [74].

Some specific enzymes and low molecular weight substances are involved in the elimination of reactive species in order to regulate the redox balance. Reduced glutathione (GSH) is the leading antioxidant synthetized by cells [75]. In healthy people and animals, most glutathione is kept in its reduced form (GSH) with low levels of its oxidized form (GSSG) [75].The antioxidant effect of GSH is mediated by different mechanisms where their disulphide form or oxidized (GSSG) can be back to its GSH form by the enzyme glutathione reductase (GR) to restore the unbalance between GSH/GSSG [76]. Glutathione peroxidase (GPx) is another enzyme involved in protecting cells against oxidative damage. It uses GSH as a cofactor to reduce H_2O_2 and organic hydroperoxides, generating GSSG and water. A stage of severe oxidative stress, as that found in HTN, can overcome the capacity of GR to reduce GSSG, causing an accumulation of GSSG [69], [74]. Thus, in HTN, a reduction of GSH levels has been observed by the overproduction of ROS [69], [74].

Moreover, an excessive ROS production also induce lipid peroxidation. These process consist in the oxidation of fatty acids polyunsaturated in low-density lipoprotein (LDL) in the cell membranes, generated during the different stages lipid peroxyl radicals or malondialdehide (MDA) [77]. MDA is currently

considered as a marker of tissue damage and failure of the antioxidant defence mechanisms, produced by an excessive oxidized LDL [78]. In HTN, the levels of MDA are increased. MDA plays an important role in the endothelial dysfunction inhibiting the expression and activity of eNOS and consequently reducing NO availability [79].

In the blood vessels, overproduction of ROS is related with a low level of vasodilators generating endothelial dysfunction. NO is the main vasodilator affected as it interacts with oxygen free radicals to generate peroxynitrite ($ONOO^-$). This leads to a reduced NO availability and increased ROS levels promoting HTN stage [66] [80].

3. Hypertension treatments

Currently, there are many treatments focused on the regulation and control of HTN. Antihypertensive therapy has been clearly shown to reduce the risk of CVD among people with vascular or renal disease, diabetes, or HTN with end-organ damage or, in the absence of these conditions [81]. Implementation of lifestyle modifications is the most recurrent method to treat HTN in patients with prehypertension and less than 10% of pre-existing cardiovascular risk [82], [83]. However, in patients with HTN or a pre-existing cardiovascular risk of 10% or higher, both lifestyle change and medication are recommended [84].

3.1. Pharmacological Treatments

Pharmacological treatments are widely used in the HTN treatment with the aim of reducing BP, acting on different targets involved in this process [85]. Pharmacological antihypertensive treatments mainly include five classes of drugs according to their targets: angiotensin II receptor blockers and ACE inhibitors, beta-blockers, diuretics, calcium-channel blockers, [86], [87].

ACE inhibitors (ACEi) are the most widely used drug for the treatment of HTN and the first choice. Indeed, five of the drugs recommended by the European Society of Cardiology in 2016 are ACEi: captopril, enalapril, ramipril and

trandolapril [88]. ACE is a metalloprotease primarily found anchored in the plasma membrane through a hydrophobic domain near its carboxy-terminal region and, therefore, it is mainly located on the tissue cells surface. However, ACE can also be released into the circulation by cleaving this carboxyl-terminal region [89]. Two homologous domains (N-terminal and C-terminal) are found in the somatic form of ACE and both contain an active site to bind zinc which active ACE. Nevertheless C domain is the dominant domain in the ACE [90]. ACEi can exert their inhibition on the RAAS system preventing the change from Ang I to Ang II or on the BK system degrading bradykinin and substance P (vasoactive factors). These vasoactive factors are degrading by other enzymes such as aminopeptidase P or carboxy-peptidase N when ACE is inhibited. Both systems present cardioprotective branch: protection to heart failure, natriuretic, antithrombotic, antihypertrophic, antifibrotic and antiarrhythmic effects, in addition to present attenuation of plaque formation and improve of vascular dysfunction [91].

Beta-blockers are in the second line of therapeutic recommendations for essential HTN [92]. Beta-blockers refer to a diverse group of drugs such as Bisoprolol or Carvedilol, which block the action of endogenous catecholamines on beta-adrenergic receptors, part of the autonomic (or sympathetic) nervous system [93]. These antihypertensive drugs are specially prescribed to reduce cardio vascular complications in patients with heart failure and reduce cardiac output [94].

Diuretics are other drugs used to decrease BP. They increase the excretion of water by modulating the reabsorption of sodium at different segment of the renal tubular system [95]. There are three main classes of diuretics in the HTN treatment: thiazides, loop diuretics and potassium-sparing agents, being thiazides the most used in the treatment of primary HTN [96]. Diuretics treatment is commonly used in different diseases such as kidney failure, congestive cardiac failure and HTN due to their ability to reduce blood volume, cardiac output and systemic vascular resistance [96]–[98].

Regarding Ca^{2+} channel blockers, they have a positive effect on kidney and cardiovascular diseases, improving the endothelial function [99]. The use of calcium channel blockers avoids changes in VSMC contractility in response to vasoactive stimuli, which modify arterial diameter and tissue blood flow and are determined by alterations in the intracellular Ca^{2+} concentration. An increment of intracellular Ca^{2+} concentration in VSMC produce vasoconstriction and leads to HTN. For this reason, calcium channel blockers are used to prevent the entry of extracellular Ca^{2+} and improve endothelial function [100]. In addition, Ca^{2+} channel blockers, including Amlodipine, are also used for treating certain types of abnormally rapid heart rhythms [101].

Another popular pharmacological therapy to improve HTN is the use of antagonists for Ang II such as Losartan or Atacand [102]. The Ang II receptor antagonists (ARBs) block AT_1R located in smooth muscle cells, liver, kidney, heart, aorta, lung and testes. This bound produces the blockade of RAAS system generating a drop of BP [103].

Nowadays, ARBs and ACE inhibitors are the most used and successful antihypertensive drugs reducing morbidity and mortality amongst patients with HTN or myocardial infarction [104], [105]. However, they can have unwanted side effects such as hypotension, dizziness, hyperkalaemia or increase creatinine levels among others [106]. Moreover, these drugs are not suitable for treating pre-hypertensive or normotensive individuals at risk of cardiovascular diseases. Indeed, they are not usually treated with pharmacological therapy and changes in life style, are normally the first step to prevent elevated BP [90]. However, is important to act at the pre-hypertensive stage with the aim to avoid the development of HTN [90]. Neither drugs such as β-blockers or diuretics are suitable for the treatment of pre-hypertension as they may have different metabolic side effects. Thus, angiotensin-receptor inhibitors and some calcium channels inhibitors are the only drugs capable of treating prehypertension in a safer way [107]. Therefore, there is a need to

develop new therapeutic antihypertensive treatments with reduced side effects and suitable for pre-hypertensive patients.

In this sense, natural products are of high interest for both scientific and food industry communities as they are a great source of bioactive compounds with several beneficial effects including antihypertensive [14], [108]. Indeed, new natural treatments aiming to reduce BP have emerged in the last years, specially ACE inhibitors [109].

3.2. Non-pharmacological Treatments

Lifestyle modifications such as salt reduction, healthy diet (dietary patterns characterized by a high consumption of fruit, vegetables, whole grains, legumes, seeds, nuts, fish, low-fat dairy, and a low consumption of meat and sweets), healthy drinks, moderation of alcohol consumption, weight reduction, smoking cessation and regular physical activity, have been shown to reduce BP in several clinical studies [16]. These changes may improve the HTN stage in patients who are being already treated pharmaceutically, which in turn allows for reducing the dosage of hypertensive drugs [110].

HTN stage shows different grades of HTN, when low grades of HTN are not treated to the same way that severe HTN grades. For example, in pre-hypertensive individuals is recommended to change their lifestyle, including diets rich in fruits and vegetables and physical activity, with the aim of alleviating this disease without the need for drugs. In this sense, the use of natural compounds as a source of antihypertensive compounds may be a great alternative as they are considered a safer option.

3.3. Natural antihypertensive compounds

Fruits and vegetables extracts are a valuable source of bioactive compounds, mainly phenolic compounds and peptides which have ACEi properties [90]. These bioactive compounds present similar or even more potent

antihypertensive effects than pharmacological therapies, inspiring many researchers to explore new therapies for HTN [111].

1.1.1. Phenolic compounds

Phenolic compounds constitute a large group of bioactive phytochemicals that present one aromatic ring at least with one or more hydroxyl group in their main structure [112]. These can be classified into flavonoids (flavanols, flavonols, anthocyanins, flavones, flavanones and isoflavones), stilbenes, phenolic acids and lignans [113]. Different studies have shown the health properties of phenolic compounds, including their antihypertensive effect [114]–[118]. A study carried out by Guerrero et al. have demonstrated the relationship between the structure of ACE and the capacity of flavonoids to inhibit it. Seventeen flavonoids were evaluated *in vitro* where their inhibitory potencies ranged from 17-95 % at a concentration of 500 µM. The better activity *in vitro* were related with sub-structures on the flavonoid skeleton, mainly the catechol group in the B-ring, the double bond between C2 and C3 at the C-ring and the cetone group in C4 at the C-ring [118]. Consumption of phenol-rich foods has been associated with a range of health benefits, including the reduction of cardiometabolic risk factors in humans [113]. Within flavonoids, flavanols of different sources such as grapes or cocoa have been proved to improve CVD by reducing BP [119], [120]. The intake of flavanols and proanthocyanidin-rich foods such as red grapes or red wine are associated with an improvement of endothelial function and the reduction of BP in hypertensive rats [121]–[123]. Furthermore, foods and extracts rich in flavanols and proanthocyanidins also improve the CVD through the reduction of BP in hypertensive and pre-hypertensive subjects [124], [125]. These beneficial effects are mainly due to the involvement of flavanols in regulating the production of the vasodilator NO, increasing NO levels in the endothelial cells [114], [126]. Furthermore, these studies are corroborated with the administration of individual flavanols such as catechin and epicatechin in

hypertensive animals, with the consequent reduction in BP [127]. Anthocyanins are the responsible of the colour of fruits and vegetables, being mainly found in blueberries and red grapes [128]. Dietary intake of anthocyanins has been studied for its health-promoting effects, more specifically with respect CVD prevention [129]. Anthocyanin-rich extracts of different sources as chokeberry, bilberry or elderberry showed the increase of NO endothelial via regulation of eNOS expression and activity, promoting vasorelaxation in pig coronary arterial rings [130]. In addition, anthocyanin-rich black soybean has demonstrated their antioxidant and antihypertensive properties acting in the prevention of oxidative damage with the increment of NO bioavailability and with the reduction of NADPH oxidase activity in lipopolisacharide (LPS)-stimulated RAW 264.7 cells [131]. Three meta-analyses of randomized controlled demonstrated the association between consumption of different sources of anthocyanins and the significant reduction of SBP and improve the vascular function [132]–[134]. One of these meta-analysis conducted with 24 clinical studies in healthy and hypertensive subjects showed that anthocyanin consumption improves the flow-mediated dilatation in acute and chronic supplementation. Additionally, the pulse was also improved in the acute supplementation, improving the vascular health [134]. The antihypertensive effect of berries rich in anthocyanins were demonstrated in a meta-analysis conducted with 22 clinical trials in healthy subjects and hypertensive subjects when berries consumption reduced SBP and LDL glucose [132]. Calfío et al. showed that the main responsible of the vasodilatation produced by anthocyanins are delphinidin-3-glucoside, petunidin-3-glucoside and malvidin-3-glucoside with the higher levels of vasodilatation *in vitro* in the endothelium [135]. Within stilbenes family, resveratrol is the found most abundant in grapes, red wine and berries with numerous beneficial effects associated with its intake, including antihypertensive effects [136]. Thus, resveratrol has been shown to exert antihypertensive properties in different models of hypertensive rats associated with the improvement of endothelium through vascular relaxation [137]–[140],

enhanced eNOS activity [137], [139], [140] and subsequently the increase of NO [140]. This antihypertensive potential of resveratrol is also related to its antioxidant capability. Hence, resveratrol administration to hypertensive rodents led to increased SOD [140]–[143], catalase [141], [142], GPx [143] and GSH [142]–[144] activity. Furthermore, resveratrol activate sirtuins such as Sirt-1 with is implicated in the deacetylation and consequent activation of eNOS [145]. However, there is some controversy regarding the effects of resveratrol on BP in humans. Thus, a meta-analysis of randomized controlled trials showed no relationship between resveratrol and pressure drop [146]. Other meta-analysis in obese or pre-hypertensive subjects showed a reduction in SBP after intake of resveratrol [147]–[149], and, in some cases, this effect was only observed at high doses [148].

Regarding the flavonol group, quercetin and kaempferol are the most widely distributed in foods with potential cardiovascular-related benefits [116]. Quercetin is found mainly in asparagus, onion and berries and kaempferol in the green leafy vegetables like spinach, but they are also found to a lesser extent in red wine [116]. Quercetin stands out for its capacity of improving endothelial function by the modulation of vasoactive agents with the increase of NO production and the reduction of ET-1 production, in addition to prevent the endothelial cell apoptosis [150], [151]. Quercetin acts via radical scavenging ability regulating enzymes such as hemeoxygenase-1 (HO-1) and reduces ROS production in macrophages [152]. In hypertensive animal model, quercetin presented the ability to inhibit ACE, reducing BP levels [152]. In subjects with metabolic syndrome, the intake of quercetin decreases SBP [153] and their intake in hypertensive subjects reduced the SBP and DBP in hypertensive subjects but not in subjects with pre-hypertension [150]. Endothelial function is also improved in healthy men as shown by a randomized, placebo-controlled, crossover trial where the administration of quercetin increased the levels of NO and reduced ET-1 [154]. In a randomised double-blinded controlled cross-over trial in pre-hypertensive subjects intake of a quercetin-rich onion skin extract

reduced the arterial BP probably by the regulation of ET-1 production [155]. In addition, a meta-analysis of 7 randomized controlled trials showed the reduction of SBP and DBP after supplementation with quercetin [156].

3.3.2. Bioactive peptides

In addition to phenolic compounds, the antihypertensive properties of bioactive peptides have been evidenced [90]. Bioactive peptides are small protein fragments that when are released to the native protein can exert a biological activity [157]. Three different ways involved in bioactive peptide release: by the action of proteolytic microorganisms, through breakdown by digestive enzymes and by hydrolysis with proteolytic enzymes from plants or microorganisms [158]. Bioactive peptides can regulate important bodily functions through their myriad activities, including antihypertensive, antimicrobial, antithrombotic, immunomodulatory, opioid, antioxidant, and mineral binding functions [159]. Antihypertensive effects are one of the most important properties attributed to bioactive peptides. This effect can be mediated by the inhibition of ACE, which is achieved after being absorbed in an intact form [157]. In recent years, the study of bioactive peptides is booming due to the great variety of foods in which they are found and their use in the revaluation of by-products [159].

The biological activity of the different protein hydrolysates is related to their composition, their amino acid sizes and sequence and their peptide configuration. Most of peptides contain 2 to 20 amino acids and are generally rich in hydrophobic amino acids [160]. The hydrolysis of food proteins is the best way to obtain an increment of bioactive peptides. In this regard, the study of bioactive peptides from dietary proteins has received great attention for their ACEi properties. Small-sized peptides, consisting of 3-12 amino acids, are most likely to exhibit ACEi activity. However, factors such as intestinal absorption, the position of amino acids within the peptide and the possible

degradation of the peptides by digestive enzymes must be taken into account [161].

4. Grape, wine and winery by-products as sources of bioactive compounds

Food-derived compounds have emerged as a potential alternative to manage HTN. In addition to foods, other interesting source of these functional compounds are agri-food industry by-products [162], [163]. Their use allows enhancing the valorisation of these wastes and making these industries more environmentally friendly. The shortage of raw materials around the world is an economic, social and environmental problem, making food products more expensive, exploiting natural resources and increasing levels of poverty, malnutrition and new diseases [164]. The current linear economic model is based on the constant need for short shelf-life products, forcing greater production to alleviate the high demand from the consumer and generating an environmental and economic crisis due to the limitation of natural resources [165], [166]. Indeed, according to the Food and Agriculture Organization (FAO) of the United Nations, about 1.3 billion tonnes of foods are lost or wasted globally [167]. Thus, a new (circular) economy model has emerged in the last decade in order to respond to high demands by generating fewer waste products and reusing them, contributing to a more sustainable and environmentally friendly economy [168].

Therefore, agri-food by-products have emerged as a novel source to obtain natural bioactive compounds with a wide range of beneficial activities, including antihypertensive properties [12], [90], [162], [169]–[172]. Hence, the significant potential of these by-products to obtain these bioactive compounds allows for increasing their value, as they can be used as functional ingredients or nutraceuticals, in addition to the aforementioned contribution to the circular economy [173] [174]. In this regard, grapes are one of the world's largest fruit

crops and the wine production process generates large amounts of by-products [11].

4.1. Grapes

Grapes are one of the most common and important fruits worldwide, and they are often consumed raw or after being converted to juice, wine, or jam [7], [175]. Five countries represent 50 % of the world vineyard, where Spain is the one with the largest vineyard area. Grape production in 2018 was 77.8 mt worldwide. Only a 36 % of this production is destined to be consumed as a fruit. The rest is destined to produce wine or make other grape products using dried grape (57% and 7%, respectively) [10]. Grape berries are rich in bioactive compounds such as phenolic compounds, being both the natural product and its by-products generated after their processing a subject of study by the scientific community. In this sense, fruits like grape, blueberry or apple have been widely studied and have shown strong cardioprotective and antihypertensive effects [115] [176]–[179]. Their beneficial effects have been mainly associated to their phenolic compounds content [180]. This fact, together with their high production and consumption in the world, has made them and its derivatives one of the most studied products to evaluate their beneficial effects.

The phenolic composition of grapes may vary depend on different factors such as soil, climate, degree of maturity or grape variety [181], [182]. Focusing on grape variety, the red ones have a higher total amount of phenolic compounds than the white varieties. This is due to the pigments contained in the red grape responsible for the different shade of the grape. These pigments are mainly anthocyanins that are absent in white grapes. **Table 2** shows an example of the phenolic composition found in the most studied grape varieties. Phenolic compounds in grapes are mostly distributed in grape seeds at 60%–70%, followed by skin at 28%–35% and pulp with less than 10% [163]. Anthocyanins are mainly found in red grapes since they are responsible for their red colour.

Indeed, they also contribute with other pigmentations (blue or purple) of many flowers, fruits and vegetables [128]. There are five main types of anthocyanins found in red grapes: malvidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, and peonidin-3-*O*-glucoside. Malvidin-3-*O*-glucoside was the most predominant [111], [183], [184].

Regarding flavanols, (+)-catechin and its isomer (-)-epicatechin are the most abundant in grapes. Frequently, flavanols are conjugated with gallic acid giving rise to the gallate flavanols which include epicatechin gallate, catechin gallate, epigallocatechin gallate and gallocatechin gallate [185]. Condensed tannins, also known as proanthocyanidins, are oligomeric and polymeric compounds which consist in couple flavanol units. Within proanthocyanidins, procyanidins are the most abundant, which contain exclusively (epi)catechin units [112].

Flavonols are less distributed in grapes than anthocyanins and are typically found as glycosides. The main flavonols found in the grapes are quercetin, kaempferol and isorhamnetin glucosides [150], [186], [187]. The disaccharide quercetin-3-O-rutinoside is a common dietary component [112].

Moreover, grapes also contain non-flavonoids, composed by two groups: phenolic acids and stilbenes. Stilbenes are compounds presenting two aromatic rings connected by ethylene bridge. Resveratrol and its structural analogue, pterostilbene, are the main stilbenes found in grapes [188]. Gallic acid (GA) is the main phenolic acid found in grapes [189]–[191].

Table 2. Phenolic composition of different grape varieties obtained from different sources ([183], [184], [209], [214]–[216], [218], [278]–[298])

	Compound	Grape variety				
		Garnacha (mg/kg)	Cabernet (mg/kg)	Tempranillo (mg/kg)	Merlot (mg/kg)	Syrah (mg/kg)
Flavanols	Catechin	13.1-1365.7	7.4-164.7	27.1-240.2	17-601	13.7-29.3
	Epicatechin	7.2-430.6	25.0-1067.4	17.1-353.6	1.56-980	10.3-74.8
	Catechin gallate	44.6-85.4	2.1-38.9	-	4.62-29	21.2
	Epicatechin gallate	0.1-34.5	2.4-75.6	9.6-30.5	0.4-2.6	3.9-25.4
	Procyanidin B2	27.3-319.4	15.8-285.1	5.1-18.3	280-366.7	-
	Procyanidin dimer	240.2-340.5	73.2-432.4	-	147.0-331.8	13.1
Flavonols	Quercetin	1.4-1.5	23.4-45.1	7.1-77.0	12.2	0.2
	Quercetin-3-O-glucoside	4.4-471.6	5.4-19.2	6.2-24.3	2.3-45.8	3.9-10.9
	Kaempferol-3-O-glucoside	1.2-57.2	0.5-76.8	0.8-8.5	0.3-97.7	0.1-0.8
	Isorhamnetin-3-O-glucoside	3.5-76.7	5.7-34.5	0.4-2.6	0.3-1.1	0.9-3.1
	Rutin	20.0-25.2	19.3-87.5	-	5.1-89.6	0.1-1.3
	Kaempferol	-	2.1-6.5	3.1-4.5	3.4	0.1
Phenolic acids	Gallic acid	2.5-19.3	0.1-216.8	0.4-9.2	3.4-153.8	0.6-89.6
	Protocatechuid acid	11.1-15.8	-	-	0.1-329.7	0.1-1.0
	p-Coumaric acid	0.4-0.9	5.1-18.3	2.7-0.9	0.2-6.1	-
	Caffeic acid	0.6	9.6-28.4	0.2-0.8	0.2-5.3	0.4-1.2
	Ferrulic acid	4.4-5.6	16.9-30.7	-	4.7-10.7	0.4-3.4

	Hydroxybenzoic acid		2.3-18.9	-	2.1-13.7	0.2-9.0
	Benzoic acid	7.5-9.6	2.2-4	-	2.2	-
Stilbenes	Resveratrol	4.5-7.8	0.2-0.4	-	-	-
	Resveratrol-O-glucoside	17.1-151.7	-	-	-	-
	trans-resveratrol	-	0.2-1.0	0.1	7.3-9.2	0.1
	Picceatanol	-	0.7-5.3	0.32-1.60	26.3	-
Anthocyanins	Malvidin-3-O-glucoside	384.1-1025.2	152.7-809.7	298.5-729.4	204.4-1065.0	324.2-963.7
	Cyanidin-3-O-glucoside	6.6-48.3	4.2-38.5	23.7-84.3	14.7-74.8	9.1-21.5
	Delphinidin-3-O-glucoside	24.9-199.2	34.0-267.4	126.2-354.0	47.7-177.9	33.0-148.5
	Petunidin-3-O-glucoside	30.4-312.5	25.2-154.4	93.1-242.2	40.4-172.8	64.8-158.3
	Peonidin-3-O-glucoside	43.4-733.3	23.6-126.8	44.0-115.7	81.3-245.1	56.3-192.4
	Malvidin-O-coumaroylglucoside	5.3-831.2	2.2-20.6	7.1-209.8	57.5-409.8	128.8-637.3
	Peonidin-O-acetyl glucoside	3.0-57.2	6.9-36.6	0.2-6.3	17.1-82.9	31.6-68.2
	Malvidin-O-acetyl glucoside	11.9-49.4	60.5-338.2	5.8-35.4	80.1-602.8	146.9-457.2

4.2. Winemaking process

The main grape-derived product is wine, being more than 50 % of grape production used to this purpose. According to 2019 statistical report from the International Organization of Vine and Wine, Spain is the third wine-producing country in the world with 44.4 million hectolitres, behind Italy and France (54.8 and 48.6 million hectolitres, respectively). However, Spain is the country with the highest number of hectares of vineyard [192], [193]. The general procedure to make wine starts with the harvest of grapes and, depending on the desired wine composition, stems can be removed (**Figure 3**). Grapes are crushed and, in the case of red wine, fermentation and maceration are carried out with the juice and pomace providing pigments, mainly anthocyanins, which are responsible of its red colour. Afterwards, the generated grape juice is pressed to remove grape bagasse, also called grape pomace, grape skin and grape seed. Then the fermentation can either continue in the tanks or finish. After the fermentation process, wine is separated from solid residues called lees (lees of first decanting step) by transferring it to another container, a process called racking. The obtained wine may continue with the malolactic fermentation in the tanks if it is desired. Lees can also be obtained from this second fermentation after sedimentation decanting. Finally, wine suffer a process of maturation in barrels and natural clarification and stabilization before bottling. In white winemaking process, the process is the same but with the exception of the first fermentation, which is not carried out [194].

4.2.1. Wine

Wine is composed mainly of water, carbohydrates, organic acids, minerals, alcohol, polyphenols and aromatics [195]. Wine may be classified in red, white and rosé wines being the red and white wines the most commonly consumed. Wine contains a large quantities of phenolic compounds responsible of its beneficial effects. Among the two main types of wine, phenolic compounds content ranges from around 189-554 mg/L in white wine and from 1,538-3,406

mg/L in red wine. This difference is mainly due to the high anthocyanins content of red wine (>700 mg/L), which are absent in white wine [196]. Thus, red wine is the most studied by its higher content in phenolic compounds.

The main phenolic compounds found in wine are: kaempferol, myricetin, quercetin and their respective glycosides in the flavonol group; catechin, epicatechin and proanthocyanidins in flavanol group; caffeic acid, ferulic acid, *p*-coumaric acid, *o*-coumaric acid, 2,3-dihydroxybenzoic acid, 2-hydroxybenzoic acid, 4-hydroxybenzoic acid, gallic acid and vanillic acid in the phenolic acids; resveratrol and piceatannol in stilbene group and cyanidin, delphinidin, malvidin, peodinin, petunidin and their derivatives (commonly linked to sugar molecules, aliphatic acids or acyl groups) in anthocyanin group [196].

Resveratrol is one of the phenolic compounds found in great quantity in wine and of great importance in research. Thus, resveratrol is the most studied phenolic compound in wine, and have been related with several healthy properties including: amelioration of renal injury, decrease of oxidative stress, improvement of cardiac function, reduction of lipogenesis and anti-inflammatory and antihypertensive properties [197]–[201].

In addition to wine, winery industries produce millions of tons of waste. Winemaking process is based on an ancestral procedure so that the winemaking process has been closely linked to artisan techniques or practices, limiting progress technology to those applications aimed at minimizing waste. Thus, the valorisation of these by-products is of great interest. The main residues of this activity are, in order of importance, organic residues (bagasse, seeds, pulp, skins, scrapes and leaves), sewage, emission of greenhouse gases (CO2, volatile organic compounds, etc.) and inorganic residues (diatomaceous earth, perlite, clays, bentonite) [162].

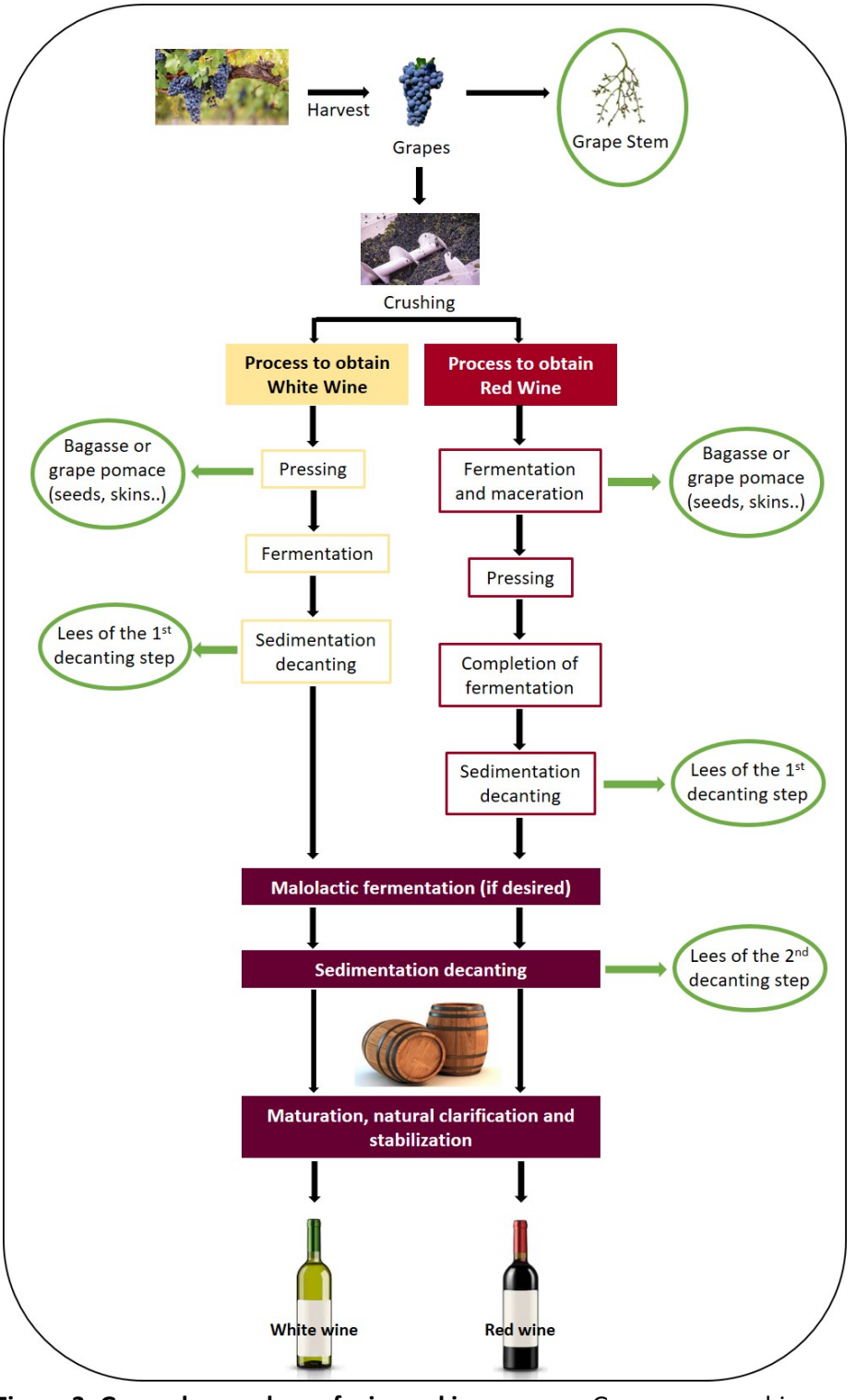

Figure 3. General procedure of winemaking process. Grapes are crushing and in the case of red wine, fermentation and maceration is carried out with the juice and pomace. Generated grape juice is pressed to remove grape bagasse

(also called grape pomace) grape skin and grape seed. Then the fermentation can continue in the tanks or end. After the fermentation process, wine is racking to be separated of solid residues called lees (lees of first decanting step). The obtained wine continues with the malolactic fermentation in the tanks and obtain lees of the second decanting step after sedimentation decanting. Finally, wine suffer a process of maturation in barrels and natural clarification and stabilization before bottling. In white winemaking process, the first fermentation process with pomace is not carried out, the rest of the process is the same that in red wine.

The characteristics and composition of these by-products are very related to the winemaking techniques used. Thus, the bioactive compounds such as phenolic compounds may vary in the same by-product depending on the winemaking process. The most interesting or investigated by-products for their bioactive compounds content are grape pomace, grape seed, grape skin, grape stem and wine lees (WL) [11], [169], [170], [202].

4.2.2. Grape pomace

Bagasse or commonly called grape pomace (GP) is an abundant by-product from the wine industry, resulting from the pressing and/or fermentation processes. GP consists of the remaining skin, seeds and stalks and represents around 25% of total grape weight used in the winemaking process [194]. It has a high content in dietary fibre with values between 172.8-887.0 mg/g of dry weight following by carbohydrates (122.0-405.3 mg/g) and protein (35.7-141.7 mg/g) [203]. In red wine the first fermentation before generation of pomace is the only step that does not occurs in white wine, however, there are not large changes in their bioactive compounds content. Therefore, significant amounts of bioactive compounds such as phenolic compounds are retained in red and white grape pomace [194]. GP phenolic composition may vary depending on the variety and maturity of grape and climatological conditions [203]. It has a wide range of phenolic compounds as it is made up of polyphenol-rich grape skin and seed. Thus, we found (+)-catechin and (-)-epicatechin like the most abundant flavonoids, followed by gallic acid, quercetin and quercetin-3-

glucoside. The stilbene compounds are low with trans-resveratrol and piceatannol. Anthocyanins, the main group in red grapes, are mainly concentrated in the skin and flavanols in the seed. Malvidin 3-O-glucoside is the anthocyanin predominant followed by peonidin, delphinidin-3-glucoside or petunidin [204].

4.2.3. Grape seed

Grape seeds are another by-product from the grape/wine industry that has been widely studied. They represent 13 % of the grape's weight and from 38% to 52 % of GP in dry weight. They are composed of around 40% dietary fibre, 16 % oil, 11 % proteins and 7% phenolic compounds and other compounds [205]. Regarding phenolic composition, flavanols are the major group found in grape seeds, being proanthocyanidins the most abundant. These dimeric compounds are composed by flavanols units of (+)-catechin and (−)-epicatechin linked together through interflavanoid bonds and by gallate esters [206]. In addition, quercetin and their derivatives are other flavonols found in high quantities in grapes seeds [207]. Moreover, these seeds contain phenolic acids, standing out the gallic acid found as gallic acid and gallic acid ethyl ester [208], [209].

Therefore, due to its high phenolic content, grape seeds have been extensively used to generate different proanthocyanidin -rich extract [210]–[212]. As an example, Quiñones et al. 2013 studied a grape seed proanthocyanidin extract (GSPE) comprised of 52 % of phenolic compounds. The analysis of the individual phenolic compounds in GSPE by reverse-phase LC-MS revealed that the most abundant phenols included GA, monomeric flavanols (-)-epicatechin and (+)-catechin, and dimer flavanols, both in their free and linked to gallate forms [120].

4.2.4. Grape skin

Grape skins can be obtained from pomaces generated in the vinification. Their phenolic content represents between 28%–35% of the total phenolic content in

grapes [163]. However, the quantity of phenolic compounds depends on different factors such as the winemaking process used. Grape skins obtained in white winemaking preserve nearly all their phenolic compounds while those obtained from red winemaking are less interesting. They loss an important part of their content in these compounds (mainly anthocyanins) in the maceration process during alcoholic fermentation [213].

Anthocyanins are the main group of phenolic compounds found in white grape skin, with malvidin 3-O-glucoside as the predominant compound mostly followed by peonidin 3-O-glucoside. This is due to the lack of alcoholic fermentation and maceration steps in white winemaking [214]. Flavanols are the second group found in the major quantities in white grape skin and the first in red grape skin, being (+)-catechin the main monomer present in grape skin, followed by (-)-epicatechin and procyanidins (B1 and B3) [215]. High levels of flavonols, specially quercetin and kaempferol derivatives glycosides (myricetin-3-O-glucoside, quercetin-3-O-galactoside, quercetin-3-O-glucoside and kaempferol-3-O-glucoside) [216]. Regarding phenolic acid group, gallic acid, protocatechuic acid, caftaric acid, coutaric acid, p-hydroxybenzoic acid and caffeic acid are identified although their content is small in comparison to the other groups [214], [215]. Trans-resveratrol and trans-polydatin are the main stilbenes found in grape skin. Many studies use the skin together with the seed of the grapes, since both by-products are extracted at the same time in the winemaking (fermentation or pressing) [208], [217], [218].

4.2.5. Grape stem

The grape stem represents 5% of the grapes processed by dry weight, being approximately 25% of the total by-products generated by the wine industry. As mentioned earlier, like other the wine industry by-products, the grape stem is rich in phenolic compounds with amounts between 187-378 mg/gallic acid by dry matter [219], [220]. Forty-two phenolic compounds have been identified in grape stem extract, including phenolic acids, stilbenes, flavonols and flavanols,

being flavanols the predominant in grape stem. Gallic and caftarc acids are the principal phenolic acids in grape stem extract. Trans-resveratrol, ε-viniferin, trans-resveratrol-glucoside (piceid), along with different dimmers and trimers of trans- and cis-resveratrol, are the main stilbenes identified. Catechin followed by epicatechin, was the main monomeric compound and procyanidin dimer B1 the highest dimer compound in the flavanols group. The most remarkable flavonols are quercetin-3-O-glucuronide, followed by quercetin-3-O-glucoside, and malvidin-3-O-glucoside is the most abundant anthocyanin [219].

4.2.6. Wine lees

According to the Council Regulation (EEC) No. 337/79, wine lees (WL) are "the residue that forms at the bottom of recipients containing wine, after fermentation, during storage or after authorized treatments, as well as the residue obtained following the filtration or centrifugation of this product" [221]. Wine lees (WL) represent between 14-25% of waste wineries [202]. They are mainly composed of yeasts, tartaric acid, inorganic matter and phenolic compounds [222]. WL has two phases, a solid and a liquid. The solid phase is rich in protein and essential free amino acids. However, the high amounts of phenolic compounds associated with these proteins make this solid fraction not assimilable [194], [223], [224].

Phenolic profile of WL have been characterized in phenolic extracts obtained from the liquid and solid fractions (**Table 3**). The most common technique for extraction of phenolic compounds from WL is solid-liquid extraction. Most of the studies are carried out on the solid fraction of WL after a centrifugation process and show a high content of phenolic compounds [225]. However, many phenolic compounds after the centrifugation process are still found in the solid phase, remaining bounded to yeast cell walls. The presence of phenolic compounds in WL is due to the great adsorption capacity of the yeast cell wall used in the winemaking process. Thus, the use of extraction methods are

required to extract all the phenolic potential contained in this by-product [226]. The phenolic compound profile of WL varies depending on the type of grape used and the winemaking process [222]. Total phenolic content shows a high level of phenolic compounds with higher quantity of non-flavonoids vs. flavonoids. Within flavonoids, anthocyanins are the most abundant in WL [202], [227]. Rutin and quercetin followed by ellagic, gallic, caffeic and p-coumaric acids have been described as the most abundant phenolic compounds in WL [227], [228]. However, full phenolic characterization of this by-product is not well known yet, leaving a field open for future research.

Table 3. Phenolic profile, identification and quantification in wine lees.

	Compound	Identification method	Quantification	Ref.
Flavanols	Catechin	HPLC-MS/MS	121 ± 6 µg/mL	[299]
	Epicatechin	LC−QqTOF-MS/MS	n.q.	[300]
	Procyanidin B2	LC−QqTOF-MS/MS	n.q.	[300]
Flavonols	Quercetin	HPLC	620 ± 42 mg/kg	[227]
		HPLC-DAD-MS	n.q.	[202]
		HPLC-MS/MS	0.85-1.42 mg/g	[301]
		HPLC-MS/MS	1216 ± 61 µg/mL	[299]
	Quercetin-3-O-glucuronide	HPLC-DAD-MS	n.q.	[202]
	Kaempferol	HPLC	59 ± 6 mg/kg	[227]
		HPLC-MS/MS	0.04-0.17 mg/g	[301]
	Kaempferol-3-O-galactoside	HPLC-DAD-MS	n.q.	[202]
	Myricetin	HPLC-DAD-MS	n.q.	[202]
	Myricetin-3-O-glucoside	HPLC-DAD-MS	n.q.	[202]
	Rutin	HPLC-MS/MS	2640 ± 132 µg/mL	[299]
Phenolic acids	Gallic acid	HPLC-DAD-MS	n.q.	[202]
		HPLC	620 ± 42 mg/kg	[227]
	Caffeic acid	HPLC	128 ± 14 mg/kg	[227]
	p-Coumaric acid	HPLC	158 ± 17 mg/kg	[227]
	Egallic acid	HPLC	219 ± 23 mg/kg	[227]
	Chlorogenic acid	HPLC	82 ± 9 mg/kg	[227]
Stilbenes	trans-resveratrol	HPLC-MS/MS	42 ± 2 µg/mL	[299]
		HPLC-MS/MS	0.03-0.13 mg/g	[301]
	trans-resveratrol-3-O-glucoside	HPLC-MS/MS	0.01-0.78 mg/g	[301]
	Delphinidin-3-O-glucoside	LC-MS/MS	n.q.	[302]

Anthocyanins	Delphinidin-3-O-(6''-p-acetyl-glucoside)	LC-MS/MS	n.q.	[302]
	Delphinidin-3-O-(6''-p-coumaroyl-glucoside)	LC-MS/MS	n.q.	[302]
	Delphinidin-3-(6-coumaroyl)-hexoside	HPLC-DAD-MS	n.q.	[202]
	Delphinidin-3-hexoside	HPLC-DAD-MS	n.q.	[202]
	Petunidin-3-O-glucoside	LC-MS/MS	n.q.	[302]
	Petunidin-3-glucoside	HPLC-MS/MS	0.97-3.41 mg/g	[301]
	Petunidin-3-(6-coumaroyl)-hexoside	HPLC-DAD-MS	n.q.	[202]
	Petunidin-3-O-(6''-p-coumaroyl-glucoside)	LC-MS/MS	n.q.	[302]
	Petunidin-3-hexoside	HPLC-DAD-MS	n.q.	[202]
	Petunidin-3-(6-acetyl)-hexoside	HPLC-DAD-MS	n.q.	[202]
	Malvidin-3-hexoside	HPLC-DAD-MS	n.q.	[202]
	Malvidin-3-glucoside	HPLC-MS/MS	2.36-4.24 mg/g	[301]
	Malvidin-3-O-glucoside	LC-MS/MS	n.q.	[302]
	Malvidin-3-(6-O-acetyl)-glucoside	HPLC-MS/MS	1.06-1.74 mg/g	[301]
	Malvidin-3-(6-acetyl)-hexoside	HPLC-DAD-MS	n.q.	[202]
	Malvidin-3-(6-coumaroyl)-hexoside	HPLC-DAD-MS	n.q.	[202]
	Malvidin-3-O-6''-acetyl-glucoside	LC-MS/MS	n.q.	[302]
	Malvidin-3-(6-O-p-coumaroyl)-glucoside	HPLC-MS/MS	0.79-1.80 mg/g	[301]
	Malvidin-3-O-(6''-p-coumaroyl-glucoside)	LC-MS/MS	n.q.	[302]
	Malvidin-3-hexoside-pyruvate	HPLC-DAD-MS	n.q.	[202]
	Cyanidin-3-hexoside	HPLC-DAD-MS	n.q.	[202]
	Cyanidin-3-(6-coumaroyl)-hexoside	HPLC-DAD-MS	n.q.	[202]
	Cyanidin-3-O-(6''-p-acetylglucoside)	LC-MS/MS	n.q.	[302]
	Peonidin-3-hexoside	HPLC-DAD-MS	n.q.	[202]
	Peonidin-3-(6-coumaroyl)-hexoside	HPLC-DAD-MS	n.q.	[202]
	Pelargonidin-3-hexoside	HPLC-DAD-MS	n.q.	[202]
	10-carboxypyranomalvidin-3-6''-p-coumaroyl-glucoside	LC-MS/MS	n.q.	[302]

n.q. (non-quantified

5. Antihypertensive effect of grapes, wine and winery by-products

Although, there are some clinical trials focus on the evaluation of the BP-lowering effect of grapes and grape-derived products in humans (**Table 4**), most of the studies are carried out using experimental models of HTN. Different animal models are used by researchers to study the HTN and their possible treatments. Spontaneously hypertensive rats (SHR) is an experimental model of HTN that was developed by inbreeding Wistar rats with the highest BP values [229]. This model is one of the most used and well-established experimental model of HTN, very similar to the HTN found in humans. The animals develop many HTN-derived features such as cardiac hypertrophy, cardiac failure and renal dysfunction [230]. In this animal model, the values of SBP reach approximately 200 mmHg SBP at 17–20 weeks of age [119]. Wistar kyoto (WKY) rats are the normotensive controls of SHR, being both animal models genetically related. Thus, this animal model is included in many studies to rule out possible undesirable hypotensive effects [231]. In addition to this model, rats fed a cafeteria diet is a well-stablished method to induce metabolic syndrome and related pathologies, including high BP and other risk factors of CVD such as elevated lipid concentrations [232].

Although the measured SBP after 10 weeks of feeding a cafeteria diet was not as high as that reached in SHR [120], the obtained values can be considered to be borderline or a state that could be designated as pre-hypertension, which thus already implies a risk factor [232]. Other experimental models include the use of different drugs, which induce HTN such as Ouabain, Nω-nitro-L-arginine methyl ester (L-NAME), Buthionine sulfoximine (BSO) or desoxycortisone acetate (DOCA)-salt, among others. Ouabain works as an endogenous regulator of blood pressure and Na^+, K^+-ATPase activity generating HTN [233]. L-NAME is an inhibitor of eNOS that reduce production of NO generating HTN [234]. BSO generate oxidative stress by GSH depletion through the inhibition of γ-gluthamylcysteine synthase, an enzyme involved in the synthesis of GSH.

Table 4. Information of decrement BP, active compound, action mechanism, model used and dose to grapes, wine and winemaking by-products.

Model used		Product	Dose (mg/kg bw)	Administration	SBP decrement	DBP decrement	Action mechanism	Ref.
Animal	SHR	Grape powder	600	10 weeks	8	11 mmHg	↑ eNOS	[237]
	Sprague-Dawley rats with BSO	Grape powder	300	3 weeks	20%	20%	↓ ERK-1/2	[238]
	Wistar rats with Ang II	Red wine	150	20 days	~20 mmHg	-	↑ Renin ↓ NADPH oxidase ↓ ROS	[240]
	Wistar rats with L-NAME	Red wine polyphenols	40	4 weeks	18%	-	↑ eNOS, NO	[241]
	SHR	Red wine pomace	300	4 weeks	30 mmHg	-	↑ NO, HO-1, SOD2 ↓ ACE, Ang II	[249]
	SHR	Grape pomace	25	4 weeks	~25 mmHg	-	-	[171]
	Wistar rats with L-NAME or DOCA-salt	Grape skin extract	100	30 days	~30 mmHg	~35 mmHg	↑ NO	[261]
	SHR	Grape skin extract	200	12 weeks	~60 mmHg	-	↑ SOD	[262]
	Wistar rats with high fat diet (HF)	Grape skin extract	200	180 days	~30 mmHg	-	↑ NO, SOD, CAT, GPx ↓ MDA	[263], [264]

Pregnancy female rats	Grape skin extract	200	Chronic	-	-	NO	[266]
SHR	Grape seed procyanidin extract	375	Acute	48 mmHg	57.4 mmHg	↑ GSH	[120]
SHR	Grape seed procyanidin extract	375	Acute	35.7 mmHg	34.5 mmHg	↑ PGFα1, NO, PGI_2	[126]
Wistar rats with cafeteria diet	Grape seed proanthocyanidins extract	375	Acute	~18 mmHg	~13 mmHg	↓ MDA	[232]
Wistar rats with cafeteria diet	Polyphenol grape seed extract	375	Acute	21 mmHg	22 mmHg	↑ NO, PGI_2	[172]
Wistar rats with cafeteria diet and SHR	Grape seed polyphenol extract	375	Acute	21 mmHg	22 mmHg	↑ eNOS, Sirt-1 ↓ NOX4, ET-1	[121]
Wistar rats with cafeteria diet	Grape seed proanthocyanidin extract	25	3 weeks	18 mmHg	17 mmHg	↑ GSH, Sirt-1 ↓ ET-1	[255]
Wistar rats with cafeteria diet	Grape seed proanthocyanidin extract	25, 100, 200	12	-	-	-	[303]

	Sprague-Dawley rats with nitric sodium or ouabain	Grape seed proanthocyanidin extract	250	5 weeks	~20 mmHg	-	↑ NO ↓ ET-1, TGF-β1	[233]
	Kunming mice with L-NAME	Grape seed proanthocyanidins	-	3 weeks	~20 mmHg	~10 mmHg	↑ eNOS, NO, SOD ↓ MDA, ET-1	[256]
Humans	Pre-hypertensive men and women	Grape seed extract	300 mg/day	6 weeks	9 mmHg	4 mmHg	-	[304]
	Adults with metabolic syndrome	Grape seed extract	150 or 300 mg/day	4 weeks	11 mmHg	6-7 mmHg	↑ eNOS	[258]
	Pre-hypertensive middle-aged Japanese men and women	Grape seed proanthocyanidin extract	400 mg/day	12 weeks	13.1 mmHg	6.5 mmHg	-	[257]
	Men with high cardiovascular risk	Dealcoholized red wine	272 mL/day	4 weeks	5.8 mmHg	2.3 mmHg	NO	[243]

Healthy men	Red wine and dealcoholized red wine	8.1± 0.9 dL	Acute	-	-	↓	ET-1	[245]
Men with metabolic syndrome	Wine grape pomace	20 g/day	16 weeks	4.3 mmHg	5.3 mmHg		-	[250]

As mentioned above, oxidative stress is involved in the HTN process, therefore, the administration of BSO is a way to induce HTN as well [235]. DOCA-salt hypertension is widely used in animal models as a classical model of cardiac remodeling, kidney hypertrophy and renal injuries. In the kidney, severe renal histopathological lesions, including glomerulosclerosis, glomerular hypertrophy, and interstitial mononuclear cell infiltration, occur in DOCA-salt hypertensive rats [236].

5.1. Antihypertensive effects of grapes and wine

The antihypertensive properties of grapes and wine have been reported by some studies (**Table 4**). In this regard, the administration of grape powder (600 mg/day) to SHR produced an improvement of the endothelial function mediated by an increment of eNOS production, which was accompanied by a BP lowering effect (10 mmHg) [237]. Moreover, administration of 330 mg of grape powder to Sprague-Dawley rats with BSO-induced hypertension, also reduced BP by 20% via extracellular signal-regulated protein kinase-1/2 (ERK-1/2) [238]. In addition, an antihypertensive effect has also been evidenced in humans after intake of grape polyphenols. In this sense a meta-analysis of 10 randomized controlled trials in subjects with HTN or pre-HTN have demonstrated to reduce SBP [239].

Regarding wine, many are its beneficial effects mostly attributed to its content in resveratrol. However, the rest of the phenolic compounds found in wine have also shown beneficial effects. Thus, different wine extracts rich in phenolic compounds have been shown to exert several beneficial bioactivities [85], [123], [240], [241]. Moderate consumption of red wine [195], [242], dealcoholized red wine [243] or the red wine extracts [123], [240] have also been related to cardioprotective and antioxidant properties, including BP lowering effects and improvement of endothelial function [240], [243], [244]. Production of NO is the main mechanism implicated in the regulation of BP mediated by phenolic compounds of red wine. In this sense, red wine

polyphenols prevent HTN by inhibiting NADPH oxidase expression and increasing the release of NO in endothelium [240]. Furthermore, red wine reduces plasmatic levels of the vasoconstrictor ET-1 at a single dose of 8.1 dL in hypertensive subjects, being improved in dealcoholized red wine [245]. A clinical trial in hypertensive subjects have shown that the moderate consumption of red wine (272 mL/day for 4 weeks) slightly reduces SBP (2.3 mmHg) and DBP (1 mmHg). Nevertheless, their effect was higher in the same red wine without alcohol with a reduction of 5.8 and 2.3 mmHg in SBP and DBP respectively, and increased the levels of plasmatic NO [243]. Pechánová et al. also reported that administration of red wine polyphenols at a dose of 40 mg/kg/day during four weeks in hypertensive rats induced by L-NAME, increased *eNos* expression and the release of NO [241]. Similar results were found *in vitro* where red wine polyphenols extract increase *eNos* expression and thus endothelial NO production [123].

In addition to phenolic compounds, wine may contain antihypertensive peptides. Pozo-Bayón et al. 2005 demonstrated that red wine contain peptides with high ACEi activity [246]. Furthermore, Alcaide-Hidalgo et al show the importance of hydrophobic peptides in the ACEi activity in sparkling wines and red wines aged on lees [247]. On the other hand, certain yeasts are used in the winemaking process that can generate bioactive peptides in the fermentation process. In this sense, it has been observed that the released peptides by *Saccharomyces cerevisiae* EC118 in the wine production could present multifunctional activity, highlighting their ACEi properties [248]. In the same way that wine contains these bioactive peptides generated by yeasts during the fermentation process, other by-products of the wine industry could contain them as long as they have participated in the fermentation process, leaving a new door open to study.

In addition to grapes and wine, given the high phenolic compound content in winery by-products, some of them have been used to obtain phenolic-enriched

extracts. These extracts have shown to exert a wide range of functionalities, including antihypertensive [169], [180].

5.2. Antihypertensive effect of grape pomace

As discussed above, GP is mainly composed by seeds and skins. Del Pino-García et al. showed that seedless red wine pomace seasoning (RWPS) reduce BP and the oxidative damage in SHR at a dose of 300 mg/kg/day bw during 4 weeks by the increase of NO bioavailability. Restoration of eNOS, mitochondrial superoxide dismutase (SOD2), HO-1 and reduction of ACE aortic gene expression were the leading mechanism involve in the reduction of BP mediated by RWPS in SHR [249]. In addition, the intake of 20 g/day of wine grape pomace flour during 16 weeks improved SBP and DBP with a reduction of 4.3 and 5.3 mmHg respectively in subjects with metabolic syndrome [250]. *In vitro* studies have demonstrated that dry GP extract exerts antioxidant effects in endothelial and muscle cells through the increment of gamma-glutamylcysteine synthetase (GCS) and glutathione S-transferase (GST) enzymes [251]. GCS is the first enzyme in the biosynthetic pathway of GSH, with a critical role for cell survival [252]. GST is induced under conditions of oxidative stress and is involved in the detoxification of organic epoxides, hydroperoxides and unsaturated aldehydes formed after lipid peroxidation. GST detoxifies these products through their conjugation with GSH [253].

5.3. Antihypertensive effect of grape seed

Grape seeds is one of the most studied grape by-products for their antihypertensive effects [122], [210]. Different extracts have been elaborated showing different bioactivities, highlighting their antihypertensive effects due to their content in phenolic compounds, especially in proanthocyanins [210]. In this sense, antihypertensive mechanisms of GSPE have been widely elucidated in many studies *in vitro* and *in vivo* (animal and human). Acute administration of GSPE (375 mg/kg bw) produced a reduction of 50 mmHg over BP in SHR and ameliorated oxidative stress. In addition, it was observed an increase of hepatic

GSH levels [120]. However, no effects were observed in Wistar Kyoto (WKY) rats after GSPE consumption. Additional studies showed the implication of NO in the lowering BP mediated by the single administration of GSPE (375 mg/kg bw) and partial mediation of the vasodilator PGI_2 with an increment of 6-keto-prostaglandin F1α (PGF1α) in plasma [126]. Sirt-1 has demonstrated to play an important role in the decrease of BP mediated by GSPE at the same dose than the study before, where GSPE increases the expression of *Sirt-1*, which in turn causes the deacetylation of eNOS increasing the bioavailability of NO [121]. In other model of hypertensive rats through cafeteria diet-fed, GSPE showed a moderate reduction of BP with around 17 and 13 mmHg in SBP and DBP, reaching normotensive values of BP. The antihypertensive effect of acute dose of GSPE (375 mg/kg bw) in this animal model was also related to an increase of NO availability and partially to PGI_2 [172]. Also, similarly to SHR, their BP lowering properties in this hypertensive model were related to their antioxidant properties since a reduction in MDA liver levels, a marker of lipid peroxidation, were observed in the animals administered GSPE [232]. A dosage study in that animal models have demonstrated that the higher dose of GSPE (500 mg/kg bw) is not the most efficient, showing the best results the intermediate dose (375 mg/kg bw) [172]. The same results have been shown in SHR models treated with extracts rich in flavanol-rich compounds such as cocoa or grape seed [120], [254]. These results could be explained by the pro-oxidant properties and the excessive production of reactive oxygen species caused by high doses of flavanols [172]. Mas-Capdevila el al. reported that chronic administration of GSPE (25 mg/kg bw per day during three weeks) to cafeteria diet-fed rats, reduced SBP and DBP by 15 and 10 mmHg, respectively. The increment of GSH liver levels observed in these animals related the long-term effect of GSPE to an improvement of the oxidative stress associated with HTN [255].

In addition, it has been shown that GSPE improves endothelial function, since a single dose of GSPE (375 mg/kg bw) restored the imbalance between vasoconstrictors and vasodilators in SHR. Pons et al. observed the increasing of

aorta *eNos* and *Sirt-1* expression levels, that could favour the production of endothelium-derived NO and reducing the expression levels of the enzyme *Nox-4* and the vasoconstrictor *Et-1* [121]. Furthermore, GSPE (250 mg/kg/day for five weeks) was shown to regulate the NO and ET-1 balance and the suppression of transforming growth factor-β1 (TGF-β1) expression, which is associated with endothelial cells remodelling, decreasing BP in Sprague-Dawley (SD) rats with HTN induced by administration of Ouabain [233]. In addition, administration of GSPE also reduced plasmatic ET-1 levels and in cardiac and renal tissue which restored endothelial dysfunction and oxidative stress in L-NAME-induced HTN pregnant mice [256].

The antihypertensive properties of GSPE have also been demonstrated in hypertensive or pre-hypertensive patients. The results of clinical trials shows that consumption of GSPE (400 mg/kg/day) for 12 weeks could ameliorate vascular stiffness and regulate the BP at a normal stage [257]. In hypertensive subjects with metabolic syndrome, reduction of oxidative stress by GSPE is one of the main pathways of BP reduction [258]. In addition, different meta-analysis have demonstrated the positive relation among grape seed extract consumption and BP reduction. A meta-analysis with 16 randomized controlled trials demonstrated the beneficial impact of grape seed extract on BP, which was more evident in younger or obese subjects as well as in people with metabolic syndrome after treatment [259]. Other meta-analysis conducted with 9 randomized controlled trials showed grape seed extract exert their antihypertensive effect over SBP and not over DBP [260]. A recent meta-analysis of 6 controlled clinical trials showed that GSPE supplementation reduces SBP and BPD in hypertensive and pre-hypertensive subjects, being interesting in the treatment of HTN or as a preventive method in pre-hypertensive subjects [260].

5.4. Antihypertensive effect of grape skin

Grape skin extracts (GSE) have also been studied for their antioxidant and antihypertensive activities. Administration of 100 mg/kg bw of GSE during 30 days showed a significant antihypertensive, vasodilator and antioxidant effects in desoxycortisone acetate (DOCA)-salt and L-NAME hypertensive rats probably mediated by restoration of NO levels [261]. De Costa et at. showed that GSE (200 mg/kg/day for 12 weeks) prevented the development of HTN in SHR due to their antioxidant effect increasing the SOD activity and decreasing MDA levels [262]. The increment of NO synthesis and restoration of SOD, catalase, GPx and MDA levels produced by chronic administration of GSE, prevented the development of HTN in rats with metabolic syndrome induced by a high-fat diet [263], [264]. Additional studies demonstrated that pre-treatment with GSE avoided the HTN stage in rats that were induced HTN by the administration of doxorubicin (Dox). Dox induce cardiomyopathy by the overproduction of ROS [265]. Furthermore, studies carry out in hypertensive-induced pregnancy female rats with L-NAME, showed the involvement of NO in the antihypertensive effect of GSE avoiding the HTN stage in rats [266].

5.5. Antihypertensive effect of grape stem

Grape stem is a great source of bioactive compounds which may be used to extract phenolic compounds with antioxidant and antihypertensive effects [219]. In this sense, antioxidant activity improves the redox system in endothelial cell cultures with an increase of GSH and decrease of MDA levels after treatment with grape stem extract. The antioxidant effect of grape stem extract depend on their qualitative phenolic composition with considerable amounts of trans-resveratrol, gallic acid, catechin, syringic acid or quercetin [251]. Grape stem extract presented an ACEI activity of 72.27 % *in vitro* at a concentration of 200 µg/mL and IC_{50} value of 69.5 µg/mL *in vitro*. Single and chronic dosage of grape stem extract reduce BP in SHR probably due to their phenolic compounds. Specifically, the phenolic compound (+)-visitin A were isolated from grape stem extract by their ACEI activity, showing

antihypertensive effects in SHR by increasing NO release from endothelial cells [267].

5.6. Antihypertensive effect of wine lees

WL is a great source of phenolic compounds and they have been used to obtain extracts with antioxidants effects [225], [268]. However, to the best of our knowledge neither WL nor extracts obtained from WL have been tested against hypertension. It has been evidenced that phenolic compounds identified in WL such as resveratrol, quercetin, gallic acid, (+)-catechin, (−)-epicatechin or malvidin-3-glucoside exhibits antihypertensive effects in hypertensive animal and human models [127], [149], [150], [189]. Considering these evidence together with the great antioxidant effect of WL extracts and the relationship between oxidative stress and HTN, WL could be a great alternative to manage HTN.

6. Extraction of bioactive compounds

As has been mentioned, winery by-products can be considered as a good source of antihypertensive compounds, mainly phenolic compounds; however, many of these phenolic compounds are bound to proteins or to the structures of the cell matrix, making their release difficult. Thus, extraction of these bioactive compounds is an important factor when using by-products.

Different extraction procedures allows for the separation of bioactive compounds from inactive compounds present in plants [269]. Factors such as temperature, pH, pressure, extraction time or solvent are decisive to achieve an effective extraction. In addition to these factors, the structure of the bioactive compounds to be extracted must also be preserved intact, being another factor to take into account when selecting the extraction method [270]. Different methods of extraction of phenolic compounds have been used in order to revalue by-products [162]. They are commonly classified in conventional techniques and green technologies [269]. Most conventional

extraction methods are based on the use of different solvents with the application of heat and/or mixing. Nevertheless, these techniques consume a lot of time, energy, and polluting solvent. Thus, in the last decade, there has been an increasing demand for new extraction techniques with the aim to reduce use of organic solvents, the time of extraction and prevent the pollution between others [269]. Within green technologies are the ultrasound-assisted extraction, microwave-assisted extraction, pressurized-liquid extraction, supercritical fluid extraction or enzyme-assisted extraction [271]. Some of these techniques have been used for the extraction of certain phenolic compounds in winery by-products or for the extraction of phenolic compounds in general. For example, microwave-assisted extraction has been used to extract quercetin, catechin or anthocyanins from grape seeds and skins and supercritical fluid extraction for release total phenolic compounds in grape seeds and pomace [272]–[275].

Enzyme-assisted extraction is one of the extraction techniques booming by its ability to improve the yield of compound extraction while the use of solvents is reduced in the process [276]. Enzymatic hydrolysis is commonly used in bioactive compounds extractions for producing softer hydrolysis when the selection of the appropriate hydrolytic enzyme depends of the source of cell walls which mainly consists of lignin, polysaccharides, cellulose, hemicellulose, pectin and proteins [276]. Winery by-products presented fiber, carbohydrates and proteins, which are susceptible to hydrolysis. Phenolic compounds, one of the main bioactive compounds presents in wine and by-products of winemaking, can be found attached to proteins or other structures of the cell matrix, being of interest the use of extraction methods to release these bioactive compounds [162], [275]. Enzymatic hydrolysis allows for the release of phenolic compounds bound to proteins, but proteins can also be hydrolyzed by releasing peptides with different bioactivities, including antihypertensive [108]. In this sense, Rodríguez-Morgado et al. have shown enzymatic extraction

of GP with protease generate a release of phenolic compounds and peptides which improve its anti-inflammatory properties [277].

CONCLUSION

In conclusion, this review emphasizes the problem generated by hypertension worldwide where new treatments based on the use of functional foods have emerged in order to reduce the use of drugs that can have several adverse effects. In addition, the wine industry generates large amounts of waste that can be reused in order to create a more sustainable environment. Waste or by-products generated by wine industry contain large amounts of bioactive compounds, being phenolic compounds and bioactive peptides the most relevant. The use of these by-products rich in bioactive compounds and their extracts elaborated by different means of extraction have been useful for the treatment of HTN. Nevertheless, some by-products rich in bioactive compounds such as WL have been little studied in the HTN treatment, opening the door to their study.

REFERENCES

[1] D. Ettehad *et al.*, "Blood pressure lowering for prevention of cardiovascular disease and death: A systematic review and meta-analysis," *Lancet*, vol. 387, no. 10022, pp. 957–967, Mar. 2016.

[2] A. Zanchetti, "Hypertension: Lower or higher blood-pressure targets for high-risk patients?," *Nature Reviews Cardiology*, vol. 13, no. 11. Nature Publishing Group, pp. 637–638, 01-Nov-2016.

[3] C. Thomopoulos, G. Parati, and A. Zanchetti, "Effects of blood pressure lowering on outcome incidence in hypertension.1. Overview, meta-analyses, and meta-regression analyses of randomized trials," *Journal of Hypertension*, vol. 32, no. 12. Lippincott Williams and Wilkins, pp. 2285–2295, 2014.

[4] J. A. E. Manson, P. J. Skerrett, P. Greenland, and T. B. VanItallie, "The Escalating Pandemics of Obesity and Sedentary Lifestyle: A Call to Action for Clinicians," *Archives of Internal Medicine*, vol. 164, no. 3. American Medical Association, pp. 249–258, 09-Feb-2004.

[5] K. T. Mills *et al.*, "Global disparities of hypertension prevalence and control," *Circulation*, vol. 134, no. 6, pp. 441–450, Aug. 2016.

[6] M. Rodríguez-Monforte, G. Flores-Mateo, and E. Sánchez, "Dietary patterns and CVD: A systematic review and meta-analysis of observational studies," *Br. J. Nutr.*, vol. 114, no. 9, pp. 1341–1359, Feb. 2013.

[7] M. M. Dohadwala and J. A. Vita, "Grapes and Cardiovascular Disease," *J. Nutr.*, vol. 139, no. 9, pp. 1788S-1793S, Sep. 2009.

[8] J. D. Wightman and R. A. Heuberger, "Effect of grape and other berries on cardiovascular health," *J. Sci. Food Agric.*, vol. 95, no. 8, pp. 1584–1597, 2015.

[9] J. Ye, X. Chen, L. Bao, and G. Tarantino, "Effects of wine on blood pressure, glucose parameters, and lipid profile in type 2 diabetes mellitus: A meta-analysis of randomized interventional trials (PRISMA Compliant)," *Med. (United States)*, vol. 98, no. 23, Jun. 2019.

[10] OIV and International Organisation of Vine and Wine, "2019 Statistical Report on World Vitiviniculture," *2019 Stat. Rep. World Vitiviniculture*, p. 23, 2019.

[11] N. Musee, L. Lorenzen, and C. Aldrich, "Cellar waste minimization in the wine industry: a systems approach," *J. Clean. Prod.*, vol. 15, no. 5, pp. 417–431, Jan. 2007.

[12] S. Ben-Othman, I. Jõudu, and R. Bhat, "Bioactives from Agri-Food Wastes: Present Insights and Future Challenges," *Molecules*, vol. 25, no. 3, p. 510, Jan. 2020.

[13] T. de Moraes Crizel, V. S. Hermes, A. de Oliveira Rios, and S. H. Flôres, "Evaluation of bioactive compounds, chemical and technological properties of fruits byproducts powder," *J. Food Sci. Technol.*, vol. 53, no. 11, pp. 4067–4075, Nov. 2016.

[14] S. Maqsood, O. Adiamo, M. Ahmad, and P. Mudgil, "Bioactive compounds from date fruit and seed as potential nutraceutical and functional food ingredients," *Food Chemistry*, vol. 308. Elsevier Ltd, p. 125522, 05-Mar-2020.

[15] "Hypertension." [Online]. Available: https://www.who.int/news-room/fact-sheets/detail/hypertension. [Accessed: 19-May-2020].

[16] T. Unger *et al.*, "2020 International Society of Hypertension Global Hypertension Practice Guidelines," *Hypertension*, pp. 1334–1357, May 2020.

[17] G. A. Roth *et al.*, "Global, regional, and national age-sex-specific mortality for 282 causes of death in 195 countries and territories, 1980–2017: a systematic analysis for the Global Burden of Disease Study 2017," *Lancet*, vol. 392, no. 10159, pp. 1736–1788, Nov. 2018.

[18] T. Witter *et al.*, "A conceptual framework for managing modifiable risk factors for cardiovascular diseases in Fiji," *Perspect. Public Health*, vol. 135, no. 2, pp. 75–84, Mar. 2015.

[19] P. Rajendran *et al.*, "The vascular endothelium and human diseases," *International Journal of Biological Sciences*, vol. 9, no. 10. Ivyspring International Publisher, pp. 1057–1069, 09-Nov-2013.

[20] J. E. Hall, A. C. Guyton, and H. L. Mizelle, "Role of the renin-angiotensin system in control of sodium excretion and arterial pressure," in *Acta Physiologica Scandinavica, Supplement*, 1990, vol. 139, no. 591, pp. 48–62.

[21] S. Atlas, "The Renin-Angiotensin Aldosterone System: Pathophysiological Role and Pharmacologic Inhibition," 2007.

[22] L. Shi, C. Mao, Z. Xu, and L. Zhang, "Angiotensin-converting enzymes and drug discovery in cardiovascular diseases," *Drug Discovery Today*, vol. 15, no. 9–10. Elsevier Current Trends, pp. 332–341, 01-May-2010.

[23] M. A. Perazella and J. F. Setaro, "Renin-angiotensin-aldosterone system: Fundamental aspects and clinical implications in renal and cardiovascular disorders," *Journal of Nuclear Cardiology*, vol. 10, no. 2. Mosby Inc., pp. 184–196, 01-Mar-2003.

[24] J. Varagic, S. Ahmad, S. Nagata, and C. M. Ferrario, "ACE2: Angiotensin II/angiotensin-(1-7) balance in cardiac and renal injury," *Current Hypertension Reports*, vol. 16, no. 3. Current Medicine Group LLC 1, 2014.

[25] M. Weir, "The renin-angiotensin-aldosterone system: a specific target for hypertension management," *Am. J. Hypertens.*, vol. 12, no. 4, pp. 205–213, Apr. 1999.

[26] A. Reaux *et al.*, "Aminopeptidase A inhibitors as potential central antihypertensive agents," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 96, no. 23, pp. 13415–13420, Nov. 1999.

[27] V. Cachofeiro *et al.*, "Aldosterone and the vascular system," *J. Steroid Biochem. Mol. Biol.*, vol. 109, no. 3–5, pp. 331–335, Apr. 2008.

[28] A. Povlsen, D. Grimm, M. Wehland, M. Infanger, and M. Krüger, "The Vasoactive

Mas Receptor in Essential Hypertension," *J. Clin. Med.*, vol. 9, no. 1, p. 267, Jan. 2020.

[29] R. A. S. Santos *et al.*, "The ACE2/Angiotensin-(1-7)/Mas axis of the renin-angiotensin system: Focus on Angiotensin-(1-7)," *Physiological Reviews*, vol. 98, no. 1. American Physiological Society, pp. 505–553, 01-Jan-2018.

[30] C. Llorens-Cortes and R. M. Touyz, "Evolution of a New Class of Antihypertensive Drugs: Targeting the Brain Renin-Angiotensin System," *Hypertension (Dallas, Tex. : 1979)*, vol. 75, no. 1. NLM (Medline), pp. 6–15, 01-Jan-2020.

[31] U. M. Steckelings, F. Rompe, E. Kaschina, and T. Unger, "The evolving story of the RAAS in hypertension, diabetes and CV disease - moving from macrovascular to microvascular targets," *Fundam. Clin. Pharmacol.*, vol. 23, no. 6, pp. 693–703, Dec. 2009.

[32] C. Manrique, G. Lastra, M. Gardner, and J. R. Sowers, "The Renin Angiotensin Aldosterone System in Hypertension: Roles of Insulin Resistance and Oxidative Stress," *Medical Clinics of North America*, vol. 93, no. 3. NIH Public Access, pp. 569–582, May-2009.

[33] V. W. M. Van Hinsbergh, "Endothelium - Role in regulation of coagulation and inflammation," *Seminars in Immunopathology*, vol. 34, no. 1. Springer, pp. 93–106, Jan-2012.

[34] S. Godo and H. Shimokawa, "Endothelial Functions," *Arterioscler. Thromb. Vasc. Biol.*, vol. 37, no. 9, pp. e108–e114, Sep. 2017.

[35] M. E. Widlansky, N. Gokce, J. F. Keaney, and J. A. Vita, "The clinical implications of endothelial dysfunction," *Journal of the American College of Cardiology*, vol. 42, no. 7. Elsevier Inc., pp. 1149–1160, 01-Oct-2003.

[36] D. Konukoglu and H. Uzun, "Endothelial dysfunction and hypertension," in *Advances in Experimental Medicine and Biology*, vol. 956, Springer New York LLC, 2016, pp. 511–540.

[37] U. Förstermann and W. C. Sessa, "Nitric oxide synthases: Regulation and function," *European Heart Journal*, vol. 33, no. 7. 2012.

[38] I. Mordi, N. Mordi, C. Delles, and N. Tzemos, "Endothelial dysfunction in human essential hypertension," *Journal of Hypertension*, vol. 34, no. 8. Lippincott Williams and Wilkins, pp. 1464–1472, 01-Aug-2016.

[39] P. W. Shaul, "REGULATION OF ENDOTHELIAL NITRIC OXIDE SYNTHASE: Location, Location, Location," *Annu. Rev. Physiol*, vol. 64, pp. 749–74, 2002.

[40] U. Fö Rstermann and W. C. Sessa, "Nitric oxide synthases: regulation and function."

[41] Y. Zhao, P. M. Vanhoutte, and S. W. S. Leung, "Vascular nitric oxide: Beyond eNOS," *Journal of Pharmacological Sciences*, vol. 129, no. 2. Japanese Pharmacological Society, pp. 83–94, 01-Oct-2015.

[42] N. A. Maniatis *et al.*, "Novel mechanism of endothelial nitric oxide synthase

activation mediated by caveolae internalization in endothelial cells," *Circ. Res.*, vol. 99, no. 8, pp. 870–877, Oct. 2006.

[43] I. Mattagajasingh *et al.*, "SIRT1 promotes endothelium-dependent vascular relaxation by activating endothelial nitric oxide synthase," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 104, no. 37, pp. 14855–14860, Sep. 2007.

[44] H. Ota *et al.*, "Induction of endothelial nitric oxide synthase, SIRT1, and catalase by statins inhibits endothelial senescence through the Akt pathway," *Arterioscler. Thromb. Vasc. Biol.*, vol. 30, no. 11, pp. 2205–2211, Nov. 2010.

[45] Q. Zhang *et al.*, "Endothelium-specific overexpression of class III deacetylase SIRT1 decreases atherosclerosis in apolipoprotein E-deficient mice."

[46] A. J. Donato, K. A. Magerko, B. R. Lawson, J. R. Durrant, L. A. Lesniewski, and D. R. Seals, "SIRT-1 and vascular endothelial dysfunction with ageing in mice and humans," *J. Physiol.*, vol. 589, no. 18, pp. 4545–4554, Sep. 2011.

[47] F. Wang, H.-Z. Chen, X. Lv, and D.-P. Liu, "SIRT1 as a Novel Potential Treatment Target for Vascular Aging and Age-Related Vascular Diseases," *Curr. Mol. Med.*, vol. 13, no. 1, pp. 155–164, Dec. 2012.

[48] N. Benjamin and J. Vane, "Nitric Oxide and Hypertension," *Circulation*, vol. 94, no. 6, pp. 1197–1198, Sep. 1996.

[49] J. T. Kielstein, S. M. Bode-Böger, J. C. Frölich, E. Ritz, H. Haller, and D. Fliser, "Asymmetric dimethylarginine, blood pressure, and renal perfusion in elderly subjects," *Circulation*, vol. 107, no. 14, pp. 1891–1895, Apr. 2003.

[50] G. M. Rubanyi and P. M. Vanhoutte, "Superoxide anions and hyperoxia inactivate endothelium-derived relaxing factor," *Am. J. Physiol. Circ. Physiol.*, vol. 250, no. 5, pp. H822–H827, May 1986.

[51] T. F. Luscher, C. M. Boulanger, Y. Dohi, and Z. Yang, "Endothelium-derived contracting factors," *Hypertension*, vol. 19, no. 2. Hypertension, pp. 117–130, 1992.

[52] J. A. Mitchell, F. Ali, L. Bailey, L. Moreno, and L. S. Harrington, "Role of nitric oxide and prostacyclin as vasoactive hormones released by the endothelium," in *Experimental Physiology*, 2008, vol. 93, no. 1, pp. 141–147.

[53] T. Nakayama, "Prostacyclin Analogues: Prevention of Cardiovascular Diseases," *Cardiovasc. Hematol. Agents Med. Chem.*, vol. 4, no. 4, pp. 351–359, Jul. 2008.

[54] J. A. Mitchell and T. D. Warner, "Cyclo-oxygenase-2: Pharmacology, physiology, biochemistry and relevance to NSAID therapy," *British Journal of Pharmacology*, vol. 128, no. 6. Nature Publishing Group, pp. 1121–1132, 1999.

[55] S. P. Herbert, S. Ponnambalam, and J. H. Walker, "Cytosolic phospholipase A2-α mediates endothelial cell proliferation and is inactivated by association with the Golgi apparatus," *Mol. Biol. Cell*, vol. 16, no. 8, pp. 3800–3809, Aug. 2005.

[56] Y. Chi *et al.*, "Inhibition of the Prostaglandin Transporter PGT Lowers Blood Pressure in Hypertensive Rats and Mice," *PLoS One*, vol. 10, no. 6, p. e0131735, Jun. 2015.

[57] M. Félétou, Y. Huang, and P. M. Vanhoutte, "Endothelium-mediated control of vascular tone: COX-1 and COX-2 products," *British Journal of Pharmacology*, vol. 164, no. 3. Wiley-Blackwell, pp. 894–912, Oct-2011.

[58] P. A. Cahill and E. M. Redmond, "Vascular endothelium - Gatekeeper of vessel health," *Atherosclerosis*, vol. 248. Elsevier Ireland Ltd, pp. 97–109, 01-May-2016.

[59] M. Yanagisawa *et al.*, "A novel potent vasoconstrictor peptide produced by vascular endothelial cells," *Nature*, vol. 332, no. 6163, pp. 411–415, 1988.

[60] T. Watanabe, N. Suzuki, N. Shimamoto, M. Fujino, and A. Imada, "Endothelin in myocardial infarction.," *Nature*, vol. 344, no. 6262, p. 114, Mar. 1990.

[61] B. L. Stauffer, C. M. Westby, and C. A. DeSouza, "Endothelin-1, aging and hypertension," *Current Opinion in Cardiology*, vol. 23, no. 4. NIH Public Access, pp. 350–355, Jul-2008.

[62] M. R. Dashwood and J. C. S. Tsui, "Endothelin-1 and atherosclerosis: Potential complications associated with endothelin-receptor blockade," *Atherosclerosis*, vol. 160, no. 2, pp. 297–304, Feb. 2002.

[63] W. G. Haynes, F. E. Strachan, and D. J. Webb, "Endothelin ETA and ETB receptors cause vasoconstriction of human resistance and capacitance vessels in vivo," *Circulation*, vol. 92, no. 3, pp. 357–363, Aug. 1995.

[64] D. Alonso and M. W. Radomski, "The nitric oxide-endothelin-1 connection," *Heart Failure Reviews*, vol. 8, no. 1. Springer, pp. 107–115, Jan-2003.

[65] T. Masaki and T. Sawamura, "Endothelin and endothelial dysfunction," *Proceedings of the Japan Academy Series B: Physical and Biological Sciences*, vol. 82, no. 1. The Japan Academy, pp. 17–24, Mar-2006.

[66] R. Rodrigo, R. Brito, G. Castillo, J. González, and N. Valls, "Oxidative Stress in Hypertension: Mechanisms and Therapeutic Opportunities," *Oxidative Stress Hypertens. … Exp Clin Endocrinol Diabetes*, vol. 123, pp. 325–335, 2015.

[67] Y. C. Cheng, J. M. Sheen, W. L. Hu, and Y. C. Hung, "Polyphenols and Oxidative Stress in Atherosclerosis-Related Ischemic Heart Disease and Stroke," *Oxidative Medicine and Cellular Longevity*, vol. 2017. Hindawi Limited, 2017.

[68] S. D. Crowley, "The Cooperative roles of inflammation and oxidative stress in the pathogenesis of hypertension," *Antioxidants Redox Signal.*, vol. 20, no. 1, pp. 102–120, 2014.

[69] D. I. Brown and K. K. Griendling, "Regulation of signal transduction by reactive oxygen species in the cardiovascular system," *Circulation Research*, vol. 116, no. 3. Lippincott Williams and Wilkins, pp. 531–549, 29-Jan-2015.

[70] R. Ray *et al.*, "Endothelial Nox4 NADPH oxidase enhances vasodilatation and reduces blood pressure in vivo," *Arterioscler. Thromb. Vasc. Biol.*, vol. 31, no. 6, pp. 1368–1376, Jun. 2011.

[71] G. Bouabout *et al.*, "Nox4 genetic inhibition in experimental hypertension and metabolic syndrome," *Arch. Cardiovasc. Dis.*, vol. 111, no. 1, pp. 41–52, Jan.

2018.

[72] A. Konior, A. Schramm, M. Czesnikiewicz-Guzik, and T. J. Guzik, "NADPH oxidases in vascular pathology," *Antioxidants and Redox Signaling*, vol. 20, no. 17. Mary Ann Liebert Inc., pp. 2794–2814, 10-Jun-2014.

[73] A. C. Montezano and R. M. Touyz, "Oxidative stress, Noxs, and hypertension: Experimental evidence and clinical controversies," *Annals of Medicine*, vol. 44, no. SUPPL. 1. Taylor & Francis, Jun-2012.

[74] M. Y. Lee and K. K. Griendling, "Redox signaling, vascular function, and hypertension," *Antioxidants and Redox Signaling*, vol. 10, no. 6. Mary Ann Liebert, Inc., pp. 1045–1059, 01-Jun-2008.

[75] H. J. Forman, H. Zhang, and A. Rinna, "Glutathione: Overview of its protective roles, measurement, and biosynthesis," *Molecular Aspects of Medicine*, vol. 30, no. 1–2. NIH Public Access, pp. 1–12, Feb-2009.

[76] J. Robaczewska *et al.*, "ROLE OF GLUTATHIONE METABOLISM AND GLUTATHIONE-RELATED ANTIOXIDANT DEFENSE SYSTEMS IN HYPERTENSION," 2016.

[77] K. Jomova and M. Valko, "Advances in metal-induced oxidative stress and human disease," *Toxicology*, vol. 283, no. 2–3. Elsevier, pp. 65–87, 10-May-2011.

[78] D. A. Jones, S. L. Prior, J. D. Barry, S. Caplin, J. N. Baxter, and J. W. Stephens, "Changes in markers of oxidative stress and DNA damage in human visceral adipose tissue from subjects with obesity and type 2 diabetes," *Diabetes Res. Clin. Pract.*, vol. 106, no. 3, pp. 627–633, Dec. 2014.

[79] D. Li and J. L. Mehta, "3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors protect against oxidized low-density lipoprotein-induced endothelial dysfunction," *Endothelium: Journal of Endothelial Cell Research*, vol. 10, no. 1. Endothelium, pp. 17–21, 2003.

[80] M. A. Incalza, R. D'Oria, A. Natalicchio, S. Perrini, L. Laviola, and F. Giorgino, "Oxidative stress and reactive oxygen species in endothelial dysfunction associated with cardiovascular and metabolic diseases," *Vascular Pharmacology*, vol. 100. Elsevier Inc., pp. 1–19, 30-Apr-2018.

[81] E. M. Lonn *et al.*, "Blood-Pressure Lowering in Intermediate-Risk Persons without Cardiovascular Disease," *N. Engl. J. Med.*, vol. 374, no. 21, pp. 2009–2020, May 2016.

[82] F. Samadian, N. Dalili, and A. Jamalian, "Lifestyle modifications to prevent and control hypertension," *Iran. J. Kidney Dis.*, vol. 10, no. 5, pp. 237–263, 2016.

[83] S. R. Collier and M. J. Landram, "Treatment of prehypertension: Lifestyle and/or medication," *Vascular Health and Risk Management*, vol. 8, no. 1. Dove Press, pp. 613–619, 2012.

[84] P. Muntner and P. K. Whelton, "Using Predicted Cardiovascular Disease Risk in Conjunction With Blood Pressure to Guide Antihypertensive Medication

Treatment," *Journal of the American College of Cardiology*, vol. 69, no. 19. Elsevier USA, pp. 2446–2456, 16-May-2017.

[85] V. Nash *et al.*, "The effects of grape and red wine polyphenols on gut microbiota – A systematic review," *Food Res. Int.*, vol. 113, no. April, pp. 277–287, 2018.

[86] G. Mancia and A. Zanchetti, "Choice of antihypertensive drugs in the European Society of Hypertension–European Society of Cardiology guidelines: specific indications rather than ranking for general usage," *J. Hypertens.*, vol. 26, no. 2, pp. 164–168, Feb. 2008.

[87] P. A. James *et al.*, "2014 Evidence-based guideline for the management of high blood pressure in adults: Report from the panel members appointed to the Eighth Joint National Committee (JNC 8)," *JAMA - Journal of the American Medical Association*, vol. 311, no. 5. American Medical Association, pp. 507–520, 05-Feb-2014.

[88] P. Ponikowski *et al.*, "2016 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure," *European Heart Journal*, vol. 37, no. 27. Oxford University Press, pp. 2129-2200m, 14-Jul-2016.

[89] K. E. Bernstein, Z. Khan, J. F. Giani, D.-Y. Cao, E. A. Bernstein, and X. Z. Shen, "Angiotensin-converting enzyme in innate and adaptive immunity," *Nat. Publ. Gr.*, vol. 14, 2018.

[90] M. Margalef, F. I. Bravo, A. Arola-Arnal, and B. Muguerza, "Natural Angiotensin Converting Enzyme (ACE) Inhibitors with Antihypertensive Properties," in *Natural Products Targeting Clinically Relevant Enzymes*, Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA, 2017, pp. 45–67.

[91] R. A. S. Santos, A. J. Ferreira, and A. C. Simões E Silva, "Recent advances in the angiotensin-converting enzyme 2-angiotensin(1-7)-Mas axis," *Experimental Physiology*, vol. 93, no. 5. Blackwell Publishing Ltd, pp. 519–527, 01-May-2008.

[92] C. A. Dézsi and V. Szentes, "The Real Role of β-Blockers in Daily Cardiovascular Therapy," *American Journal of Cardiovascular Drugs*, vol. 17, no. 5. Springer International Publishing, pp. 361–373, 01-Oct-2017.

[93] C. S. Wiysonge, H. A. Bradley, J. Volmink, B. M. Mayosi, and L. H. Opie, "Beta-blockers for hypertension," *Cochrane Database of Systematic Reviews*, vol. 2017, no. 1. John Wiley and Sons Ltd, 20-Jan-2017.

[94] J. J. DiNicolantonio *et al.*, "β-Blockers in hypertension, diabetes, heart failure and acute myocardial infarction: a review of the literature," *Open Hear.*, vol. 2, no. 1, p. e000230, Mar. 2015.

[95] D. Wile, "Diuretics: a review," *Ann. Clin. Biochem.*, vol. 49, no. 5, pp. 419–431, Sep. 2012.

[96] M. E. Ernst and S. J. Mann, "Diuretics in the treatment of hypertension," *Semin. Nephrol.*, vol. 31, no. 6, pp. 495–502, Nov. 2011.

[97] M. S. Islam, "The art and science of using diuretics in the treatment of heart failure in diverse clinical settings," in *Advances in Experimental Medicine and*

Biology, vol. 1067, Springer New York LLC, 2018, pp. 47–65.

[98] J. C. Trullàs, J. L. Morales-Rull, and F. Formiga, "[Diuretic therapy in acute heart failure].," *Med. Clin. (Barc).*, vol. 142 Suppl 1, no. SUPPL. 1, pp. 36–41, Mar. 2014.

[99] J. Tamargo and L. M. Ruilope, "Investigational calcium channel blockers for the treatment of hypertension," *Expert Opinion on Investigational Drugs*, vol. 25, no. 11. Taylor and Francis Ltd, pp. 1295–1309, 01-Nov-2016.

[100] A. Low, N. Kotecha, T. Neild, C. Kwan, and E. Daniel, "RELATIVE CONTRIBUTIONS OF EXTRACELLULAR Ca^{2+} AND Ca^{2+} STORES TO SMOOTH MUSCLE CONTRACTION IN ARTERIES AND ARTERIOLES OF RAT, GUINEA-PIG DOG AND RABBIT," *Clin. Exp. Pharmacol. Physiol.*, vol. 23, no. 4, pp. 310–316, Apr. 1996.

[101] H. Fares, J. J. DiNicolantonio, J. H. O'Keefe, and C. J. Lavie, "Amlodipine in hypertension: A first-line agent with efficacy for improving blood pressure and patient outcomes," *Open Hear.*, vol. 3, no. 2, 2016.

[102] W. H. Frishman, A. Cheng-Lai, and J. Newarskas, *Current cardiovascular drugs*. Current Medicine, 2005.

[103] A. Helmer, N. Slater, and S. Smithgall, "A Review of ACE Inhibitors and ARBs in Black Patients With Hypertension.," *Ann. Pharmacother.*, vol. 52, no. 11, pp. 1143–1151, Nov. 2018.

[104] P. Zhang *et al.*, "Association of Inpatient Use of Angiotensin Converting Enzyme Inhibitors and Angiotensin II Receptor Blockers with Mortality Among Patients With Hypertension Hospitalized With COVID-19," *Circ. Res.*, Apr. 2020.

[105] S. Lim *et al.*, "Angiotensin receptor blockers as an alternative to angiotensin-converting enzyme inhibitors in patients with acute myocardial infarction undergoing percutaneous coronary intervention," *J. Korean Med. Sci.*, vol. 34, no. 45, 2019.

[106] S. Bœuf-Gibot *et al.*, "Benefits and adverse effects of ACE inhibitors in patients with heart failure with reduced ejection fraction: a systematic review and meta-analysis," *European Journal of Clinical Pharmacology*, vol. 77, no. 3. Springer Science and Business Media Deutschland GmbH, pp. 321–329, 01-Mar-2020.

[107] F. H. Messerli, B. Williams, and E. Ritz, "Essential hypertension," *Lancet*, vol. 370, no. 9587. Elsevier, pp. 591–603, 18-Aug-2007.

[108] A. Jakubczyk, M. Karas, K. Rybczynska-Tkaczyk, E. Zielinska, and D. Zielinski, "Current trends of bioactive peptides - New sources and therapeutic effect," *Foods*, vol. 9, no. 7. MDPI Multidisciplinary Digital Publishing Institute, 01-Jul-2020.

[109] E. D. Sturrock *et al.*, "Structural basis for the C-domain-selective angiotensin-converting enzyme inhibition by bradykinin-potentiating peptide b (BPPb)," *Biochem. J.*, vol. 476, no. 10, pp. 1553–1570, May 2019.

[110] B. Jankowska-Polańska, K. Zamęta, I. Uchmanowicz, A. Szymańska-Chabowska, D. Morisky, and G. Mazur, "Adherence to pharmacological and non-

pharmacological treatment of frail hypertensive patients," *J. Geriatr. Cardiol.*, vol. 15, no. 2, pp. 153–161, 2018.

[111] C. N. Zhao *et al.*, "Fruits for prevention and treatment of cardiovascular diseases," *Nutrients*, vol. 9, no. 6. MDPI AG, 13-Jun-2017.

[112] D. Del Rio, A. Rodriguez-Mateos, J. P. E. Spencer, M. Tognolini, G. Borges, and A. Crozier, "Dietary (poly)phenolics in human health: Structures, bioavailability, and evidence of protective effects against chronic diseases," *Antioxidants and Redox Signaling*, vol. 18, no. 14. Mary Ann Liebert, Inc., pp. 1818–1892, 10-May-2013.

[113] C. G. Fraga, K. D. Croft, D. O. Kennedy, and F. A. Tomás-Barberán, "The effects of polyphenols and other bioactives on human health," *Food Funct.*, vol. 10, no. 2, pp. 514–528, Feb. 2019.

[114] V. B. Schini-Kerth, C. Auger, J. H. Kim, N. Étienne-Selloum, and T. Chataigneau, "Nutritional improvement of the endothelial control of vascular tone by polyphenols: Role of NO and EDHF," *Pflugers Archiv European Journal of Physiology*, vol. 459, no. 6. Pflugers Arch, pp. 853–862, May-2010.

[115] A. Durazzo *et al.*, "Polyphenols: A concise overview on the chemistry, occurrence, and human health," *Phytotherapy Research*, vol. 33, no. 9. John Wiley and Sons Ltd, pp. 2221–2243, 01-Sep-2019.

[116] W. M. Dabeek and M. V. Marra, "Dietary quercetin and kaempferol: Bioavailability and potential cardiovascular-related bioactivity in humans," *Nutrients*, vol. 11, no. 10. MDPI AG, 01-Oct-2019.

[117] D. Maaliki, A. A. Shaito, G. Pintus, A. El-Yazbi, and A. H. Eid, "Flavonoids in hypertension: a brief review of the underlying mechanisms," *Current Opinion in Pharmacology*, vol. 45. Elsevier Ltd, pp. 57–65, 01-Apr-2019.

[118] L. Guerrero *et al.*, "Inhibition of Angiotensin-Converting Enzyme Activity by Flavonoids: Structure-Activity Relationship Studies," *PLoS One*, vol. 7, no. 11, p. 49493, Nov. 2012.

[119] M. Quiñones *et al.*, "Long-term intake of CocoanOX attenuates the development of hypertension in spontaneously hypertensive rats," *Food Chem.*, vol. 122, no. 4, pp. 1013–1019, Oct. 2010.

[120] M. Quiñones *et al.*, "Low-molecular procyanidin rich grape seed extract exerts antihypertensive effect in males spontaneously hypertensive rats," *Food Res. Int.*, vol. 51, no. 2, pp. 587–595, May 2013.

[121] Z. Pons, M. Margalef, F. I. Bravo, A. Arola-Arnal, and B. Muguerza, "Grape seed flavanols decrease blood pressure via Sirt-1 and confer a vasoprotective pattern in rats," *J. Funct. Foods*, vol. 24, pp. 164–172, Jun. 2016.

[122] C. Rodríguez-Pérez, B. García-Villanova, E. Guerra-Hernández, and V. Verardo, "Grape seeds proanthocyanidins: An overview of in vivo bioactivity in animal models," *Nutrients*, vol. 11, no. 10. MDPI AG, 01-Oct-2019.

[123] J. F. Leikert, T. R. Räthel, P. Wohlfart, V. Cheynier, A. M. Vollmar, and V. M.

Dirsch, "Red wine polyphenols enhance endothelial nitric oxide synthase expression and subsequent nitric oxide release from endothelial cells," *Circulation*, vol. 106, no. 13, pp. 1614–1617, Sep. 2002.

[124] C. Heiss *et al.*, "Improvement of endothelial function with dietary flavanols is associated with mobilization of circulating angiogenic cells in patients with coronary artery disease," *J. Am. Coll. Cardiol.*, vol. 56, no. 3, pp. 218–224, Jul. 2010.

[125] J. Ren, J. An, M. Chen, H. Yang, and Y. Ma, "Effect of proanthocyanidins on blood pressure: A systematic review and meta-analysis of randomized controlled trials," *Pharmacological Research*, vol. 165. Academic Press, p. 105329, 01-Mar-2021.

[126] M. Quiñones *et al.*, "Involvement of nitric oxide and prostacyclin in the antihypertensive effect of low-molecular-weight procyanidin rich grape seed extract in male spontaneously hypertensive rats," *J. Funct. Foods*, vol. 6, no. 1, pp. 419–427, Jan. 2014.

[127] M. Quiñones, M. Margalef, A. Arola-Arnal, B. Muguerza, M. Miguel, and A. Aleixandre, "The blood pressure effect and related plasma levels of flavan-3-ols in spontaneously hypertensive rats," *Food Funct.*, vol. 6, no. 11, pp. 3479–3489, Nov. 2015.

[128] H. E. Khoo, A. Azlan, S. T. Tang, and S. M. Lim, "Anthocyanidins and anthocyanins: Colored pigments as food, pharmaceutical ingredients, and the potential health benefits," *Food and Nutrition Research*, vol. 61, no. 1. Swedish Nutrition Foundation, 2017.

[129] T. C. Wallace, "Anthocyanins in cardiovascular disease," *Advances in Nutrition*, vol. 2, no. 1. Adv Nutr, pp. 1–7, Jan-2011.

[130] D. R. Bell and K. Gochenaur, "Direct vasoactive and vasoprotective properties of anthocyanin-rich extracts," *J. Appl. Physiol.*, vol. 100, no. 4, pp. 1164–1170, Apr. 2006.

[131] J. N. Kim, S. N. Han, T. J. Ha, and H. K. Kim, "Black soybean anthocyanins attenuate inflammatory responses by suppressing reactive oxygen species production and mitogen activated protein kinases signaling in lipopolysaccharide-stimulated macrophages," *Nutr. Res. Pract.*, vol. 11, no. 5, pp. 357–364, Oct. 2017.

[132] H. Huang, G. Chen, D. Liao, Y. Zhu, and X. Xue, "Effects of Berries Consumption on Cardiovascular Risk Factors: A Meta-analysis with Trial Sequential Analysis of Randomized Controlled Trials," *Sci. Rep.*, vol. 6, Mar. 2016.

[133] L. P. Yang *et al.*, "Effects of anthocyanins on cardiometabolic health: A systematic review and meta-analysis of randomized controlled trials," *Advances in Nutrition*, vol. 8, no. 5. American Society for Nutrition, pp. 684–693, 01-Sep-2017.

[134] L. Fairlie-Jones, K. Davison, E. Fromentin, and A. M. Hill, "The effect of anthocyanin-rich foods or extracts on vascular function in adults: A systematic review and meta-analysis of randomised controlled trials," *Nutrients*, vol. 9, no.

8. MDPI AG, 20-Aug-2017.

[135] C. Calfío and J. P. Huidobro-Toro, "Potent vasodilator and cellular antioxidant activity of endemic patagonian calafate berries (berberis microphylla) with nutraceutical potential," *Molecules*, vol. 24, no. 15, Jul. 2019.

[136] D. Bonnefont-Rousselot, "Resveratrol and cardiovascular diseases," *Nutrients*, vol. 8, no. 5. MDPI AG, 01-May-2016.

[137] R. Miatello, M. Vázquez, N. Renna, M. Cruzado, A. P. Zumino, and N. Risler, "Chronic administration of resveratrol prevents biochemical cardiovascular changes in fructose-fed rats," *Am. J. Hypertens.*, vol. 18, no. 6, pp. 864–870, Jun. 2005.

[138] K. Mizutani, K. Ikeda, Y. Kawai, and Y. Yamori, "Resveratrol attenuates ovariectomy-induced hypertension and bone loss in stroke-prone spontaneously hypertensive rats," *J. Nutr. Sci. Vitaminol. (Tokyo).*, vol. 46, no. 2, pp. 78–83, 2000.

[139] F. Akar *et al.*, "High-fructose corn syrup causes vascular dysfunction associated with metabolic disturbance in rats: Protective effect of resveratrol," *Food Chem. Toxicol.*, vol. 50, no. 6, pp. 2135–2141, Jun. 2012.

[140] S. R. Bhatt, M. F. Lokhandwala, and A. A. Banday, "Resveratrol prevents endothelial nitric oxide synthase uncoupling and attenuates development of hypertension in spontaneously hypertensive rats," *Eur. J. Pharmacol.*, vol. 667, no. 1–3, pp. 258–264, Sep. 2011.

[141] J. G. Franco *et al.*, "Resveratrol attenuates oxidative stress and prevents steatosis and hypertension in obese rats programmed by early weaning," *J. Nutr. Biochem.*, vol. 24, no. 6, pp. 960–966, Jun. 2013.

[142] P. K. Bagul *et al.*, "Attenuation of insulin resistance, metabolic syndrome and hepatic oxidative stress by resveratrol in fructose-fed rats," *Pharmacol. Res.*, vol. 66, no. 3, pp. 260–268, Sep. 2012.

[143] M. Ramar *et al.*, "Protective effect of ferulic acid and resveratrol against alloxan-induced diabetes in mice," *Eur. J. Pharmacol.*, vol. 690, no. 1–3, pp. 226–235, Sep. 2012.

[144] A. A. Alturfan, A. Tozan-Beceren, A. Ö. Şehirli, E. Demiralp, G. Şener, and G. Z. Omurtag, "Resveratrol ameliorates oxidative DNA damage and protects against acrylamide-induced oxidative stress in rats," *Mol. Biol. Rep.*, vol. 39, no. 4, pp. 4589–4596, Apr. 2012.

[145] M. Gertz *et al.*, "A Molecular Mechanism for Direct Sirtuin Activation by Resveratrol," *PLoS One*, vol. 7, no. 11, Nov. 2012.

[146] A. Sahebkar *et al.*, "Lack of efficacy of resveratrol on C-reactive protein and selected cardiovascular risk factors - Results from a systematic review and meta-analysis of randomized controlled trials," *Int. J. Cardiol.*, vol. 189, no. 1, pp. 47–55, Jun. 2015.

[147] S. Asgary, R. Karimi, S. Momtaz, R. Naseri, and M. H. Farzaei, "Effect of

resveratrol on metabolic syndrome components: A systematic review and meta-analysis," *Reviews in Endocrine and Metabolic Disorders*, vol. 20, no. 2. Springer New York LLC, pp. 173–186, 15-Jun-2019.

[148] H. Huang, G. Chen, D. Liao, Y. Zhu, R. Pu, and X. Xue, "The effects of resveratrol intervention on risk markers of cardiovascular health in overweight and obese subjects: a pooled analysis of randomized controlled trials," *Obes. Rev.*, vol. 17, no. 12, pp. 1329–1340, Dec. 2016.

[149] F. Fogacci, G. Tocci, V. Presta, A. Fratter, C. Borghi, and A. F. G. Cicero, "Effect of resveratrol on blood pressure: A systematic review and meta-analysis of randomized, controlled, clinical trials," *Critical Reviews in Food Science and Nutrition*, vol. 59, no. 10. Taylor and Francis Inc., pp. 1605–1618, 31-May-2019.

[150] Y. Marunaka *et al.*, "Actions of quercetin, a polyphenol, on blood pressure," *Molecules*, vol. 22, no. 2. MDPI AG, 01-Feb-2017.

[151] O. Dayoub, R. Andriantsitohaina, and N. Clere, "Pleiotropic Beneficial Effects of Epigallocatechin Gallate, Quercetin and Delphinidin on Cardiovascular Diseases Associated with Endothelial Dysfunction," *Cardiovasc. Hematol. Agents Med. Chem.*, vol. 11, no. 4, pp. 249–264, Apr. 2014.

[152] M. Ibarra *et al.*, "Effects of the Flavonoid Quercetin and its Methylated Metabolite Isorhamnetin in Isolated Arteries from Spontaneously Hypertensive Rats," *Planta Med.*, vol. 69, no. 11, pp. 995–1000, Nov. 2003.

[153] S. Egert *et al.*, "Quercetin reduces systolic blood pressure and plasma oxidised low-density lipoprotein concentrations in overweight subjects with a high-cardiovascular disease risk phenotype: A double-blinded, placebo-controlled cross-over study," *Br. J. Nutr.*, vol. 102, no. 7, pp. 1065–1074, Oct. 2009.

[154] W. M. Loke, J. M. Hodgson, J. M. Proudfoot, A. J. McKinley, I. B. Puddey, and K. D. Croft, "Pure dietary flavonoids quercetin and (-)-epicatechin augment nitric oxide products and reduce endothelin-1 acutely in healthy men," *Am. J. Clin. Nutr.*, vol. 88, no. 4, pp. 1018–1025, Oct. 2008.

[155] V. Brüll *et al.*, "Effects of a quercetin-rich onion skin extract on 24 h ambulatory blood pressure and endothelial function in overweight-to-obese patients with (pre-)hypertension: A randomised double-blinded placebo-controlled cross-over trial," *Br. J. Nutr.*, vol. 114, no. 8, pp. 1263–1277, Aug. 2015.

[156] M. C. Serban *et al.*, "Effects of Quercetin on Blood Pressure: A Systematic Review and Meta-Analysis of Randomized Controlled Trials," *J. Am. Heart Assoc.*, vol. 5, no. 7, Jul. 2016.

[157] M. Margalef, F. I. Bravo, A. Arola-Arnal, and B. Muguerza, "Natural Angiotensin Converting Enzyme (ACE) Inhibitors with Antihypertensive Properties," in *Natural Products Targeting Clinically Relevant Enzymes*, Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA, 2017, pp. 45–67.

[158] S. Sultan, N. Huma, M. S. Butt, M. Aleem, and M. Abbas, "Therapeutic potential of dairy bioactive peptides: A contemporary perspective," *Crit. Rev. Food Sci. Nutr.*, vol. 58, no. 1, pp. 105–115, Jan. 2018.

[159] S. Chakrabarti, S. Guha, and K. Majumder, "Food-derived bioactive peptides in human health: Challenges and opportunities," *Nutrients*, vol. 10, no. 11. MDPI AG, 12-Nov-2018.

[160] N. P. Möller, K. E. Scholz-Ahrens, N. Roos, and J. Schrezenmeir, "Bioactive peptides and proteins from foods: Indication for health effects," *European Journal of Nutrition*, vol. 47, no. 4. Springer, pp. 171–182, 27-Jun-2008.

[161] K. Erdmann, B. W. Y. Cheung, and H. Schröder, "The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease," *Journal of Nutritional Biochemistry*, vol. 19, no. 10. Elsevier, pp. 643–654, 01-Oct-2008.

[162] A. Teixeira *et al.*, "Natural bioactive compounds from winery by-products as health promoters: A review," *International Journal of Molecular Sciences*, vol. 15, no. 9. MDPI AG, pp. 15638–15678, 04-Sep-2014.

[163] J. P. Trigo, E. M. C. Alexandre, J. A. Saraiva, and M. E. Pintado, "High value-added compounds from fruit and vegetable by-products–Characterization, bioactivities, and application in the development of novel food products," *Critical Reviews in Food Science and Nutrition*, vol. 60, no. 8. Taylor and Francis Inc., pp. 1388–1416, 27-Apr-2020.

[164] J. M. Tomiyama, D. Takagi, and M. B. Kantar, "The effect of acute and chronic food shortage on human population equilibrium in a subsistence setting," *Agric. Food Secur.*, vol. 9, no. 1, p. 6, Sep. 2020.

[165] J. A. Garza-Reyes, V. Kumar, L. Batista, A. Cherrafi, and L. Rocha-Lona, "From linear to circular manufacturing business models," *Journal of Manufacturing Technology Management*, vol. 30, no. 3. Emerald Group Publishing Ltd., pp. 554–560, 15-Apr-2019.

[166] K. R. Subramanian, "The Crisis of Consumption of Natural Resources SUSTAINING GLOBALIZATION IN AN ENVIRONMENT OF RISING NATIONALISM View project," 2018.

[167] "FAO - News Article: Food wastage: Key facts and figures." [Online]. Available: http://www.fao.org/news/story/en/item/196402/icode/. [Accessed: 30-Jan-2021].

[168] A. Del Borghi, L. Moreschi, and M. Gallo, "Circular economy approach to reduce water–energy–food nexus," *Current Opinion in Environmental Science and Health*, vol. 13. Elsevier B.V., pp. 23–28, 01-Feb-2020.

[169] A. J. Shrikhande, "Wine by-products with health benefits," *Food Research International*, vol. 33, no. 6. Elsevier Ltd, pp. 469–474, 01-Jul-2000.

[170] C. Di Lorenzo, L. P. de Sousa, R. F. Pastor, F. Colombo, G. Frigerio, and P. Restani, "Beneficial effects of non-alcoholic grape-derived products on human health: A literature review," *BIO Web Conf.*, vol. 5, no. 2015, p. 04002, 2015.

[171] Z. Rasines-Perea, I. Ky, G. Cros, A. Crozier, and P.-L. Teissedre, "Grape Pomace: Antioxidant Activity, Potential Effect Against Hypertension and Metabolites Characterization after Intake," *Diseases*, vol. 6, no. 3, p. 60, Jul. 2018.

[172] Z. Pons, M. Margalef, F. I. Bravo, A. Arola-Arnal, and B. Muguerza, "Acute administration of single oral dose of grape seed polyphenols restores blood pressure in a rat model of metabolic syndrome: role of nitric oxide and prostacyclin," *Eur. J. Nutr.*, vol. 55, no. 2, pp. 749–758, Mar. 2016.

[173] J. Banerjee, R. Singh, R. Vijayaraghavan, D. MacFarlane, A. F. Patti, and A. Arora, "Bioactives from fruit processing wastes: Green approaches to valuable chemicals," *Food Chemistry*, vol. 225. Elsevier Ltd, pp. 10–22, 15-Jun-2017.

[174] M. Iriondo-Dehond, E. Miguel, and M. D. Del Castillo, "Food byproducts as sustainable ingredients for innovative and healthy dairy foods," *Nutrients*, vol. 10, no. 10. MDPI AG, 01-Oct-2018.

[175] M. Sovak, "Grape extract, resveratrol, and its analogs: A review," *J. Med. Food*, vol. 4, no. 2, pp. 93–105, 2001.

[176] A. Leibowitz *et al.*, "Red grape berry-cultured cells reduce blood pressure in rats with metabolic-like syndrome," *Eur. J. Nutr.*, vol. 53, no. 3, pp. 973–980, Apr. 2014.

[177] N. Vaisman and E. Niv, "Daily consumption of red grape cell powder in a dietary dose improves cardiovascular parameters: A double blind, placebo-controlled, randomized study," *Int. J. Food Sci. Nutr.*, vol. 66, no. 3, pp. 342–349, May 2015.

[178] N. P. Bondonno *et al.*, "Flavonoid-Rich Apple Improves Endothelial Function in Individuals at Risk for Cardiovascular Disease: A Randomized Controlled Clinical Trial," *Mol. Nutr. Food Res.*, vol. 62, no. 3, Feb. 2018.

[179] S. A. Johnson *et al.*, "Daily blueberry consumption improves blood pressure and arterial stiffness in postmenopausal women with pre- and stage 1-hypertension: A randomized, double-blind, placebo-controlled clinical trial," *J. Acad. Nutr. Diet.*, vol. 115, no. 3, pp. 369–377, Mar. 2015.

[180] Z. Rasines-Perea and P. L. Teissedre, "Grape Polyphenols' effects in human cardiovascular diseases and diabetes," *Molecules*, vol. 22, no. 1. MDPI AG, 01-Jan-2017.

[181] M. U. Josep, X. Sort, A. Zayas, and M. P. Rosa, "Effects of soil and climatic conditions on grape ripening and wine quality of Cabernet Sauvignon," *J. Wine Res.*, vol. 21, no. 1, pp. 1–17, 2010.

[182] J. Garrido and F. Borges, "Wine and grape polyphenols - A chemical perspective," *Food Research International*, vol. 54, no. 2. Elsevier, pp. 1844–1858, 01-Dec-2013.

[183] L. Iglesias-Carres, A. Mas-Capdevila, F. I. Bravo, L. Arola, B. Muguerza, and A. Arola-Arnal, "Exposure of Fischer 344 rats to distinct photoperiods influences the bioavailability of red grape polyphenols," *J. Photochem. Photobiol. B Biol.*, vol. 199, p. 111623, Oct. 2019.

[184] L. Iglesias-Carres *et al.*, "Optimized Extraction by Response Surface Methodology Used for the Characterization and Quantification of Phenolic Compounds in Whole Red Grapes (Vitis vinifera)," *Nutrients*, vol. 10, no. 12, p. 1931, Dec. 2018.

[185] C. Braicu, V. Pilecki, O. Balacescu, A. Irimie, and I. B. Neagoe, "The relationships between biological activities and structure of flavan-3-ols," *Int. J. Mol. Sci.*, vol. 12, no. 12, pp. 9342–9353, Dec. 2011.

[186] A. J. Larson, J. David Symons, and T. Jalili, "Therapeutic potential of quercetin to decrease blood pressure: Review of efficacy and mechanisms," *Advances in Nutrition*, vol. 3, no. 1. American Society for Nutrition, pp. 39–46, Jan-2012.

[187] Y. Li *et al.*, "Quercetin, inflammation and immunity," *Nutrients*, vol. 8, no. 3. MDPI AG, 15-Mar-2016.

[188] R.-M. Peng, G.-R. Lin, Y. Ting, and J.-Y. Hu, "Oral delivery system enhanced the bioavailability of stilbenes: Resveratrol and pterostilbene," *BioFactors*, vol. 44, no. 1, pp. 5–15, Jan. 2018.

[189] L. Jin *et al.*, "Gallic Acid Reduces Blood Pressure and Attenuates Oxidative Stress and Cardiac Hypertrophy in Spontaneously Hypertensive Rats," *Sci. Rep.*, vol. 7, no. 1, Dec. 2017.

[190] P. V. Dludla *et al.*, "Inflammation and oxidative stress in an obese state and the protective effects of gallic acid," *Nutrients*, vol. 11, no. 1. MDPI AG, 01-Jan-2019.

[191] S. S. Mirvish, A. Cardesa, L. Wallcave, and P. Shubik, "Induction of mouse lung adenomas by amines or ureas plus nitrite and by N nitroso compounds: effect of ascorbate, gallic acid, thiocyanate, and caffeine," *J. Natl. Cancer Inst.*, vol. 55, no. 3, pp. 633–636, 1975.

[192] OIV and International Organisation of Vine and Wine, "2019 Statistical Report on World Vitiviniculture," *2019 Stat. Rep. World Vitiviniculture*, p. 23, 2019.

[193] G. V. Jones and L. B. Webb, "Climate change, viticulture, and wine: Challenges and opportunities," *J. Wine Res.*, vol. 21, no. 2, pp. 103–106, Nov. 2010.

[194] C. Beres *et al.*, "Towards integral utilization of grape pomace from winemaking process: A review," *Waste Management*, vol. 68. Elsevier Ltd, pp. 581–594, 01-Oct-2017.

[195] L. Snopek *et al.*, "Contribution of red wine consumption to human health protection," *Molecules*, vol. 23, no. 7, pp. 1–16, 2018.

[196] F. Visioli, S. A. Panaite, and J. Tomé-Carneiro, "Wine's phenolic compounds and health: A pythagorean view," *Molecules*, vol. 25, no. 18. MDPI AG, 01-Sep-2020.

[197] M. Izdebska *et al.*, "Resveratrol limits lipogenesis and enhance mitochondrial activity in HepG2 cells," *J. Pharm. Pharm. Sci.*, vol. 21, no. 1, pp. 504–515, 2018.

[198] N. Matsumura *et al.*, "Resveratrol improves cardiac function and exercise performance in MI-induced heart failure through the inhibition of cardiotoxic HETE metabolites," *J. Mol. Cell. Cardiol.*, vol. 125, pp. 162–173, Dec. 2018.

[199] T. Rafe *et al.*, "Preventive Role of Resveratrol Against Inflammatory Cytokines and Related Diseases," *Curr. Pharm. Des.*, vol. 25, no. 12, pp. 1345–1371, Apr. 2019.

[200] S. R, K. A, H. R, H. T, and H. Y, "Resveratrol Decreases Oxidative Stress by

Restoring Mitophagy and Improves the Pathophysiology of Dystrophin-Deficient mdx Mice," *Oxid. Med. Cell. Longev.*, vol. 2018, 2018.

[201] E. N. Kim *et al.*, "Resveratrol, an Nrf2 activator, ameliorates aging-related progressive renal injury," *Aging (Albany. NY).*, vol. 10, no. 1, pp. 83–99, Jan. 2018.

[202] A. Giacobbo *et al.*, "Wine lees from the 1st and 2nd rackings: valuable by-products," *J. Food Sci. Technol.*, vol. 56, no. 3, pp. 1559–1566, Mar. 2019.

[203] B. Antonić, S. Jančíková, D. Dordević, and B. Tremlová, "Grape Pomace Valorization: A Systematic Review and Meta-Analysis," *Foods*, vol. 9, no. 11, p. 1627, Nov. 2020.

[204] J. García-Lomillo and M. L. González-SanJosé, "Applications of Wine Pomace in the Food Industry: Approaches and Functions," *Compr. Rev. Food Sci. Food Saf.*, vol. 16, no. 1, pp. 3–22, Jan. 2017.

[205] F. Pasini, F. Chinnici, M. F. Caboni, and V. Verardo, "Recovery of oligomeric proanthocyanidins and other phenolic compounds with established bioactivity from grape seed by-products," *Molecules*, vol. 24, no. 4, Feb. 2019.

[206] I. I. Rockenbach *et al.*, "Characterization of flavan-3-ols in seeds of grape pomace by CE, HPLC-DAD-MS n and LC-ESI-FTICR-MS," *Food Res. Int.*, vol. 48, no. 2, pp. 848–855, Oct. 2012.

[207] S. Kadri *et al.*, "Characterization, anti-oxidative effect of grape seed powder and in silico affinity profiling of polyphenolic and extra-phenolic compounds for calpain inhibition," *J. Pharm. Biomed. Anal.*, vol. 164, pp. 365–372, Feb. 2019.

[208] Y. Yilmaz and R. T. Toledo, "Major Flavonoids in Grape Seeds and Skins: Antioxidant Capacity of Catechin, Epicatechin, and Gallic Acid," *J. Agric. Food Chem.*, vol. 52, no. 2, pp. 255–260, Jan. 2004.

[209] M. M. Pantelić *et al.*, "Identification and quantification of phenolic compounds in berry skin, pulp, and seeds in 13 grapevine varieties grown in Serbia," *Food Chem.*, vol. 211, pp. 243–252, Nov. 2016.

[210] M. Gupta, S. Dey, D. Marbaniang, P. Pal, S. Ray, and B. Mazumder, "Grape seed extract: having a potential health benefits," *Journal of Food Science and Technology*, vol. 57, no. 4. Springer, pp. 1205–1215, 01-Apr-2020.

[211] S. K. Katiyar, "Proanthocyanidins from grape seeds inhibit UV-radiation-induced immune suppression in mice: Detection and analysis of molecular and cellular targets," *Photochemistry and Photobiology*, vol. 91, no. 1. Blackwell Publishing Inc., pp. 156–162, 2015.

[212] Y. Liang, J. Wang, H. Gao, Q. Wang, J. Zhang, and J. Qiu, "Beneficial effects of grape seed proanthocyanidin extract on arterial remodeling in spontaneously hypertensive rats via protecting against oxidative stress," *Mol. Med. Rep.*, vol. 14, no. 4, pp. 3711–3718, Oct. 2016.

[213] L. Luo *et al.*, "Preparative separation of grape skin polyphenols by high-speed counter-current chromatography," *Food Chem.*, vol. 212, pp. 712–721, Dec.

2016.

[214] D. Kammerer, A. Claus, R. Carle, and A. Schieber, "Polyphenol screening of pomace from red and white grape varieties (Vitis vinifera L.) by HPLC-DAD-MS/MS," *J. Agric. Food Chem.*, vol. 52, no. 14, pp. 4360–4367, Jul. 2004.

[215] R. Rodríguez Montealegre, R. Romero Peces, J. L. Chacón Vozmediano, J. Martínez Gascueña, and E. García Romero, "Phenolic compounds in skins and seeds of ten grape Vitis vinifera varieties grown in a warm climate," *J. Food Compos. Anal.*, vol. 19, no. 6–7, pp. 687–693, Sep. 2006.

[216] A. Pea-Neira, A. Cceres, and C. Pastenes, "Low molecular weight phenolic and anthocyanin composition of grape skins from cv. Syrah (Vitis vinifera L.) in the Maipo Valley (Chile): Effect of clusters thinning and vineyard yield," *Food Sci. Technol. Int.*, vol. 13, no. 2, pp. 153–158, 2007.

[217] M. Mahmoudi, K. Charradi, F. Limam, and E. Aouani, "Grape seed and skin extract as an adjunct to xenical therapy reduces obesity, brain lipotoxicity and oxidative stress in high fat diet fed rats," *Obes. Res. Clin. Pract.*, vol. 12, no. 1, pp. 115–126, Jan. 2018.

[218] L. Sochorova *et al.*, "Health Effects of Grape Seed and Skin Extracts and Their Influence on Biochemical Markers," *Molecules (Basel, Switzerland)*, vol. 25, no. 22. NLM (Medline), 14-Nov-2020.

[219] J. A. Nieto, S. Santoyo, M. Prodanov, G. Reglero, and L. Jaime, "Valorisation of grape stems as a source of phenolic antioxidants by using a sustainable extraction methodology," *Foods*, vol. 9, no. 5, May 2020.

[220] N. Goutzourelas *et al.*, "Polyphenolic composition of grape stem extracts affects antioxidant activity in endothelial and muscle cells," *Mol. Med. Rep.*, vol. 12, no. 4, pp. 5846–5856, Oct. 2015.

[221] J. A. Pérez-Serradilla and M. D. L. de Castro, "Role of lees in wine production: A review," *Food Chemistry*, vol. 111, no. 2. Elsevier, pp. 447–456, 15-Nov-2008.

[222] J. A. Pérez-Serradilla and M. D. Luque de Castro, "Microwave-assisted extraction of phenolic compounds from wine lees and spray-drying of the extract," *Food Chem.*, vol. 124, no. 4, pp. 1652–1659, Feb. 2011.

[223] M. A. Bustamante, R. Moral, C. Paredes, A. Pérez-Espinosa, J. Moreno-Caselles, and M. D. Pérez-Murcia, "Agrochemical characterisation of the solid by-products and residues from the winery and distillery industry," *Waste Manag.*, vol. 28, no. 2, pp. 372–380, Jan. 2008.

[224] A. De Iseppi, G. Lomolino, M. Marangon, and A. Curioni, "Current and future strategies for wine yeast lees valorization," *Food Research International*, vol. 137. Elsevier Ltd, p. 109352, 01-Nov-2020.

[225] M. J. Jara-Palacios, "Wine lees as a source of antioxidant compounds," *Antioxidants*, vol. 8, no. 2. MDPI AG, 01-Feb-2019.

[226] A. Morata, M. C. Gómez-Cordovés, B. Colomo, and J. A. Suárez, "Cell wall anthocyanin adsorption by different Saccharomyces strains during the

fermentation of Vitis vinifera L. cv Graciano grapes," *Eur. Food Res. Technol.*, vol. 220, no. 3–4, pp. 341–346, Nov. 2005.

[227] I. Landeka Jurčević, M. Dora, I. Guberović, M. Petras, S. Rimac Brnčić, and D. Đikić, "Wine Lees Polyphenols as a Novel Functional Bioactive Compound in the Protection against Oxidative Stress and Hyperlipidemia," *Food Technol. Biotechnol.*, vol. 55, no. 1, p. 109, 2017.

[228] M. Caro, A. Sansone, J. Amezaga, V. Navarro, C. Ferreri, and I. Tueros, "Wine lees modulate lipid metabolism and induce fatty acid remodelling in zebrafish," *Food Funct.*, vol. 8, no. 4, pp. 1652–1659, 2017.

[229] K. OKAMOTO and K. AOKI, "Development of a Strain of Spontaneously Hypertensive Rats," *Jpn. Circ. J.*, vol. 27, no. 3, pp. 282–293, Mar. 1963.

[230] Y. M. Pinto, M. Paul, and D. Ganten, "Lessons from rat models of hypertension: From Goldblatt to genetic engineering," *Cardiovascular Research*, vol. 39, no. 1. Oxford Academic, pp. 77–88, 01-Jul-1998.

[231] Y. Zhang-James, F. A. Middleton, and S. V. Faraone, "Genetic architecture of Wistar-Kyoto rat and spontaneously hypertensive rat substrains from different sources," *Physiol. Genomics*, vol. 45, no. 13, pp. 528–538, Jul. 2013.

[232] Z. Pons, L. Guerrero, M. Margalef, L. Arola, A. Arola-Arnal, and B. Muguerza, "Effect of low molecular grape seed proanthocyanidins on blood pressure and lipid homeostasis in cafeteria diet-fed rats," *J. Physiol. Biochem.*, vol. 70, no. 2, pp. 629–637, 2014.

[233] X. Liu *et al.*, "Grape seed proanthocyanidin extract alleviates ouabain-induced vascular remodeling through regulation of endothelial function," *Mol. Med. Rep.*, vol. 6, no. 5, pp. 949–954, Nov. 2012.

[234] S. MONCADA and E. A. HIGGS, "Endogenous nitric oxide: physiology, pathology and clinical relevance," *European Journal of Clinical Investigation*, vol. 21, no. 4. John Wiley & Sons, Ltd, pp. 361–374, 01-Aug-1991.

[235] N. D. Vaziri, X. Q. Wang, F. Oveisi, and B. Rad, "Induction of oxidative stress by glutathione depletion causes severe hypertension in normal rats," *Hypertension*, vol. 36, no. 1, pp. 142–146, 2000.

[236] C. Z. Lan *et al.*, "Grape seed proanthocyanidins prevent DOCA-salt hypertension-induced renal injury and its mechanisms in rats," *Food Funct.*, vol. 6, no. 7, pp. 2179–2186, Jul. 2015.

[237] S. J. Thandapilly, J. L. LeMaistre, X. L. Louis, C. M. Anderson, T. Netticadan, and H. D. Anderson, "Vascular and Cardiac Effects of Grape Powder in the Spontaneously Hypertensive Rat," *Am. J. Hypertens.*, vol. 25, no. 10, pp. 1070–1076, Oct. 2012.

[238] F. Allam *et al.*, "Grape powder supplementation prevents oxidative stress-induced anxiety-like behavior, memory impairment, and high blood pressure in rats," *J. Nutr.*, vol. 143, no. 6, pp. 835–842, Jun. 2013.

[239] S.-H. Li, P. Zhao, H.-B. Tian, L.-H. Chen, and L.-Q. Cui, "Effect of Grape

Polyphenols on Blood Pressure: A Meta-Analysis of Randomized Controlled Trials," *PLoS One*, vol. 10, no. 9, p. e0137665, Sep. 2015.

[240] M. Sarr *et al.*, "Red wine polyphenols prevent angiotensin II-induced hypertension and endothelial dysfunction in rats: Role of NADPH oxidase," *Cardiovasc. Res.*, vol. 71, no. 4, pp. 794–802, Sep. 2006.

[241] O. Pech??nov?? *et al.*, "Red wine polyphenols prevent cardiovascular alterations in L-NAME-induced hypertension," *J. Hypertens.*, vol. 22, no. 8, pp. 1551–1559, Aug. 2004.

[242] A. Artero, A. Artero, J. J. Tarín, and A. Cano, "The impact of moderate wine consumption on health," *Maturitas*, vol. 80, no. 1, pp. 3–13, 2015.

[243] G. Chiva-Blanch *et al.*, "Dealcoholized red wine decreases systolic and diastolic blood pressure and increases plasma nitric oxide: Short communication," *Circ. Res.*, vol. 111, no. 8, pp. 1065–1068, Sep. 2012.

[244] E. A. de Figueiredo *et al.*, "Antioxidant and antihypertensive effects of a chemically defined fraction of syrah red wine on spontaneously hypertensive rats," *Nutrients*, vol. 9, no. 6, pp. 10–23, Jun. 2017.

[245] T. O. Kiviniemi *et al.*, "Decreased endothelin-1 levels after acute consumption of red wine and de-alcoholized red wine," *Atherosclerosis*, vol. 211, no. 1, pp. 283–286, Jul. 2010.

[246] M. Á. Pozo-Bayón, J. M. Alcaíde, M. C. Polo, and E. Pueyo, "Angiotensin I-converting enzyme inhibitory compounds in white and red wines," *Food Chem.*, vol. 100, no. 1, pp. 43–47, Jan. 2007.

[247] J. M. Alcaide-Hidalgo, A. J. Martínez-Rodríguez, P. J. Martín-Álvarez, and E. Pueyo, "Influence of the elaboration process on the peptide fraction with angiotensin I-converting enzyme inhibitor activity in sparkling wines and red wines aged on lees," *Food Chem.*, vol. 111, no. 4, pp. 965–969, Dec. 2008.

[248] J. M. Alcaide-Hidalgo, E. Pueyo, M. C. Polo, and A. J. Martiánez-Rodriáguez, "Bioactive peptides released from Saccharomyces cerevisiae under accelerated autolysis in a wine model system," *J. Food Sci.*, vol. 72, no. 7, Sep. 2007.

[249] R. Del Pino-García, M. D. Rivero-Pérez, M. L. González-Sanjosé, K. D. Croft, and P. Muñiz, "Antihypertensive and antioxidant effects of supplementation with red wine pomace in spontaneously hypertensive rats," *Food Funct.*, vol. 8, no. 7, pp. 2444–2454, 2017.

[250] I. Urquiaga *et al.*, "Wine grape pomace flour improves blood pressure, fasting glucose and protein damage in humans: A randomized controlled trial," *Biol. Res.*, vol. 48, no. 1, p. 49, Sep. 2015.

[251] N. Goutzourelas *et al.*, "Grape pomace extract exerts antioxidant effects through an increase in GCS levels and GST activity in muscle and endothelial cells," *Int. J. Mol. Med.*, vol. 36, no. 2, pp. 433–441, Aug. 2015.

[252] T. P. Dalton, Y. Chen, S. N. Schneider, D. W. Nebert, and H. G. Shertzer, "Genetically altered mice to evaluate glutathione homeostasis in health and

disease," *Free Radical Biology and Medicine*, vol. 37, no. 10. Free Radic Biol Med, pp. 1511–1526, 15-Nov-2004.

[253] A. Fernández-Iglesias *et al.*, "Combination of grape seed proanthocyanidin extract and docosahexaenoic acid-rich oil increases the hepatic detoxification by GST mediated GSH conjugation in a lipidic postprandial state," *Food Chem.*, vol. 165, pp. 14–20, Dec. 2014.

[254] E. Cienfuegos-Jovellanos, M. Del Mar Quiñones, B. Muguerza, L. Moulay, M. Miguel, and A. Aleixandre, "Antihypertensive effect of a polyphenol-rich cocoa powder industrially processed to preserve the original flavonoids of the cocoa beans," *J. Agric. Food Chem.*, vol. 57, no. 14, pp. 6156–6162, Jul. 2009.

[255] A. Mas-capdevila, L. Iglesias-carres, A. Arola-arnal, M. Suárez, and I. Francisca, "Changes in arterial blood pressure caused by long-term administration of grape seed proanthocyanidins in rats with established hypertension," *Food Funct.*, pp. 0–17, 2020.

[256] F. Zhu, J. Hu, F. Lv, P. Cheng, and S. Gao, "Effects of oligomeric grape seed proanthocyanidins on *L*- NAME - induced hypertension in pregnant mice: Role of oxidative stress and endothelial dysfunction," *Phyther. Res.*, vol. 32, no. 9, pp. 1836–1847, Sep. 2018.

[257] T. Odai, M. Terauchi, K. Kato, A. Hirose, and N. Miyasaka, "Effects of grape seed proanthocyanidin extract on vascular endothelial function in participants with prehypertension: A randomized, double-blind, placebo-controlled study," *Nutrients*, vol. 11, no. 12, Dec. 2019.

[258] B. Sivaprakasapillai, I. Edirisinghe, J. Randolph, F. Steinberg, and T. Kappagoda, "Effect of grape seed extract on blood pressure in subjects with the metabolic syndrome," *Metabolism.*, vol. 58, no. 12, pp. 1743–1746, Dec. 2009.

[259] H. Zhang *et al.*, "The impact of grape seed extract treatment on blood pressure changes: A meta-analysis of 16 randomized controlled trials," *Medicine (United States)*, vol. 95, no. 33. Lippincott Williams and Wilkins, 01-Aug-2016.

[260] H. H. H. Feringa, D. A. Laskey, J. E. Dickson, and C. I. Coleman, "The Effect of Grape Seed Extract on Cardiovascular Risk Markers: A Meta-Analysis of Randomized Controlled Trials," *J. Am. Diet. Assoc.*, vol. 111, no. 8, pp. 1173–1181, Aug. 2011.

[261] R. S. de Moura *et al.*, "Antihypertensive, vasodilator and antioxidant effects of a vinifera grape skin extract," *J. Pharm. Pharmacol.*, vol. 54, no. 11, pp. 1515–1520, Nov. 2002.

[262] G. F. De Costa *et al.*, "Vitis vinifera L grape skin extract prevents development of hypretension and altered lipid profile in sponteneously hypertensive tats: Role of oxidative stress.," *Prev. Nutr. Food Sci.*, vol. 25, no. 1, pp. 25–31, Mar. 2020.

[263] A. F. Emiliano *et al.*, "Metabolic Disorders and Oxidative Stress Programming in Offspring of Rats Fed a High-fat Diet During Lactation: Effects of a Vinifera Grape Skin (ACH09) Extract," *J. Cardiovasc. Pharmacol.*, vol. 58, no. 3, pp. 319–328, Sep. 2011.

[264] A. C. Resende *et al.*, "Grape skin extract protects against programmed changes in the adult rat offspring caused by maternal high-fat diet during lactation," *J. Nutr. Biochem.*, vol. 24, no. 12, pp. 2119–2126, Dec. 2013.

[265] M. Mokni, S. Hamlaoui-Guesmi, M. Amri, L. Marzouki, F. Limam, and E. Aouani, "Grape seed and skin extract protects against acute chemotherapy toxicity induced by doxorubicin in rat heart," *Cardiovasc. Toxicol.*, vol. 12, no. 2, pp. 158–165, Jun. 2012.

[266] R. S. De Moura, Â. C. Resende, A. S. Moura, and M. F. Maradei, "Protective action of a hydroalcoholic extract of a vinifera grape skin on experimental preeclampsia in rats," *Hypertens. Pregnancy*, vol. 26, no. 1, pp. 89–100, Jan. 2007.

[267] Y. S. Lin, Y. L. Lu, G. J. Wang, L. G. Chen, C. L. Wen, and W. C. Hou, "Ethanolic extracts and isolated compounds from small-leaf grape (Vitis thunbergii var. taiwaniana) with antihypertensive activities," *J. Agric. Food Chem.*, vol. 60, no. 30, pp. 7435–7441, Aug. 2012.

[268] R. Romero-Díez, S. Rodríguez-Rojo, M. J. Cocero, C. M. M. Duarte, A. A. Matias, and M. R. Bronze, "Phenolic characterization of aging wine lees: Correlation with antioxidant activities," *Food Chem.*, vol. 259, pp. 188–195, Sep. 2018.

[269] S. Pimentel-Moral, M. de la L. Cádiz-Gurrea, C. Rodríguez-Pérez, and A. Segura-Carretero, "Recent advances in extraction technologies of phytochemicals applied for the revaluation of agri-food by-products," in *Functional and Preservative Properties of Phytochemicals*, Elsevier, 2020, pp. 209–239.

[270] C. L. Okolie, T. O. Akanbi, B. Mason, C. C. Udenigwe, and A. N. A. Aryee, "Influence of conventional and recent extraction technologies on physicochemical properties of bioactive macromolecules from natural sources: A review," *Food Research International*, vol. 116. Elsevier Ltd, pp. 827–839, 01-Feb-2019.

[271] R. A. Carciochi, L. G. D'Alessandro, P. Vauchel, M. M. Rodriguez, S. M. Nolasco, and K. Dimitrov, "Valorization of Agrifood By-Products by Extracting Valuable Bioactive Compounds Using Green Processes," in *Ingredients Extraction by Physicochemical Methods in Food*, Elsevier, 2017, pp. 191–228.

[272] Z. Fang and B. Bhandari, "Encapsulation of polyphenols - A review," *Trends in Food Science and Technology*, vol. 21, no. 10. Elsevier, pp. 510–523, 01-Oct-2010.

[273] J. L. Marqués, G. Della Porta, E. Reverchon, J. A. R. Renuncio, and A. M. Mainar, "Supercritical antisolvent extraction of antioxidants from grape seeds after vinification," *J. Supercrit. Fluids*, vol. 82, pp. 238–243, Oct. 2013.

[274] J. Ahn, I. U. Grün, and A. Mustapha, "Antimicrobial and Antioxidant Activities of Natural Extracts in Vitro and in Ground Beef," *J. Food Prot.*, vol. 67, no. 1, pp. 148–155, 2004.

[275] V. Louli, N. Ragoussis, and K. Magoulas, "Recovery of phenolic antioxidants from wine industry by-products," *Bioresour. Technol.*, vol. 92, no. 2, pp. 201–208, Apr. 2004.

[276] M. Puri, D. Sharma, and C. J. Barrow, "Enzyme-assisted extraction of bioactives from plants," *Trends in Biotechnology*, vol. 30, no. 1. Elsevier Current Trends, pp. 37–44, 01-Jan-2012.

[277] B. Rodríguez-Morgado *et al.*, "Obtaining from Grape Pomace an Enzymatic Extract with Anti-inflammatory Properties," *Plant Foods Hum. Nutr.*, vol. 70, no. 1, pp. 42–49, Mar. 2015.

[278] I. Nicoletti, C. Bello, A. De Rossi, and D. Corradini, "Identification and quantification of phenolic compounds in grapes by HPLC-PDA-ESI-MS on a semimicro separation scale," *J. Agric. Food Chem.*, vol. 56, no. 19, pp. 8801–8808, Oct. 2008.

[279] M. Pantelić *et al.*, "Impact of Clonal Variability on Phenolics and Radical Scavenging Activity of Grapes and Wines: A Study on the Recently Developed Merlot and Cabernet Franc Clones (Vitis vinifera L.)," *PLoS One*, vol. 11, no. 10, p. e0163823, Oct. 2016.

[280] E. Gómez-Plaza, A. B. Bautista-Ortín, Y. Ruiz-García, J. I. Fernández-Fernández, and R. Gil-Muñoz, "Effect of elicitors on the evolution of grape phenolic compounds during the ripening period," *J. Sci. Food Agric.*, vol. 97, no. 3, pp. 977–983, Feb. 2017.

[281] G. Gutiérrez-Gamboa, E. Gómez-Plaza, A. B. Bautista-Ortín, T. Garde-Cerdán, Y. Moreno-Simunovic, and A. M. Martínez-Gil, "Rootstock effects on grape anthocyanins, skin and seed proanthocyanidins and wine color and phenolic compounds from *Vitis vinifera* L. Merlot grapevines," *J. Sci. Food Agric.*, vol. 99, no. 6, pp. 2846–2854, Apr. 2019.

[282] M. Pantelić *et al.*, "Impact of clonal variability on phenolics and radical scavenging activity of grapes and wines: A study on the recently developed Merlot and Cabernet Franc clones (Vitis vinifera L.)," *PLoS One*, vol. 11, no. 10, Oct. 2016.

[283] G. E. Pereira *et al.*, "Trunk Girdling Increased Stomatal Conductance in Cabernet Sauvignon Grapevines, Reduced Glutamine, and Increased Malvidin-3-Glucoside and Quercetin-3-Glucoside Concentrations in Skins and Pulp at Harvest," *Front. Plant Sci.*, vol. 11, Jun. 2020.

[284] Y. Fang *et al.*, "Influence of shriveling on berry composition and antioxidant activity of Cabernet Sauvignon grapes from Shanxi vineyards," *J. Sci. Food Agric.*, vol. 91, no. 4, pp. 749–757, Mar. 2011.

[285] J. Gabaston *et al.*, "Characterization of Stilbene Composition in Grape Berries from Wild Vitis Species in Year-To-Year Harvest," *J. Agric. Food Chem.*, vol. 68, no. 47, pp. 13408–13417, Nov. 2020.

[286] Y. Wang *et al.*, "Effects of cluster thinning on vine photosynthesis, berry ripeness and flavonoid composition of Cabernet Sauvignon," *Food Chem.*, vol. 248, pp. 101–110, May 2018.

[287] G. E. Pereira *et al.*, "Impact of grapevine red blotch disease on primary and secondary metabolites in 'Cabernet Sauvignon' grape tissues," *Food Chem.*, vol. 342, p. 128312, Apr. 2020.

[288] J. Portu, R. López, E. Baroja, P. Santamaría, and T. Garde-Cerdán, "Improvement of grape and wine phenolic content by foliar application to grapevine of three different elicitors: Methyl jasmonate, chitosan, and yeast extract," *Food Chem.*, vol. 201, pp. 213–221, Jun. 2016.

[289] T. Garde-Cerdán, G. Gutiérrez-Gamboa, B. Ayestarán, M. González-Lázaro, P. Rubio-Bretón, and E. P. Pérez-Álvarez, "Influence of seaweed foliar application to Tempranillo grapevines on grape and wine phenolic compounds over two vintages," *Food Chem.*, vol. 345, p. 128843, May 2021.

[290] J. Portu, P. Santamaría, I. López-Alfaro, R. López, and T. Garde-Cerdán, "Methyl jasmonate foliar application to tempranillo vineyard improved grape and wine phenolic content," *J. Agric. Food Chem.*, vol. 63, no. 8, pp. 2328–2337, Mar. 2015.

[291] G. Gutiérrez-Gamboa, T. Garde-Cerdán, L. Martínez-Lapuente, B. S. Costa, P. Rubio-Bretón, and E. P. Pérez-Álvarez, "Phenolic composition of Tempranillo Blanco (*Vitis vinifera* L.) grapes and wines after biostimulation via a foliar seaweed application," *J. Sci. Food Agric.*, vol. 100, no. 2, pp. 825–835, Jan. 2020.

[292] J. F. Meng, Y. Yu, T. C. Shi, Y. S. Fu, T. Zhao, and Z. W. Zhang, "Melatonin treatment of pre-veraison grape berries modifies phenolic components and antioxidant activity of grapes and wine," *Food Sci. Technol.*, vol. 39, no. 1, pp. 35–42, Jan. 2019.

[293] F. Mattivi, R. Guzzon, U. Vrhovsek, M. Stefanini, and R. Velasco, "Metabolite profiling of grape: Flavonols and anthocyanins," *J. Agric. Food Chem.*, vol. 54, no. 20, pp. 7692–7702, Oct. 2006.

[294] E. P. Pérez-Álvarez, J. M. Martínez-Vidaurre, and T. Garde-Cerdán, "Anthocyanin composition of grapes from three different soil types in cv. Tempranillo A.O.C. Rioja vineyards," *J. Sci. Food Agric.*, vol. 99, no. 10, pp. 4833–4841, Aug. 2019.

[295] M. S. Lingua, M. P. Fabani, D. A. Wunderlin, and M. V. Baroni, "From grape to wine: Changes in phenolic composition and its influence on antioxidant activity," *Food Chem.*, vol. 208, pp. 228–238, Oct. 2016.

[296] M. Figueiredo-Gonzalez, B. Cancho-Grande, and J. Simal-Gandara, "Evolution of colour and phenolic compounds during Garnacha Tintorera grape raisining," *Food Chem.*, vol. 141, no. 3, pp. 3230–3240, Dec. 2013.

[297] J. Portu, R. López, P. Santamaría, and T. Garde-Cerdán, "Elicitation with methyl jasmonate supported by precursor feeding with phenylalanine: Effect on Garnacha grape phenolic content," *Food Chem.*, vol. 237, pp. 416–422, Dec. 2017.

[298] E. Obreque-Slier, R. López-Solís, L. Castro-Ulloa, C. Romero-Díaz, and Á. Peña-Neira, "Phenolic composition and physicochemical parameters of Carménère, Cabernet Sauvignon, Merlot and Cabernet Franc grape seeds (Vitis vinifera L.) during ripening," *LWT - Food Sci. Technol.*, vol. 48, no. 1, pp. 134–141, Sep. 2012.

[299] M. Caro, A. Sansone, J. Amezaga, V. Navarro, C. Ferreri, and I. Tueros, "Wine

lees modulate lipid metabolism and induce fatty acid remodelling in zebrafish," *Food Funct.*, vol. 8, no. 4, pp. 1652–1659, Apr. 2017.

[300] M. P. Delgado De La Torre, F. Priego-Capote, and M. D. Luque De Castro, "Characterization and comparison of wine lees by liquid chromatography-mass spectrometry in high-resolution mode," *J. Agric. Food Chem.*, vol. 63, no. 4, pp. 1116–1125, Feb. 2015.

[301] F. Dujmić *et al.*, "Non-thermal ultrasonic extraction of polyphenolic compounds from red wine lees," *Foods*, vol. 9, no. 4, p. 472, Apr. 2020.

[302] R. Romero-Díez *et al.*, "Microwave and ultrasound pre-treatments to enhance anthocyanins extraction from different wine lees," *Food Chem.*, vol. 272, pp. 258–266, Jan. 2019.

[303] Z. Pons, M. Margalef, F. I. Bravo, A. Arola-Arnal, and B. Muguerza, "Chronic administration of grape-seed polyphenols attenuates the development of hypertension and improves other cardiometabolic risk factors associated with the metabolic syndrome in cafeteria diet-fed rats," *Br. J. Nutr.*, vol. 117, no. 2, pp. 200–208, Jan. 2017.

[304] E. Park, I. Edirisinghe, Y. Y. Choy, A. Waterhouse, and B. Burton-Freeman, "Effects of grape seed extract beverage on blood pressure and metabolic indices in individuals with pre-hypertension: A randomised, double-blinded, two-arm, parallel, placebo-controlled trial," *Br. J. Nutr.*, vol. 115, no. 2, pp. 226–238, Jan. 2016.

HYPOTHESIS AND OBJECTIVES

HYPOTHESIS AND OBJECTIVES

HTN is one of the major risk factors of CVD and the reduction of its prevalence by 25 % is a global target to be attained for 2025. Pharmacological treatments for HTN are effective in the majority of hypertensive patients, but some of synthetic drugs can produce several side effects. Thus, natural antihypertensive agents are highly searched. Moreover, these alternative compounds could be especially useful for pre-hypertensive subjects, since this population is not usually clinically treated. In this regard, an interesting source of natural BP-lowering compounds are agri-food by-products, since their valorization makes to these industries more environmentally friendly.

Wine industry has a high socio-economic impact in different regions of the world, but also generates an elevate amount of wastes. Winery by-products are rich in grape phenolic compounds with evidenced antihypertensive activities. In this regards, some winery by-products have already been used to elaborate phenolic-rich extracts with different functional activities, including antihypertensive. However, some of these by-products, as WL, have not been considered so far as potential sources of antihypertensive compounds. In addition to grape phenolic compounds, they have a high amount of proteins coming from grape and yeasts used in the winemaking process. Hydrolysis of these proteins could release antihypertensive peptides. In addition, enzyme-assisted extraction has been also efficiently used to release phenolic compounds entrapped in the vegetal matrix. Moreover, since WL also contain ethanol and it can modify BP, the WL dealcoholization could enhance their potential antihypertensive effects.

Therefore, ***WL could be a good source of natural compounds with BP-lowering properties after their oral administration.***

Thus, the main objective of this thesis was to obtain WL-derived products with antihypertensive activities after their oral administration to hypertensive rats

and identify their bioactive compounds and the underlying mechanisms responsible of its bioactivity.

To achieve these general objectives, the following specific objectives were proposed:

1. **To evaluate the potential of WL as a source of antihypertensive compounds** (*Chapter 1*)**.**

WL are rich in grape phenolic compound and their composition is depending on grape variety and harvest conditions. However, although some of the phenolic compounds identified in WL have demonstrated antihypertensive activities, the potential WL antihypertensive effect remains unexplored.

In addition, enzyme-assisted extraction has been recently reported as a good methodology to release phenolic compounds from food matrix. However, the use of this methodology can generate or release other bioactive compounds different to phenolic compounds, including peptides with antihypertensive properties.

Considering these facts, the following aims were proposed:

a) **To evaluate the potential antihypertensive effect of WL and to identify the compounds responsible of their activity**

   The potential *in vitro* ACEi and antihypertensive activities of WL from different single grape varieties will be investigated. In addition, the phenolic compounds involved in their bioactivity will be identified. Furthermore, a potential hypotensive effect of WL in normotensive rats will be ruled out and the reproducibility of the functionality of the selected WL obtained in different harvests will be evaluated **[Manuscript 1]**.

b) **To obtain WL enriched in antihypertensive compounds**

b.1. To release non-soluble phenolic compounds from WL by using an enzyme-assisted extraction to maximize the phenolic yield and their functional properties **[Manuscript 2]**.

b.2. To identify the ACE inhibitors and/or antihypertensive peptides released during the enzyme-assisted extraction process carried out in WL **[Manuscript 3].**

2. **To evaluate the antihypertensive effect of WL dealcoholization and stablish the mechanisms involved in their BP-lowering effect** *(Chapter 2).*

Biological functionalities of phenolic compounds not always act in a dose-dependent manner. Therefore, the establishment of the most effective dose of a functional ingredient is essential to optimize the effectivity and manufacturing of this product. In addition, the study of the dried WL is essential to commercialize the WL as functional ingredient or nutraceutical. Nevertheless, WL contain ethanol, which could modify BP and will disappear after the drying process. Therefore, the dealcoholized WL could change their BP-lowering properties. Thus, the antihypertensive effect of the dealcoholized WL will be investigated. In addition, in the development of a functional ingredient is recommendable to know the underlying mechanisms taking part of this activity.

Therefore, the following goals were proposed:

a) To determinate the most effective antihypertensive dose of WL after an acute administration to SHR, evaluate the role of alcohol in their antihypertensive effect administrating dried WL to the animals and discard a possible hypotensive effect of the free-alcohol WL in normotensive WKY rats **[Manuscript 4]**.

b) To establish the mechanisms underlying in the BP-lowering effect of the alcohol-fee WL in SHR. Since it has been evidenced that phenolic compounds can exhibit a BP-lowering effect by acting on components of ACE system, improving endothelial function and reducing ROS, these

antihypertensive mechanisms will be investigated **[Manuscript 4]** and **[Manuscript 5]**.

3. **To assay the effect of dealcoholized wine lees after a long-term administration in hypertensive rats** *(Chapter 3)***.**

The large majority of patients showing HTN requires lifelong treatments to control their BP levels. Thus, natural antihypertensive compounds should be effective reducing BP after a long-term administration. SHR is one of the most commonly used animal models to evaluate antihypertensive compounds after their acute and chronic administrations. In addition, different methodologies are used to record BP. Methods as telemetry allows for the continuous and stress-free measurement of SBP and DBP and other parameters such as heart rate, locomotor activity and body temperature yield more information about the bioactivity of the compounds.

Considering these facts, the following aim was proposed:

a) To investigate the effect of WL after long-term administration to SHR on BP and other cardiovascular parameters using a telemetry system **[Manuscript 6]**.

This thesis was performed in the Nutrigenomics Research Group of the Universitat Rovira i Virgili from October 2018 to March 2020. In addition, an international research stay was carried out in the Institute of Public Health and Clinical Nutrition at University of Eastern Finland (UEF) (Kuopio, Findland) under the supervision of Dr. Carlos Gómez Gallego and Dr. Hani El-Nezami from 15th August 2020-2nd December 2020.

The research carried out in this Ph.D. has been developed within a collaborative framework among a university "Universitat Rovira i Virgili", a technological center "Eurecat", a winery "Grandes Vinos y Viñedos S.A" and a food industry association "Cluster Aragonés de Alimentación". It has been supported by the

grant numbers RETOS COLABORACIÓN: RTC-2017-6044-2 from the Spanish Ministry of Economy and Competitiveness and European Regional Development Fund (FEDER) and AEI-010300-2013-254, AEI-010500-2014-201, AEI-010500-2015-328 and IDI-20180101 from the Spanish Centre for the Development of industrial Technology (CDTI). As a result of the research, an antihypertensive ingredient has been developed. An international patent request titled "WINE LEES, DERIVATIVES THEREOF AND THEIR USES" was registered at the Spanish *Patent and Trademark Office with the numbers EP20382358.8 and PCT/EP2021/053051 on 20th April 2020 and 9th February 2021, respectively being the applicant Grandes Vinos y Viñedos SA.*

EXPERIMENTAL DESIGNS

EXPERIMENTAL DESIGNS

Different experimental designs were used to assess the main hypothesis and reach the experimental objectives previously described in this thesis.

1. Evaluation of the potential antihypertensive effect of WL and identification of the compounds responsible of their activity.

WL from different red grape varieties (Mazuela, Cabernet, Garnacha and Merlot) and from one white grape variety (Macabeo) were obtained and submitted to centrifugation process. The ACEi activity of the obtained soluble fractions were determined. Mazuela, Cabernet and Garnacha were selected according to their ACEi properties. Their phenolic profile by UHPLC-ESI(+/-)-Q-TOF-MS and their in vivo antihypertensive effect at an acute dose of 5 mL/kg bw in SHR were determined. Captopril (50 mg/kg bw) and water were used as positive and negative controls. BP was recorded before administration and at 2, 4, 6, 8, 24 and 48 h post-administration by *tail cuff* method. Cabernet WL were selected according their BP-lowering effects and were tested in normotensive rats (WKY) at the same dose (5 mL/kg bw) in order to rule out a hypotensive effect, Additionally, reproducibility of antihypertensive effect of Cabernet WL from different vintage were evaluated in SHR at a single dose of 5 mL/kg bw (**Figure 1**).

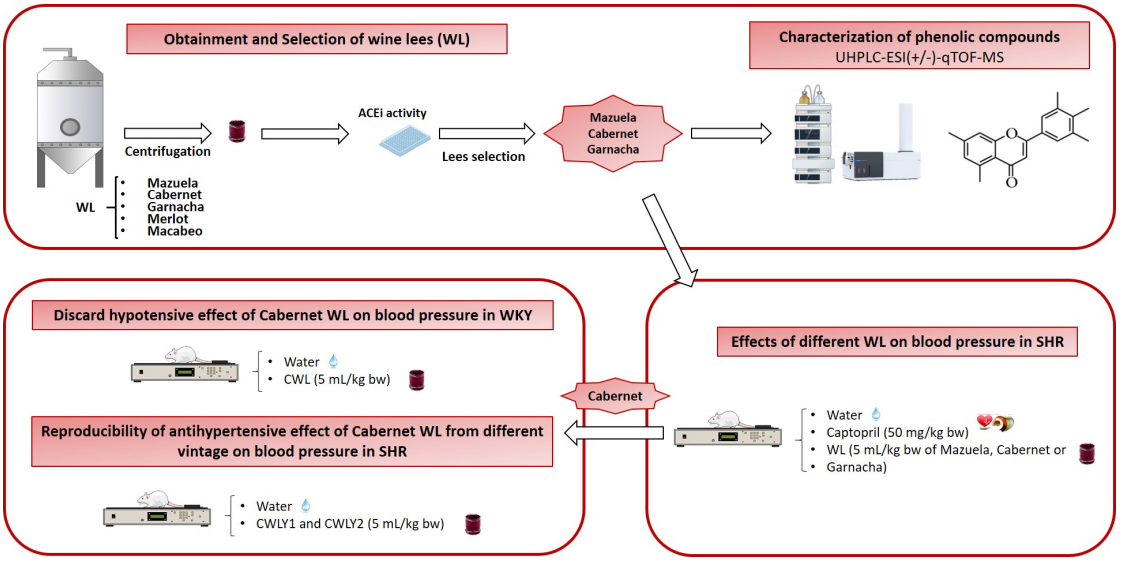

Figure 1. Experimental procedure to evaluate the potential antihypertensive effect of WL and identify the compounds responsible of their activity.

2. Obtainment of a wine lees enriched in antihypertensive bioactive compounds

To obtain a wine lees enriched in bioactive compounds, an enzyme-assisted extraction was used, which is based on the hydrolysis of WL compounds. WL were submitted to a hydrolysis process with Flavourzyme at 25 ºC, pH 4 for 2h. A control of the process was done submitting the WL to the same procedure but without enzyme. Phenolic and amino acid profile were determined in both samples by UHPLC-ESI(+/-)-Q-TOF-MS. ACEi activity and DPPH radical scavenging activity were determined in the samples. In addition, their antihypertensive effect was evaluated in SHR at a single dose of 5 mL/kg bw. Captopril (50 mg/kg bw) and water were used as positive and negative controls. BP were recorded before administration and at 2, 4, 6, 8, 24 and 48 h post-administration by *tail cuff* method (**Figure 2**).

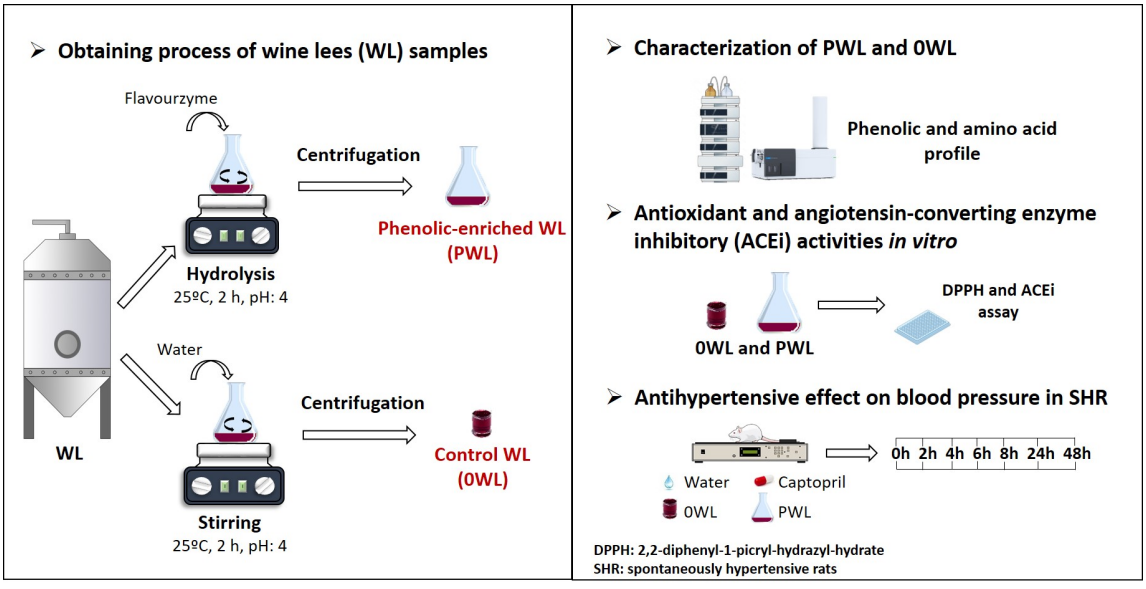

Figure 2. Experimental procedure to the elaboration of wine lees enriched in antihypertensive compounds.

In addition, these WL enriched in antihypertensive compounds were further analyzed to identify the ACE inhibitors and/or antihypertensive peptides

released during the enzyme-assisted extraction process carried out in WL. WL peptides were separated by RP-HPLC. The ACEi activity were measured in the RP-HPLC fractions and the most active were further analyzed by a nanoLC-LTQ-Orbitap HPCL-MS to identify the peptides. The identified peptides were synthesized and their ACEi and antihypertensive were determined. The synthetized peptides were administered to SHR at a dose of 10 mg/kg bw. SBP and DBP were measured before administration and at 2, 4, 6, 8, 24 and 48 h post-administration by *tail cuff* method. Moreover, peptides were submitted to a gastrointestinal digestion using an *in silico* approach with ExPASy PeptideCutter to evaluate the peptide digestion after their oral administration. The peptide-derived fragments were searched in the Biopep database to find the potential reported antihypertensive effect of these peptides (**Figure 3**).

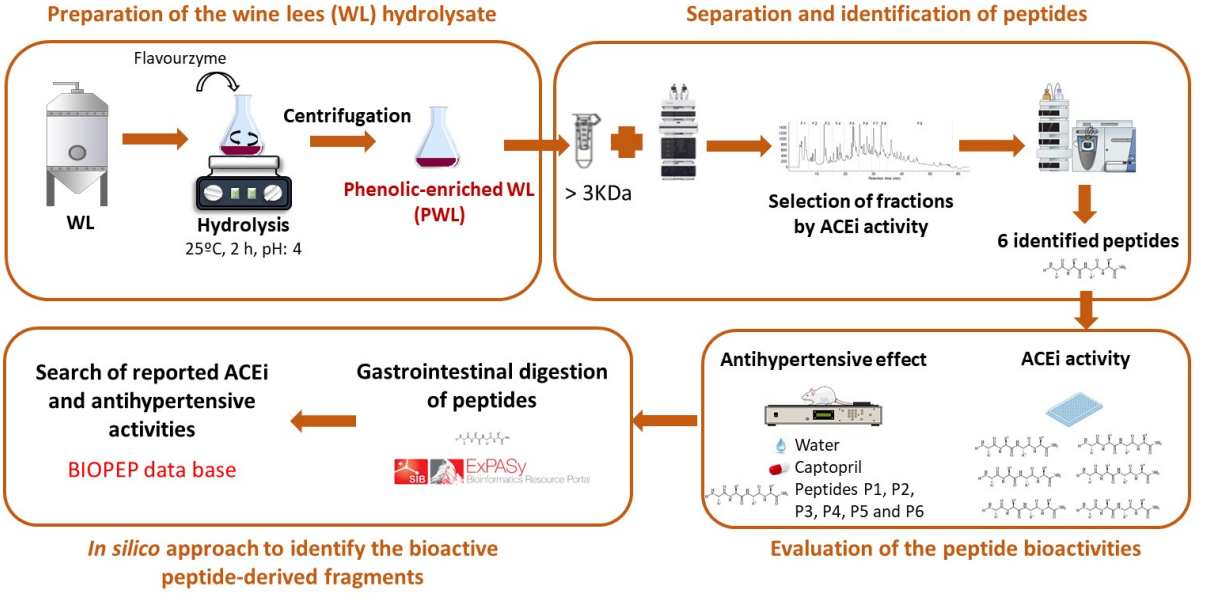

Figure 3. Representation of the methodology used to prepare WL hydrolysate, separate and identify the peptides contained in WL hydrolysate by their angiotensin-converting enzyme inhibitory activity and/or their antihypertensive effect *in vivo* and their possible gastrointestinal digestion *in silico*.

3. Determination of the most effective dose of the WL and evaluation of the antihypertensive effect of dealcoholized WL

To determinate the most effective antihypertensive dose of the WL, three different acute doses (2.5, 5.0 and 7.5 mL/kg bw) were administered to SHR. The dose of 5.0 ml/kg was set as the most effective and selected to evaluate the antihypertensive effect of WL powder (WLPW). For that, WL were lyophilized, removing the alcohol in the process, and were administered to SHR at a dose of 125 mg/kg bw (equivalent to the dose of 5 mL/kg bw). In addition, its potential hypotensive effect was evaluated in the normotensive rats at the same dose to discard this undesirable effect. In all in vivo studies, BP was recorded before administration and at 2, 4, 6, 8, 24 and 48 h post-administration by *Tail cuff* method (**Figure 4**).

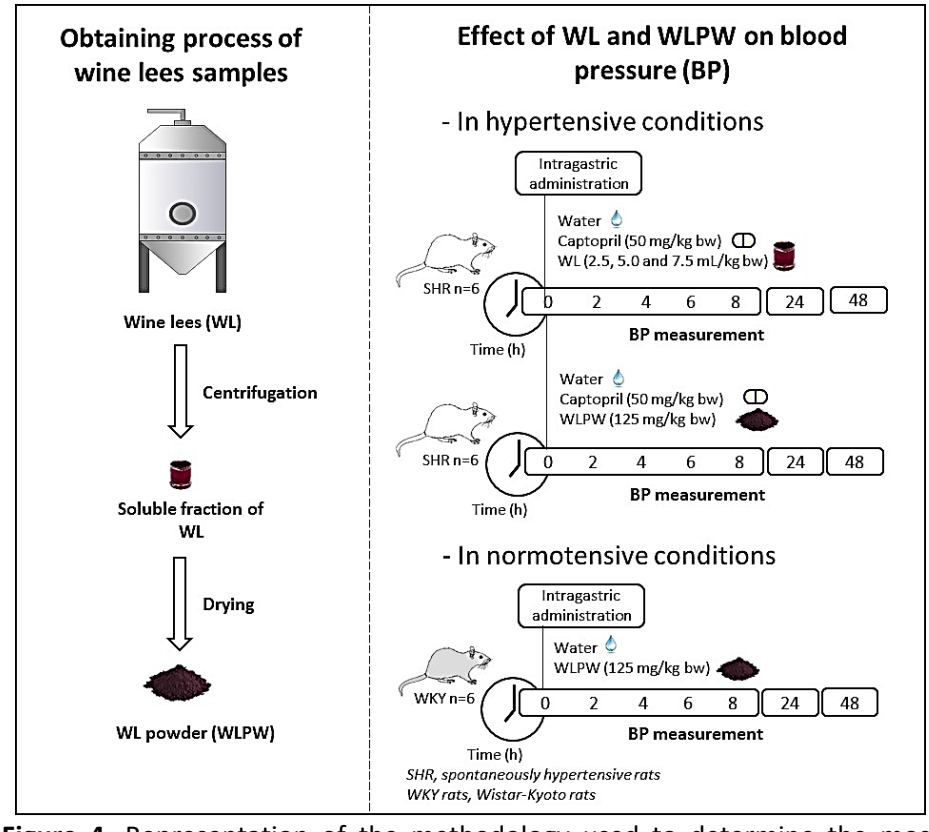

Figure 4. Representation of the methodology used to determine the most effective dose of the wine lees and evaluate the antihypertensive and hypotensive effect of dealcoholized WL powder (WLPW).

4. Evaluate the possible antihypertensive mechanisms involve in the lowering BP effect of WLPW

In order to identify the underlying mechanisms taking part of the BP-lowering effect of WLPW, an acute dose of 125 mg/kg bw WLPW or water were orally administered to SHR. Animals were sacrificed at 6 h post-administration, time point of their maximum antihypertensive effect and plasma and liver were collected. Plasma ACE activity was determined to evaluate the involvement of the renin-angiotensin system in the antihypertensive effect of these WLPW. Moreover, plasma nitric oxide metabolites (NOx) and MDA and hepatic ROS and GSH were measured in animals to evaluate the antioxidant effect as mechanism involved in the WLPW antihypertensive effect (**Figure 5**).

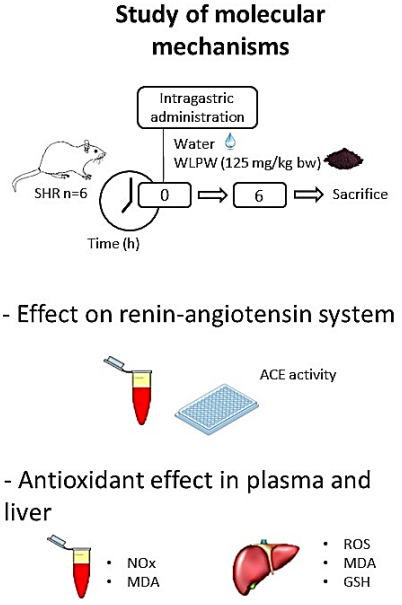

Figure 4. Schematic representation of methodology followed to evaluate the involvement of the renin-angiotensin system and antioxidant effect in the antihypertensive effect of wine lees powder (WLPW).

Moreover, in order to elucidate the implication of NO, PGI_2 and Sirt-1 in the WLPW antihypertensive effect, rats administered WLPW (125 mg/kg bw) or water were treated with L-NAME (30 mg/kg bw, inhibitor of NO synthesis), indomethacin (1 mg/kg bw, inhibitor of PGI_2 synthesis) or sirtinol (1 mg/kg bw, inhibitor of sirtuins synthesis) 4 h after WLPW administration. BP was recorded before and 6 h after WLPW administration. Moreover, an additional experiment was carried using also SHR that were administered WLPW (125 mg/kg bw) or

water. 6 h post-administration rats were sacrificed and aorta was extracted to determine the endothelial gene expression of *eNos*, *Sirt1*, *Nox4* and *Et1* (**Figure 6**).

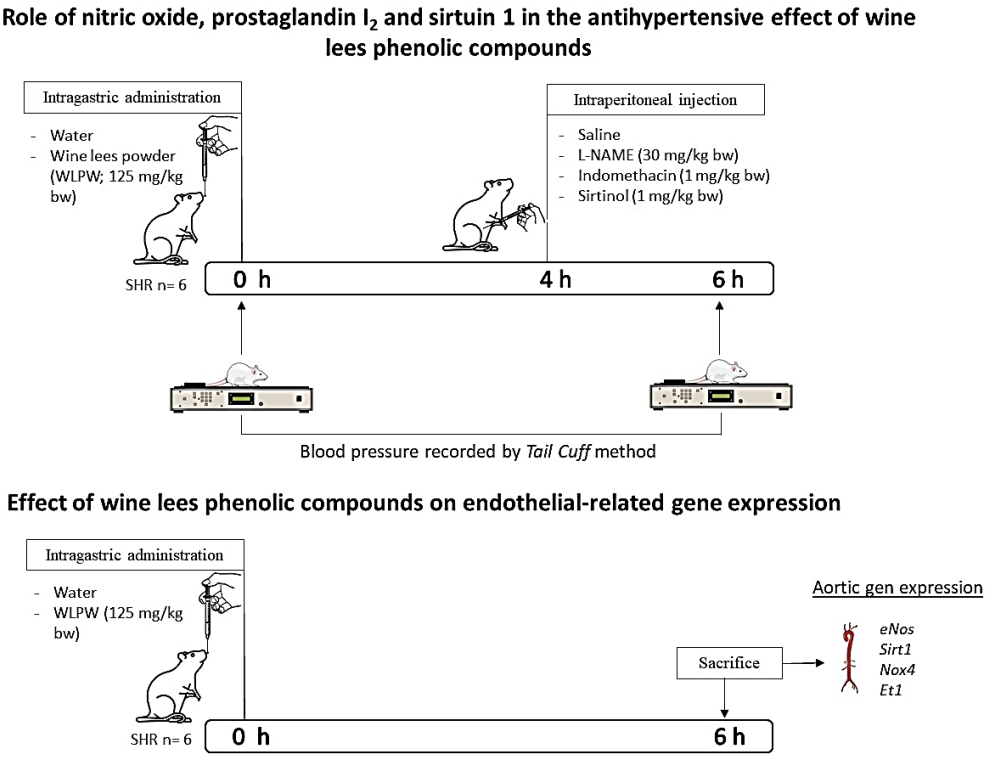

Figure 5. Experimental procedure followed to evaluate the role of the endothelium-derived factors in the blood pressure-lowering effect of WLPW.

5. Evaluation of the long-term administration effects of wine lees powder on BP and other cardiovascular parameters using a telemetry system in SHR.

In order to evaluate the long-term effects of WLPW, an oral dose of WLPW (125 mg/kg/day) or vehicle were administered during five weeks to SHR. The 6th week of the study animals did not receive any treatments. Body weight and food and water intake were measured once a week during all the experiment. BP, heart rate, locomotor activity and body temperature were monitored before, after treatments (24 h/3 times/week) during the first 5 weeks and at the end of the 6th week. These parameters were monitored by a telemetry

system through the insertion of a HD-S10 telemetry transmitters in the aorta of the animals (**Figure 7**).

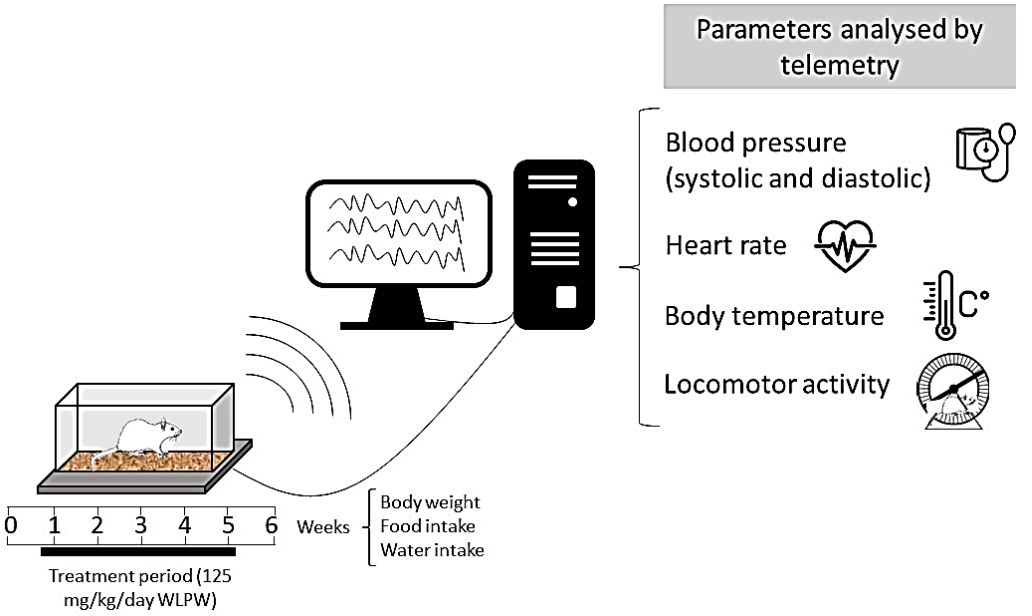

Figure 6. Experimental design carried out to evaluate the effect of the long-term administration of the dried wine lees on blood pressure, heart rate, temperature and locomotor activity by telemetry system to SHR.

RESULTS

CHAPTER 1:

To evaluate the potential of WL as a source of antihypertensive compounds

Manuscript 1:

Objective:

To evaluate the potential antihypertensive effect of WL and to identify the compounds responsible of their activity

ACE inhibitory and antihypertensive activities of wine lees and relationship among bioactivity and phenolic profile

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ACE Inhibitory and Antihypertensive Activities of Wine Lees and Relationship among Bioactivity and Phenolic Profile

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Abstract: Wine lees (WL) are by-products generated in the winemaking process. The aim of this study was to investigate the angiotensin-converting enzyme inhibitory (ACEi) activity, and the blood pressure (BP) lowering effect of WL from individual grape varieties. The relationship among their activities and phenolic profiles was also studied. Three WL, from Cabernet, Mazuela, and Garnacha grape varieties, were firstly selected based on their ACEi properties. Their phenolic profiles were fully characterized by UHPLC-ESI-Q-TOF-MS. Then, their potential antihypertensive effects were evaluated in spontaneously hypertensive rats (SHR). BP was recorded before and after their oral administrations (2, 4, 6, 8, 24, and 48 h) at a dose of 5 mL/kg bw. Cabernet WL (CWL) exhibited a potent antihypertensive activity, similar to that obtained with the drug Captopril. This BP-lowering effect was related to the high amount of anthocyanins and flavanols present in these lees. In addition, a potential hypotensive effect of CWL was discarded in normotensive Wistar–Kyoto rats. Finally, the ACEi and antihypertensive activities of CWL coming from a different harvest were confirmed. Our results suggest the potential of CWL for controlling arterial BP, opening the door to commercial use within the wine industry.

Keywords: blood pressure; Cabernet grape variety; hypertension; polyphenols; SHR; winery by-product

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1. Introduction

Nowadays, the leading cause of death worldwide is cardiovascular disease (CVD), being hypertension (HTN) one of their major risk factors [1]. The global prevalence of HTN is high since it is suffered by one in four adults [2]. In fact, a 25% reduction of its prevalence is one of the global targets to be attained by 2025 [3]. The adoption of healthy lifestyles in combination with pharmacological therapy has been shown to be effective for controlling blood pressure (BP) and improving CVD [4]. In this sense, angiotensin-converting enzyme (ACE) inhibitors, such as Captopril or Enalapril, are the first-choice treatments for HTN [5]. These drugs act blocking the ACE, which plays an important role in the BP regulation within the renin-angiotensin system [6]. In fact, its inhibition exerts a clear BP-lowering effect since ACE catalyzes the hydrolysis of the peptide angiotensin I (Ang I) to generate the vasoconstrictor Ang II. ACE is also involved in the kallikrein–kinin system, degrading the vasodilator bradykinin. Despite the effectiveness in controlling the BP of ACE inhibitors, new natural compounds are being investigated since drugs can cause certain side effects in some patients [7]. These alternatives could result in the reduction of HTN at the early or mid-stages of the disease [8]. Thus, antihypertensive compounds from natural sources have emerged as an excellent alternative to synthetic drugs, and are highly demanded, and researched.

Agri-food by-products have emerged as a novel source to obtain these natural anti-hypertensive agents since they can contain compounds with a wide range of biological

properties [9]. The use of these waste products as a source of bioactive compounds allows their revaluation, making the food and agricultural industries more sustainable and environmentally friendly [10,11]. Grapes are one of the world's largest fruit crops and the wine production process generates large amounts of by-products [12]. Some winery by-products, such as grape seeds and skin, have already been used as a source for the extraction of phenolic compounds with numerous health benefits, including antihypertensive properties [13–17]. However, the presence of antihypertensive compounds in other winery by-products as wine lees (WL) remains unexplored.

According to the Council Regulation (EEC) No. 337/79, WL are "the residue that forms at the bottom of recipients containing wine, after fermentation, during storage or after authorized treatments, as well as the residue obtained following the filtration or centrifugation of this product" [18]. The potential application in the food, cosmetics, and pharmaceutical industries of WL has been suggested [19]. In fact, some studies have reported antioxidant, antimicrobial, anti-inflammatory, and cardioprotective properties of WL [19–21]. Differently from other winemaking by-products, WL have been least studied and exploited, and only the extraction of ethanol and tartaric acid is performed on a large scale [22]. However, WL could present ACEi and/or antihypertensive properties since their use as a source of phenolic compounds has been suggested [20]. In this sense, some studies have detected anthocyanins, flavonols, flavanols, and phenolic acids in WL [19,23–26].

Therefore, the aim of this study was to investigate the potential antihypertensive effect of WL. Thus, the ACEi activity was evaluated in five different WL generated from the winemaking process using single grape varieties. Three of these WL were selected according to their ACEi activity. Their phenolic profile was fully characterized and their antihypertensive activities were tested in spontaneously hypertensive rats (SHR). In addition, we evaluated BP-lowering effect of the selected WL in normotensive rats Wistar–Kyoto (WKY) to rule out a potential hypotensive effect. Furthermore, their ACEi and antihypertensive activities were evaluated using WL from a different harvest.

2. Materials and Methods

2.1. Chemicals and Reagents

Human Angiotensin-converting enzyme (ACE, EC 3.4.15.1, 5.1 U/mg), Captopril (PubChem CID: 44093) and N-Hippuryl-His-Leu (Hip-His-Leu), were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). O-aminobenzoylglicil-p-nitrofenilalanilprolina (o-Abz-Gly-p-Phe(NO2)-Pro-OH, PubChem CID: 128860) was provided by Bachem Feinchemikalien (Bubendorf, Switzerland). Acetonitrile and trifluoroacetic acid HPLC grade were purchased from Sigma-Aldrich (Madrid, Spain). Gallic acid, (−)-epicatechin, p-coumaric acid, and (+)-catechin were purchased from Fluka/Sigma-Aldrich; chlorogenic acid, caffeic acid, malvidin-3-O-glucoside, (−)-epigallocatechin gallate, and procyanidin dimer B2 were purchased from Extrasynthése (Lyon, France); cyanidin-3-O-rutinoside was purchased from PhytoLab (Vestenbergsgreuth, Germany); resveratrol was purchased from Carl Roth (Karlsruhe, Germany); and rutin was kindly provided by Nutrafur S.A. (Murcia, Spain). All other chemical solvents used were of analytical grade.

2.2. Wine Lees

WL were provided by Grandes Vinos y Viñedos, S.A located in the Cariñena P.O.D area (Zaragoza, Spain). They were collected after racking the wines. All the wines were elaborated with a single grape variety and following the same manufacturing procedure. The selected grape varieties were Cabernet, Garnacha, Mazuela, Merlot (all of them red grape varieties), and Macabeo (white grape variety). Moreover, lees obtained in the elaboration of wine with Cabernet grapes were supplied from two different harvests (CWL and CWL2). WL were centrifuged at 3000 × g for 15 min at 4 °C to remove solid particles. Supernatants were collected and kept at 4 °C until their analysis or administration to animals.

2.3. Measurement of the ACEi Activity

ACEi activity was measured by a fluorescence technique according to Mas-Capdevila et al. [27]. This technique is based on the ability of ACE to hydrolyze the fluorescence compound o-Abz-Gly-p-Phe(NO_2)-Pro-OH. Inhibition of this enzyme produces a decrease in fluorescence values. Thus, an aliquot of 40 µL of WL was added to a microtiter-plate well and mixed with 160 µL of 0.45 mM o-Abz-Gly-p-Phe(NO_2)-Pro-OH dissolved in 150 mM Tris-base buffer (pH 8.3), containing 1.125 M NaCl. The enzymatic reaction started by adding 40 µL of an ACE solution prepared in 0.15 M Tris buffer (pH 8.3) containing 0.1 µM of $ZnCl_2$ (enzyme concentration in the well was 0.04 U/mL). The reaction was carried out at 37 °C during 30 min. At this time point, fluorescence measurements using λex 360 nm and λem 400 nm were recorded and used to determine the inhibitory activity. The ACEi activity was calculated using the following formula:

$$\text{ACEi activity } (\%) : 1 - \frac{S - Bs}{Pc - B} \times 100$$

where S is the fluorescence emitted after the action of ACE on the substrate, with inhibitor (sample), Bs is the fluoresce emitted by the substrate and the sample, Pc is the fluoresce emitted after the action of ACE on the substrate, without inhibitor, and B is the fluoresce emitted by the substrate and the sample.

ACEi activity was expressed as a percentage (%) or IC_{50} (µL). Percentage of ACEi activity was determined at WL volume of 0.16 µL in order to compare the effects of different WL on ACE activity. IC_{50} was calculated by linear approximation regression. Data are represented as the mean value of three determinations ± SD.

2.4. Detection and Quantification of the Phenolic Compounds from Wine Lees

The individual phenolic profile of WL from Garnacha, Cabernet, and Mazuela WL was carried out by high-performance liquid chromatography coupled to electrospray ionisation and quadrupole time-of-flight mass spectrometry (UHPLC-ESI-Q-TOF-MS). WL samples were diluted twice with water:methanol with 1% of formic acid (50:50, v:v), centrifuged for 5 min at 17,150 × g at room temperature and supernatants were directly analyzed using a 1290 UHPLC Infinity II series coupled to a Q-TOF/MS 6550 (Agilent Technologies, Palo Alto, CA, USA). Two different methodologies based on UHPLC-ESI-Q-TOF-MS systems were used to separate, detect, and quantify the non-anthocyanin and anthocyanin phenolic compounds. For the separation of non-anthocyanin compounds, an Acquity HSST3 C18 column (150 mm × 2.1 mm i.d., 1.8 µm particle size) (Waters, Milford, MA, USA) was used and the mobile phase consisted of (A) water:acetic acid (95:5, v:v) and (B) acetonitrile. The gradient mode was as follows: initial conditions, 0% B; 0–0.5 min, 0% B; 0.5–18 min, 0–30% B; 18–21 min, 30–95% B; 21–24 min, 95% B; and 24–25 min, 100–0% B. A post-run of 6 min was required for column re-equilibration. The flow rate was set at 0.550 mL/min and column temperature was 45 ºC. The injection volume was 2.5 µL for all runs. Electrospray ionization (ESI) operating in negative mode was conducted with a gas temperature at 200 °C and the flow rate was 14 L/min. Nebulizer gas pressure was 20 psi, sheath gas temperature was 350 ºC, sheath gas flow was 11 L/min, and the capillary voltage was 3000 V. The anthocyanins compounds were separated on an Acquity BEH C18 column (100 mm × 2.1 mm, 1.7 µm particle size) (Waters) and the mobile phase consisted on water:formic acid (9:1, v:v) (A) and acetonitrile (B). The gradient mode was as follows: initial conditions, 0% B; 0–0.5 min, 0% B; 0.5–5 min, 0–9% B; 5–7 min, 9–15% B; 7–9.5 min, 15–30% B; 9.5–10 min, 30–100% B; 10–12 min, 100% B; and 12–12.1 min, 100–0% B. A post-run of 5 min was required for column re-equilibration. The flow rate was set at 0.4 mL/min and column temperature was 25 ºC. The injection volume was 2.5 µL for all runs. ESI operating in positive mode was conducted with a gas temperature set at 200 °C and the flow rate was 14 L/min. Nebulizer gas pressure was 20 psi, sheath gas temperature was 350 ºC, sheath gas flow was 11 L/min and the capillary voltage was 3000 V. The mass spectra were recorded between 100–1000 m/z at 2.5 spectra/s for both methodologies.

The assignment of the phenolic compounds was performed by direct comparison with the commercial standards available or by bibliographic information using chromatographic behavior, mass accurate molecular ion ([M-H]- or [M-H]+), and fragmentation patterns [19,28,29]. The obtained calibration curves of commercial standards available were used for the quantification of their corresponding phenolic compounds. When commercial standards were not available, a tentative quantification was carried out by using the calibration curve of the standard more similar.

2.5. Experimental Procedure in Rats

Male SHR and WKY rats (17–20-week-old, weighing 310–350 g) were purchased from Charles River Laboratories España S.A. (Barcelona, Spain). The animals were housed at a temperature of 23 °C with 12/12 h light/dark cycles and 50% of humidity. After quarantine and a training period of 2 weeks, animals were given tap water and a standard diet (A04 Panlab, Barcelona, Spain) ad libitum during the experiments. The initial values of the systolic blood pressure (SBP) and diastolic blood pressure (DBP) in the SHR were 186.6 ± 1.7 and 153.6 ± 2.6 mmHg, respectively.

Figure 1 shows a graphical representation of the three experimental designs used in this study. A first study was carried out in SHR in order to evaluate antihypertensive effect of three WL obtained in the winemaking process with three different grapes varieties: Cabernet, Garnacha, and Mazuela (Figure 1A). For that, a single dose of 5 mL/kg bw of the WL was administered to SHR rats. Water and Captopril (50 mg/kg bw, dissolved in water) were used as a negative and positive control, respectively. A second study was carried out in WKY rats to discard a possible hypotensive effect of the Cabernet WL (CWL, Figure 1B). These WL were administered to animals in a single dose (5 mL/kg bw). Water was used as a negative control. In both studies, SBP and DBP were recorded in the animals before and 2, 4, 6, 8, 24, and 48 h after treatment administration to rats using the tail-cuff method, according to Quiñones et al. [30]. ΔSBP and ΔDBP were calculated as the difference between the mean values of SBP or DBP after and before treatment administration for each rat. Data were expressed as the mean values ± SEM for a minimum of six experiments.

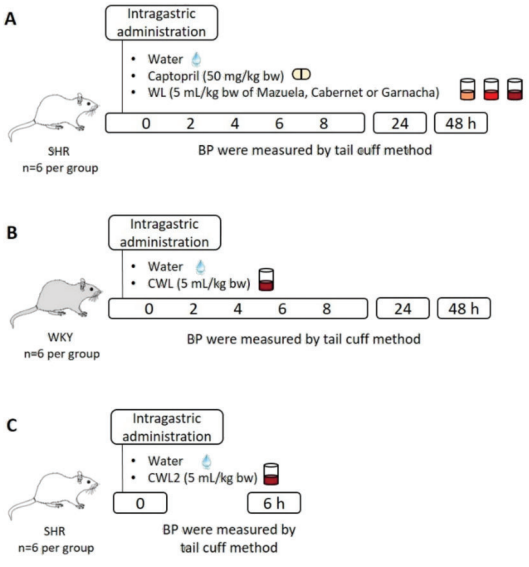

Figure 1. Graphical representation of the three in vivo studies carried out to investigate (**A**) the effect of three wine lees (WL) on blood pressure (BP) in spontaneously hypertensive rats (SHR), (**B**) the effect of Cabernet WL (CWL) on BP in normotensive Wistar–Kyoto rats (WKY) and (**C**) the effect of CWL from a different harvest (CWL2) at 6 h post-administration in SHR.

For the evaluation of the effect of different harvests of CWL on the decrease in BP, an additional trial was conducted with SHR (Figure 1C). The study was carried out by administering a dose of 5 mL/kg bw of CWL2 to SHR ($n = 6$ per group). Water was used as a negative control. BP was recorded before and 6 h after administration.

In all the in vivo studies, treatments were administered by gastric intubation between 9 and 10 am in a volume between 1.5 and 2 mL by oral gavage.

All animal protocols followed in this study were approved by the Animal Ethics Review Committee for Animal Experimentation of the Universitat Rovira i Virgili and further approved by Generalitat de Catalunya (permission number 10780).

2.6. Statistical Analysis

BP differences produced by the administration of the different WL were analyzed by a two-way analysis of variance (ANOVA) for the studies with SHR and WKY rats. Student's T-test was used to evaluate differences between CWL from different campaigns in both IC_{50} analysis and antihypertensive study. A one-way ANOVA was used to evaluate differences between phenolic compounds in WL. All the analyses were performed using GraphPad Prism 7.04 for Windows (GraphPad Software, San Diego, California). Outliers were determined by using Grubbs' test. Differences between groups were considered significant when $p < 0.05$.

3. Results

3.1. Selection of the Wine Lees

Table 1 shows the ACEi activity of the five WL used in this study. As it is shown, WL obtained from red grape varieties (Cabernet, Garnacha, Mazuela, and Merlot) showed a greater ACEi activity than the one related to the white grape variety (Macabeo). Specifically, red grapes showed a percentage of ACEi activity between 28% and 56%. In addition, the concentration of WL needed to inhibit 50% of the ACE activity (IC_{50}) was also determined. They ranged between 0.15 ± 0.01 and 3.74 ± 0.05 µL. Cabernet, Garnacha, and Mazuela WL were the ones with the highest activities (lower than 0.5 µL, Table 1) and were selected for further studies. The dose-response of ACE inhibition of some of the tested WL is represented in Figure 2.

Table 1. Angiotensin-converting enzyme inhibitory (ACEi) activity of the wine lees obtained in the winemaking process using different individual grape varieties.

Grape Variety	ACEi activity	
	%*	IC_{50} (µL)
Cabernet	55.69 ± 1.92	0.15 ± 0.01
Garnacha	44.16 ± 2.54	0.22 ± 0.01
Mazuela	50.70 ± 8.10	0.21 ± 0.03
Merlot	28.76 ± 0.34	0.32 ± 0.02
Macabeo	< 10	3.74 ± 0.05

*ACEi activity showed by a wine lees volume of 0.16 µL.

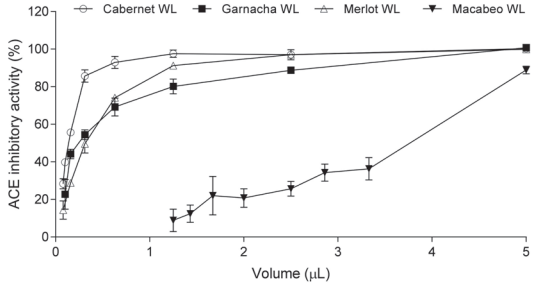

Figure 2. Dose–response curves (effect as a function of the dose in µL) for Cabernet, Garnacha, Merlot, and Macabeo wine lees (WL). Values are the average of three replicates ± SD.

3.2. Determination of the Phenolic Profile of the Three Selected Wine Lees

Phenolic composition of the selected WL was determined using commercial standards. As standards of all the phenolic compounds were not always available, a tentative quantification of these other compounds was carried out using the calibration curve of the most similar available structures. Figure 3 shows the results of the overlapped extract ion chromatograms (EIC) of non-anthocyanin phenolic compounds analyzed by UHPLC-(ESI-)-Q-TOF-MS (Figure 3A) and anthocyanin phenolic compounds analyzed by UHPLC-(ESI +)-Q-TOF-MS (Figure 3B). Table 2 shows the total phenolic content and total content of flavanols, flavonols, phenolic acids, stilbenes, and anthocyanins of Cabernet, Mazuela, and Garnacha WL. The total content of phenolic compounds in the CWL was almost double than the content measured in Mazuela and Garnacha WL (690.6, 395.3, and 379.6 mg/L, respectively). In addition, the contribution of the different phenolic families to the total phenolic content was different depending on the type of WL. Flavanols was the main family in the CWL distantly followed by anthocyanins and phenolic acids (311.1, 153.5, and 133.5 mg/L, respectively). However, flavanols and phenolic acids, in the same proportion, were the main groups in Mazuela and Garnacha WL. The main difference found between CWL and both Mazuela and Garnacha WL was the highest content of flavanols and anthocyanins showed by CWL (Table 2).

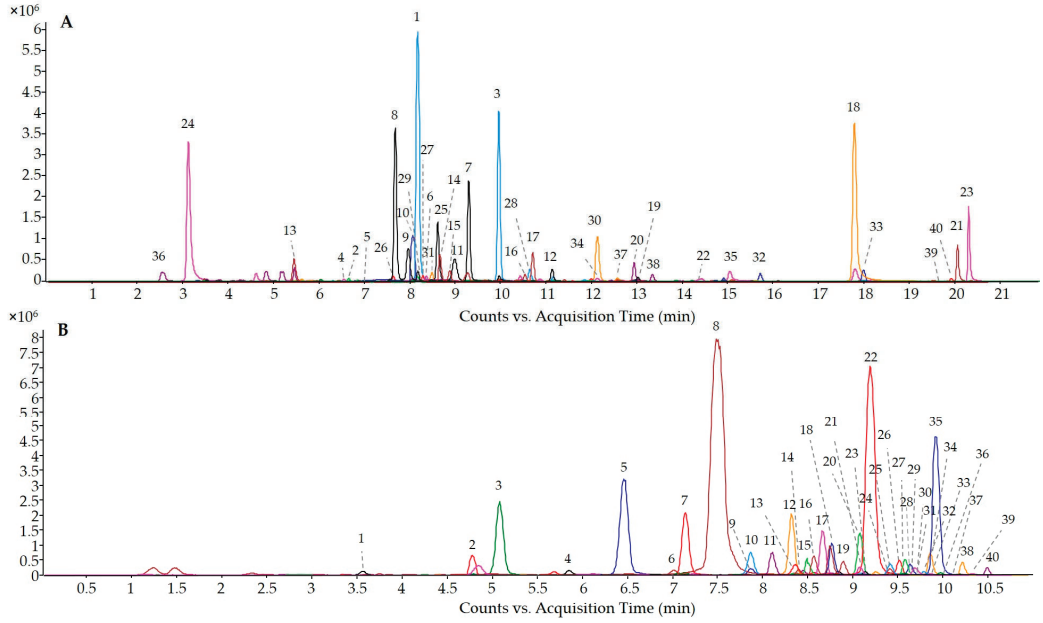

Figure 3. Overlapped extract ion chromatograms (EIC) of (**A**) non-anthocyanin wine lees (WL)-phenolic compounds analyzed by UHPLC-(ESI-)-Q-TOF-MS and (**B**) anthocyanin WL-phenolic compounds analyzed by UHPLC-(ESI +)-Q-TOF-MS. Chromatographic peaks are numbered according to Tables 3 and 4.

Table 2. Total composition of flavanols, flavonols, phenolic acids, stilbenes, and anthocyanins in studied wine lees (WL).

Phenolic Compounds	Cabernet WL (mg/L)	Mazuela WL (mg/L)	Garnacha WL (mg/L)
Flavanols	331.11	122.63	154.60
Flavonols	57.62	44.83	57.28
Phenolic acids	133.54	132.75	103.05
Stilbenes	14.73	20.08	13.59
Anthocyanins	153.53	74.99	51.12
Total	690.63	395.28	379.64

Table 3. Characterization of phenolic compounds in Cabernet, Mazuela, and Garnacha wine lees (WL) by UHPLC-(ESI-)-Q-TOF-MS.

	Compounds	R.T. (min)	[M-H]-	Fragment (*m*/*z*)	Cabernet WL (mg/L)	Mazuela WL (mg/L)	Garnacha WL (mg/L)
	Flavanols						
1	Catechin	8.17	289.0718		97.63 ± 0.62 ^{a}	39.27 ± 0.25 ^{b}	56.65 ± 0.36 ^{c}
2	Catechin gallate [1]	6.66	441.0827	289.07209	0.80 ± 0.01 ^{a}	1.53 ± 0.03 ^{b}	0.79 ± 0.01 ^{a}
3	Epicatechin	9.96	289.0718		43.48 ± 0.23 ^{a}	12.94 ± 0.07 ^{b}	20.16 ± 0.11 ^{b}
4	(Epi)catechin O-glucoside iso1 [2]	6.55	451.1246	289.0721	0.50 ± 0.00 ^{a}	0.50 ± 0.00 ^{a}	0.90 ± 0.00 ^{a}
5	(Epi)catechin O-glucoside iso2 [2]	7.41	451.1246	289.0721	0.33 ± 0.00 ^{a}	0.29 ± 0.00 ^{a}	0.43 ± 0.00 ^{a}
6	(Epi)catechin O-glucoside iso3 [2]	8.37	451.1246	289.0721	1.47 ± 0.03 ^{a}	0.77 ± 0.01 ^{b}	1.64 ± 0.03 ^{a}
7	Procyanidin dimer B2	9.30	577.1387	289.0733	34.60 ± 0.01 ^{a}	9.61 ± 0.00 ^{b}	9.09 ± 0.00 ^{c}
8	Procyanidin dimer iso1 [3]	7.68	577.1387	289.0733	64.21 ± 0.34 ^{a}	32.11 ± 0.17 ^{b}	32.37 ± 0.17 ^{b}
9	Procyanidin dimer iso2 [3]	7.97	577.1387	289.0733	14.26 ± 0.09 ^{a}	4.19 ± 0.03 ^{b}	5.81 ± 0.04 ^{c}
10	Procyanidin dimer iso3 [3]	8.18	577.1387	289.0733	2.96 ± 0.02 ^{a}	0.84 ± 0.01 ^{b}	1.50 ± 0.01 ^{c}
11	Procyanidin dimer iso4 [3]	8.99	577.1387	289.0733	12.80 ± 0.00 ^{a}	2.71 ± 0.00 ^{b}	3.79 ± 0.00 ^{c}
12	Procyanidin dimer iso5 [3]	11.14	577.1387	289.0733	4.32 ± 0.04 ^{a}	1.54 ± 0.02 ^{b}	1.97 ± 0.02 ^{b}
13	Procyanidin trimer iso1 [3]	5.46	865.2016	577.1369	16.28 ± 0.29 ^{a}	5.14 ± 0.09 ^{b}	7.91 ± 0.14 ^{c}
14	Procyanidin trimer iso2 [3]	8.67	865.2016	577.1369	14.35 ± 0.71 ^{a}	4.62 ± 0.23 ^{b}	5.15 ± 0.25 ^{c}
15	Procyanidin trimer iso3 [3]	8.89	865.2016	577.1369	6.11 ± 0.03 ^{a}	2.37 ± 0.01 ^{b}	2.55 ± 0.01 ^{b}
16	Procyanidin trimer iso4 [3]	10.55	865.2016	577.1369	3.22 ± 0.14 ^{a}	1.38 ± 0.06 ^{b}	1.19 ± 0.06 ^{b}
17	Procyanidin trimer iso5 [3]	10.71	865.2016	577.1369	13.79 ± 0.23 ^{a}	2.82 ± 0.05 ^{b}	2.70 ± 0.05 ^{b}
	Flavonols						
18	Quercetin	17.80	301.0372		36.78 ± 0.17 ^{a}	28.62 ± 0.13 ^{b}	25.35 ± 0.12 ^{c}
19	Quercetin-3-O-glucoside [4]	13.00	463.0904	301.0361	1.63 ± 0.04 ^{a}	2.73 ± 0.08 ^{b}	7.68 ± 0.21 ^{c}
20	Quercetin-3-O-glucuronide [4]	12.95	477.0702	301.0369	2.42 ± 0.01 ^{a}	5.62 ± 0.03 ^{b}	4.86 ± 0.02 ^{c}
21	Kaempferol [4]	20.07	285.0405		5.15 ± 0.02 ^{a}	1.63 ± 0.01 ^{b}	9.35 ± 0.04 ^{c}
22	kaempferol-3-O-glucuronide [4]	14.22	461.0763	285.0412	0.48 ± 0.01 ^{a}	1.31 ± 0.02 ^{b}	3.24 ± 0.05 ^{c}
23	Isorhamnetin [4]	20.31	315.0531		11.16 ± 0.12 ^{a}	4.92 ± 0.05 ^{b}	6.80 ± 0.07 ^{c}
	Phenolic acids						
24	Gallic acid	3.13	169.0193		120.87 ± 3.67 ^{a}	121.19 ± 3.68 ^{a}	96.11 ± 2.92 ^{b}
25	Caffeic acid	8.63	179.0401		3.27 ± 0.04 ^{a}	2.18 ± 0.02 ^{a}	1.09 ± 0.01 ^{a}
26	Caffeic acid O-glucoside iso1 [5]	7.64	341.0878	179.0350	0.55 ± 0.02 ^{a}	1.21 ± 0.05 ^{a}	0.13 ± 0.01 ^{a}
27	Caffeic acid O-glucoside iso2 [5]	8.29	341.0878	179.0350	0.66 ± 0.03 ^{a}	1.13 ± 0.05 ^{a}	0.20 ± 0.01 ^{a}
28	p-Coumaric acid	10.65	163.0439		3.44 ± 0.03 ^{a}	3.73 ± 0.04 ^{a}	1.15 ± 0.01 ^{a}
29	4-Hydroxybenzoic acid	8.17	137.0243		1.67 ± 0.05 ^{a}	0.89 ± 0.03 ^{a}	1.19 ± 0.04 ^{a}
30	Ferulic acid	12.00	193.0506		0.75 ± 0.01 ^{a}	0.27 ± 0.00 ^{a}	0.51 ± 0.01 ^{a}
31	Vanillic acid	8.51	167.0350		2.33 ± 0.07 ^{a}	2.15 ± 0.08 ^{a}	2.67 ± 0.04 ^{a}
	Stilbenes						
32	trans-Resveratrol [6]	15.73	227.0714		4.60 ± 0.02 ^{a}	3.63 ± 0.02 ^{b}	3.12 ± 0.01 ^{c}
33	Resveratrol iso1[6]	18.00	227.0714		2.95 ± 0.01 ^{a}	2.56 ± 0.01 ^{b}	0.97 ± 0.00 ^{c}
34	Resveratrol O-glucoside iso1 [6]	12.44	389.1242	227.0721	0.27 ± 0.00 ^{a}	1.22 ± 0.02 ^{b}	1.17 ± 0.02 ^{b}
35	Resveratrol O-glucoside iso2 [6]	14.92	389.1242	227.0721	1.35 ± 0.02 ^{a}	6.01 ± 0.10 ^{b}	3.32 ± 0.05 ^{c}
36	Piceatannol [6]	2.59	243.0663	203.0727	4.20 ± 0.05 ^{a}	4.96 ± 0.06 ^{b}	4.04 ± 0.05 ^{c}
37	Piceatannol 3-O-glucoside iso1 [6]	12.89	405.1208	243.0670	0.22 ± 0.01 ^{a}	0.14 ± 0.00 ^{b}	0.04 ± 0.00 ^{c}
38	Piceatannol 3-O-glucoside iso2 [6]	13.15	405.1208	243.0670	0.06 ± 0.00 ^{a}	0.18 ± 0.00 ^{b}	0.05 ± 0.00 ^{a}
39	Viniferin-iso1 [6]	19.53	453.1344	116.9291	0.27 ± 0.01 ^{a}	0.33 ± 0.01 ^{a}	0.15 ± 0.00 ^{b}
40	Viniferin-iso2 [6]	19.92	453.1344	116.9291	0.81 ± 0.02 ^{a}	1.05 ± 0.03 ^{b}	0.73 ± 0.02 ^{c}

Abbreviations: Retention time (R.T.). Wine lees (WL). ^{a,b,c} Different letters indicate significant differences in the content of each individual phenolic compound between the different WL ($p < 0.05$; one-way ANOVA).[1] Tentatively quantified using the catechin calibrating curve. [2] Tentatively quantified using the epicatechin calibrating curve. [3] Tentatively quantified using the procyanidin dimer B2 calibrating curve. [4] Tentatively quantified using the quercetin calibrating curve. [5] Tentatively quantified using the caffeic acid calibrating curve. [6] Tentatively quantified using the resveratrol calibrating curve.

Table 4. Characterization of anthocyanin in Cabernet, Mazuela, and Garnacha wine lees (WL) by UHPLC- (ESI +)-Q-TOF-MS.

	Anthocyanins	R.T. (min)	[M-H]+	Fragment (*m/z*)	Cabernet WL (mg/L)	Mazuela WL (mg/L)	Garnacha WL (mg/L)
1	Gallocatechin-Malvidin-3-glucoside dimer [1]	3.58	797.2035		0.25 ± 0.01 [a]	0.10 ± 0.00 [a]	0.16 ± 0.00 [a]
2	Malvidin-3-glucoside-(epi) catechin [1]	4.84	781.1974		1.11 ± 0.01 [a]	0.53 ± 0.00 [b]	0.50 ± 0.00 [b]
3	Delphinidin-3-glucoside [2]	5.06	465.1028	303.0511	3.69 ± 0.04 [a]	2.98 ± 0.03 [b]	1.39 ± 0.01 [c]
4	Cyanidin-3-glucoside [2]	5.85	449.1078	287.0531	0.23 ± 0.01 [a]	0.20 ± 0.01 [a]	0.16 ± 0.01 [a]
5	Petunidin-3-glucoside [3]	6.47	479.1184	317.0669	5.03 ± 0.06 [a]	4.90 ± 0.06 [a]	2.18 ± 0.03 [b]
6	Petunidin-3-glucoside-pyruvic acid [3]	7.05	547.1082	385.0547	0.09 ± 0.00 [a]	0.06 ± 0.00 [a]	0.03 ± 0.00 [a]
7	Peonidin-3-glucoside [3]	7.14	463.1235	301.0717	2.72 ± 0.04 [a]	1.83 ± 0.03 [b]	2.48 ± 0.04 [c]
8	Malvidin-3-glucoside [1]	7.48	493.1341	331.0843	60.67 ± 0.68 [a]	43.90 ± 0.49 [b]	26.78 ± 0.30 [c]
9	Peonidin-3-glucoside-pyruvic acid [3]	7.81	531.1133	369.0607	0.04 ± 0.00 [a]	0.02 ± 0.00 [a]	0.02 ± 0.00 [a]
10	Delphinidin-(6-acetyl)-3-glucoside [2]	7.87	507.1133	303.0496	0.91 ± 0.02 [a]	0.09 ± 0.00 [b]	0.02 ± 0.00 [b]
11	Visitin A (malvidin-3-glucoside-pyruvic acid) [1]	8.11	561.1239	399.0730	1.23 ± 0.01 [a]	0.63 ± 0.01 [b]	0.35 ± 0.00 [c]
12	Visitin B (malvidin-3-glucoside-acetaldehyde) [1]	8.32	517.1341	355.0826	3.06 ± 0.08 [a]	4.92 ± 0.12 [b]	5.11 ± 0.00 [b]
13	Malvidin-3-glucoside-ethyl-(epi) catechin [1]	8.40	809.2287		0.37 ± 0.00 [a]	0.09 ± 0.00 [b]	0.31 ± 0.13 [a]
14	Cyanidin-(6-acetyl)-3-glucoside [2]	8.45	491.1184	491.1189	0.20 ± 0.00 [a]	0.02 ± 0.00 [b]	0.01 ± 0.00 [b]
15	Acetylvisitin A [1]	8.50	603.1344	399.0718	0.79 ± 0.03 [a]	0.10 ± 0.00 [b]	0.15 ± 0.00 [b]
16	Malvidin-3-glucoside-ethyl-(epi) catechin [1]	8.57	809.2287		1.38 ± 0.02 [a]	0.51 ± 0.01 [b]	1.65 ± 0.00 [c]
17	Petunidin-(6-acetyl)-3-glucoside [3]	8.66	521.1378	317.0667	1.29 ± 0.04 [a]	0.16 ± 0.01 [b]	0.04 ± 0.02 [b]
18	Malvidin-3-glucoside-ethyl-(epi) catechin [1]	8.75	809.2287		2.04 ± 0.06 [a]	0.80 ± 0.03 [b]	2.63 ± 0.00 [c]
19	Acetylvisitin B [1]	8.77	559.1446	355.0813	1.66 ± 0.05 [a]	0.47 ± 0.01 [b]	0.27 ± 0.08 [b]
20	Peonidin-(6-acetyl)-3-glucoside [3]	9.08	505.1341	301.0714	1.32 ± 0.03 [a]	0.13 ± 0.00 [b]	0.08 ± 0.01 [b]
21	Delphinidin-(6-coumaroyl)-3-glucoside [2]	9.08	611.1395	303.0508	0.44 ± 0.01 [a]	0.55 ± 0.01 [a]	0.09 ± 0.00 [b]
22	Malvidin-(6-acetyl)-3-glucoside [1]	9.13	535.1446	331.0836	28.39 ± 0.03 [a]	2.57 ± 0.00 [b]	0.79 ± 0.00 [c]
23	Coumaroylvisitin A [1]	9.29	707.1607	399.0718	0.20 ± 0.00 [a]	0.13 ± 0.00 [b]	0.04 ± 0.00 [b]
24	Malvidin-(6-caffeoyl)-3-glucoside [1]	9.41	655.1657	331.0808	0.36 ± 0.02 [a]	0.10 ± 0.00 [b]	0.04 ± 0.00 [b]
25	Cyanidin-(6-coumaroyl)-3-glucoside [2]	9.42	595.1446	287.0560	0.10 ± 0.00 [a]	0.11 ± 0.00 [a]	0.03 ± 0.00 [b]
26	Catechin-ethyl-Malvidin-3-acetylglucoside dimer [1]	9.43	851.2511		0.88 ± 0.03 [a]	0.03 ± 0.00 [b]	0.06 ± 0.00 [b]
27	Petunidin-(6-coumaroyl)-3-glucoside [3]	9.52	625.1552	317.0662	0.74 ± 0.03 [a]	0.78 ± 0.01 [a]	0.16 ± 0.00 [b]
28	Pinotin A (malvidin-3-glucoside-vinylcatechol) [1]	9.53	625.1552	463.0998	0.84 ± 0.02 [a]	0.88 ± 0.02 [a]	0.18 ± 0.00 [b]
29	Malvidin-glucoside-vinyl-catechin [1]	9.56	805.1974		0.15 ± 0.00 [a]	0.08 ± 0.00 [b]	0.16 ± 0.00 [a]
30	Coumaroylvisitin B [1]	9.58	663.1708	355.0822	0.91 ± 0.03 [a]	1.08 ± 0.04 [b]	1.12 ± 0.04 [b]
31	Malvidin-3-glucoside-vinylguaiacol [1]	9.63	639.1708	331.0823	0.59 ± 0.01 [a]	0.37 ± 0.01 [b]	0.17 ± 0.00 [b]
32	Catechin-ethyl-malvidin-3-coumaroylglucoside dimer [1]	9.70	955.2785		0.68 ± 0.01 [a]	0.21 ± 0.00 [b]	0.51 ± 0.01 [a]
33	Catechin-ethyl-malvidin-3-acetylglucoside dimer [1]	9.81	851.2511		0.14 ± 0.00 [a]	0.02 ± 0.00 [b]	0.02 ± 0.00 [b]
34	Peonidin-(6coumaroyl)-3-glucoside [3]	9.87	609.1603	301.0716	0.94 ± 0.03 [a]	0.60 ± 0.02 [b]	0.42 ± 0.01 [c]
35	Malvidin-(6-coumaroyl)-3-glucoside [1]	9.92	639.1708	331.0823	10.77 ± 0.02 [a]	4.43 ± 0.01 [b]	2.31 ± 0.01 [c]
36	Malvidin-glucoside-vinyl-catechin [1]	9.99	805.1974		0.16 ± 0.00 [a]	0.06 ± 0.00 [b]	0.14 ± 0.00 [a]
37	Acetyl-pinotin A [1]	10.19	667.1657		0.01 ± 0.00 [a]	0.00 ± 0.00 [b]	0.01 ± 0.00 [a]
38	Malvidin 3-O-glucoside 4-vinylphenol (Pigment A) [1]	10.22	609.1603	447.1079	0.64 ± 0.01 [a]	0.44 ± 0.00 [b]	0.44 ± 0.00 [b]
39	Catechin-ethyl-malvidin-3-coumaroylglucoside dimer [1]	10.33	955.2785		0.12 ± 0.00 [a]	0.04 ± 0.00 [b]	0.10 ± 0.00 [a]
40	Malvidin acetyl 3-O-glucoside 4-vinylphenol (Acetyl-pigment A) [1]	10.50	651.1708	447.1076	0.38 ± 0.01 [a]	0.03 ± 0.00 [b]	0.02 ± 0.00 [b]

Abbreviations: Retention time (R.T.). Wine lees (WL). [a,b,c] Different letters indicate significant differences in the content of each individual phenolic compound between the different WL ($p < 0.05$; one-way ANOVA). [1] Tentatively quantified using the calibrating curve of malvidin glucoside. [2] Tentatively quantified using the calibrating curve of cyaniding rutinoside. [3] Tentatively quantified using the calibrating curve of peonidin rutinoside.

The sample individual phenolic profile on flavanols, flavonols, phenolic acids, and stilbenes is shown in Table 3. The major compounds in all samples were catechin, epicatechin, procyanidin dimer B2, and procyanidin dimer iso1, with higher levels found in CWL compared to Mazuela and Garnacha WL. Regarding the content of the other phenolic families, the major compounds found were: quercetin and isorhamnetin in the flavonols group, gallic acid in the phenolic acid group, and trans-resveratrol and piceatannol in the stilbene group.

Regarding the anthocyanin composition (Table 4), a total of forty different anthocyanins were identified in the WL with malvidin-3-glucoside > malvidin-(6-acetyl)-3-glucoside > malvidin-(6-coumaroyl)-3-glucoside as the major compounds. The content of these three compounds was notably higher in the CWL compared to the other WL.

3.3. Effect of Different Wine Lees on Blood Pressure in Hypertensive Rats

The antihypertensive effect of Cabernet, Garnacha, and Mazuela WL was evaluated in SHR rats after an acute oral dose (5 mL/kg bw). SBP and DBP results of this study are shown in Figure 4A,B, respectively. As expected, animals that received water did not show changes in their BP. In contrast, Captopril administration (50 mg/kg bw) led to a continuous decrease in the animals' SBP and DBP 2 h post-treatment. The maximum decreases were observed at 6 h (-43.2 ± 3.9 and -47.2 ± 1.5 mmHg for SBP and DBP,

respectively). Regarding the WL, only CWL showed an antihypertensive effect on both SBP and DBP in SHR, being their behavior similar to the one observed by Captopril. The maximum decrease in BP was also observed at 6 h post-administration (-36.4 ± 3.4 and -38.8 ± 4.6 mmHg for SBP and DBP, respectively). Initial BP values were recovered at 24 or 48 h for SBP and DBP, respectively. No significant changes in BP were found between the Garnacha or Mazuela WL groups and water group (Figure 4A,B). CWL were selected according their antihypertensive effect for further studies.

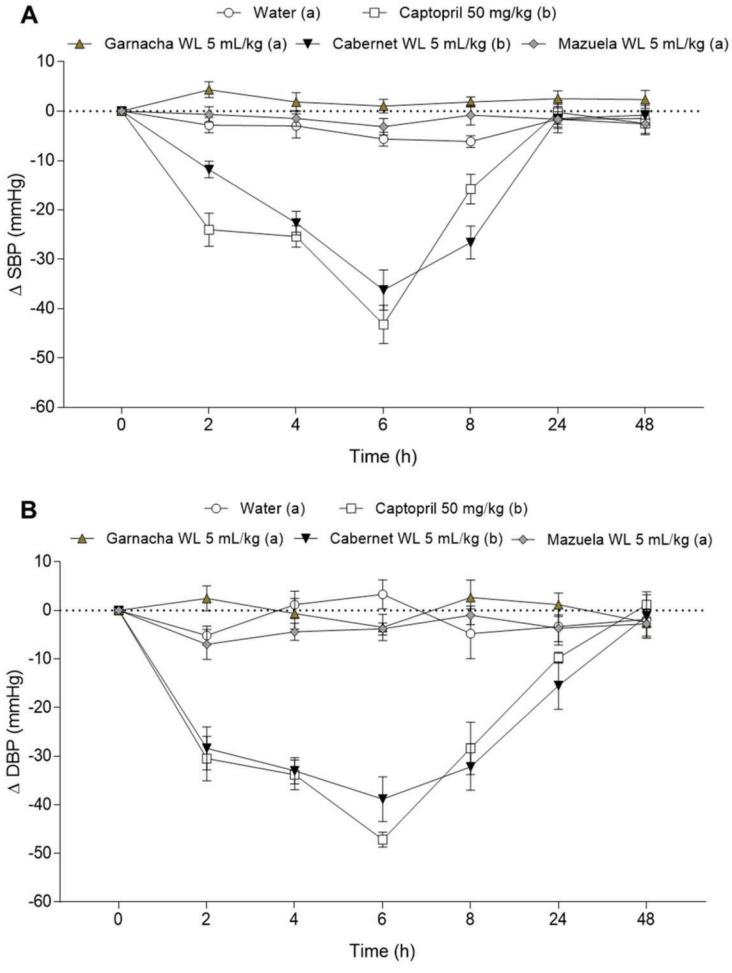

Figure 4. Decrease of systolic blood pressure (SBP, **A**) and diastolic blood pressure (DBP, **B**) in spontaneous hypertensive rats after the administration of water, Captopril (50 mg/kg bw), and the three selected wine lees (WL; 5 mL/kg bw): Garnacha WL, Cabernet WL, and Mazuela WL. Data are expressed as mean ($n = 6$) $\pm$ SEM. Significant differences ($p < 0.05$) between treatments are represented by different letters in the legend. p value was estimated by two-way ANOVA and Tukey test was used as post hoc.

3.4. Effect of Cabernet Wine Lees on Blood Pressure in Normotensive Rats

The effect of CWL on BP was also evaluated in normotensive rats (WKY) in order to discard possible hypotensive effects. Initial values of SBP and DBP were 119.1 ± 4.2 and 87.9 ± 8.8 mmHg, respectively. The administration of a single dose of CWL (5 mL/kg bw) did not modify SBP or DBP values in the animals during the experiment (Figure 5). BP values were significantly similar to those showed by the animals that ingested water.

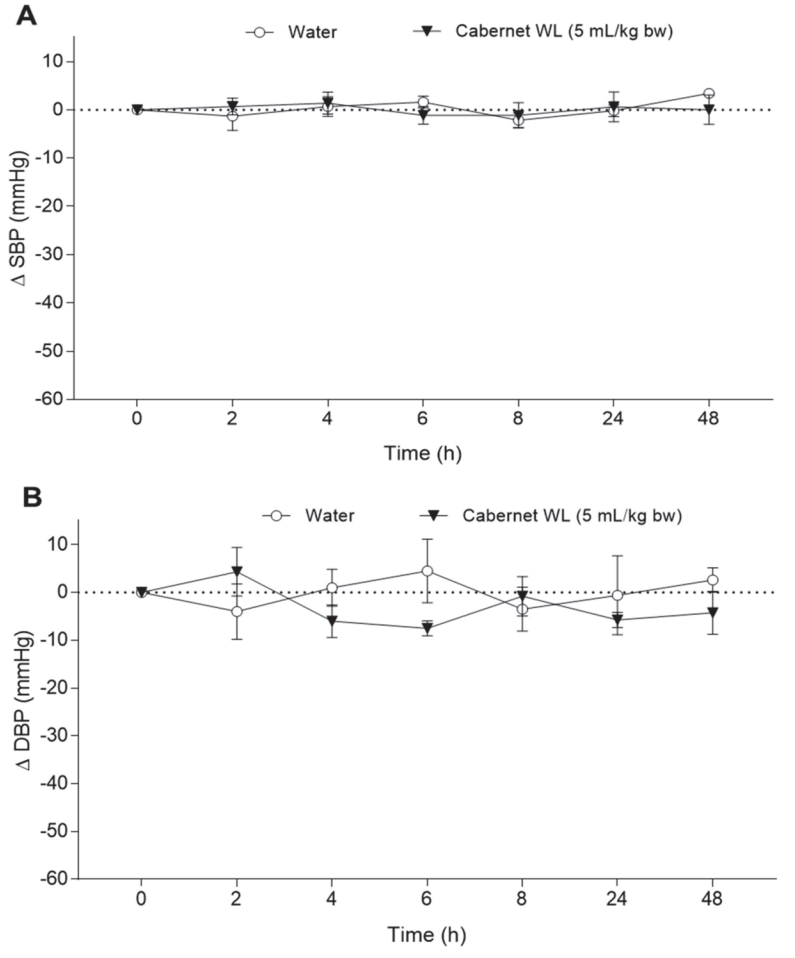

Figure 5. Decrease in systolic blood pressure (SBP, **A**) and diastolic blood pressure (DBP, **B**) caused in Wistar–Kyoto rats after the acute administration of water or Cabernet WL (5 mL/g bw). Data are expressed as mean ($n = 6$) ± SEM. No significant differences ($p < 0.05$) were found. p was estimated by two-way ANOVA.

3.5. Variability between Cabernet Wine Lees from Two Different Harvests

Finally, the variability of ACEi and antihypertensive activities of CWL harvested in two different years (CWL and CWL2) were evaluated. ACEi activity (%) and IC_{50} did not show differences between CWL from different grape harvests (Figure 6A,B). The antihypertensive properties of CWL and CWL2 were also evaluated in SHR at a single dose of 5 mL/kg bw at 6 h post-administration. No differences were found in SBP and DBP between different harvests (Figure 6C,D).

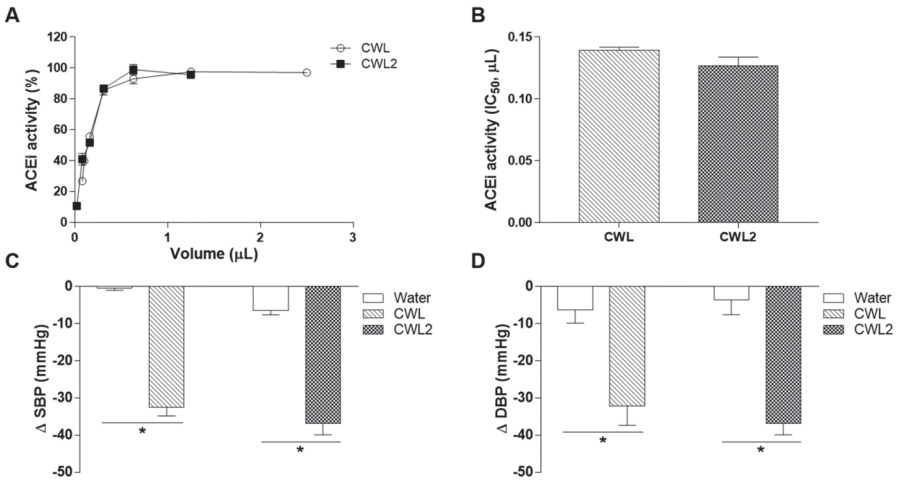

Figure 6. Variability between Cabernet wine lees (CWL) from two different harvests (CWL and CWL2). ACEi activity for both harvests is represented as dose–response curve (%, **A**) and IC_{50} (**B**). Data are shown as mean ± SD (n = 3). No significant differences ($p < 0.05$) were found between IC_{50} values (Student's T-test). Decrease in systolic blood pressure (SBP, **C**) and diastolic blood pressure (DBP, **D**) caused in spontaneous hypertensive rats by the acute administration of water, CWL, or CWL2 (both 5 mL/kg bw). Data are shown as mean ± SEM (n = 6). No significant differences ($p < 0.05$) were found between CWL and CWL2 (Student's T-test). * represents significant differences ($p < 0.05$) between CWL and CWL2 and their respective water control groups estimated by Student's T-test.

4. Discussion

Annually, a large number of agri-food by-products are generated during food processing and their valorization has attracted a great deal of attention over the past few years [9]. In fact, one of the most emerging purposes is to be used as a source of bioactive compounds. These compounds are highly valued by the food, pharmaceutical, and cosmetic industries because they show a wide range of beneficial health effects. In this sense, winery by-products have been successfully used to obtain antioxidant [31], antimicrobial [32], anti-inflammatory [33], antihyperglycemic [34], or antihypertensive compounds [14,16,17,35]. For instance, extracts rich in phenolic compounds (proanthocyanidins or resveratrol), with antihypertensive properties in both rats and humans, have been extracted from stem, grape seeds, or skin, respectively [13,15–17,36–38]. However, to our knowledge, no studies have been performed to evaluate whether WL can present ACEi or antihypertensive properties. Thus, the aim of this study was to evaluate the ACEi and antihypertensive activities in several WL. For this, five WL samples were obtained in the elaboration of wine with a single grape variety (red grapes varieties: Cabernet, Garnacha, Mazuela, Merlot, and white grape variety: Macabeo) were selected to determine their ability to inhibit ACE. ACE inhibitors, as Captopril, Enalapril, or Lisinopril, are usually used to treat hypertension [39]. In fact, ACEi activity is commonly used as a screening tool in the search for natural antihypertensive compounds. The determination of the ACEi activity of the five WL showed that lees obtained from red grape varieties exerted higher activity than those obtained from the white grape variety (Table 1; Figure 2). Phenolic compounds are present in red grapes in larger quantities than in white grapes [40]. Therefore, these compounds could be responsible for the ACEi effects since in vitro studies have demonstrated inhibitory properties of phenolic compounds on ACE [8]. Similar results were reported by Pozo-Bayón et al. and Alcaide-Hidalgo et al., who studied the ACEi activity of red (Tempranillo) and white (Airén, Verdejo and Sauvignon Blanc) wines, respectively [41,42]. The ACEi activity of the WL was also calculated as IC_{50}. The expression of this value in volume is indicative of the microliters of the WL necessary to inhibit the enzyme by 50% under the assay conditions, where the total volume is 240 µL. Therefore, it is a measure of the pharmacological potency, given that the

lower the IC_{50}, expressed in volume, the higher the potency of ACE inhibition of the WL assayed [43]. All WL obtained from red grapes showed a potent ACEi activity, although differences in the IC_{50} values can be noted (Table 1). ACEi activities of red WL ranged among 0.15 ± 0.0 and 0.32 ± 0.0 µL. These inhibitory potencies are higher than those reported by other authors in milk fermented with *Enterococcus faecalis* and *Lactobacillus helveticus* [43,44]. These results clearly reveal important ACEi potency in WL obtained from red grapes. Although ACEi activity is frequently used to select antihypertensive compounds, it does not always correspond to an *in vivo* effect. Gastrointestinal digestion could produce variations over different WL compounds. In addition, first pass metabolism and microbiota metabolism will significantly modify the ingested phenolic compounds [45]. All these modifications could lead to changes in their ACEi properties. Therefore, *in vivo* studies must be carried out to demonstrate the antihypertensive effect of WL. In the present study, the antihypertensive effect of Cabernet, Garnacha, and Mazuela WL, selected by their ACEi activity, was evaluated in SHR after a single oral dose of 5 mL/kg bw (Figure 4). While Garnacha and Mazuela WL did not exhibit BP-lowering effects, CWL showed a clear antihypertensive effect. The maximum antihypertensive effect was reached at 6 h post-administration. The ACE inhibitor Captopril also exhibited a similar response time. A maximum decrease in BP at 4 or 6 h post-administration has been also reported by our group for other natural ACE inhibitors such as phenolic rich cocoa or grape seed extracts [30,46] or bioactive peptides [47]. Furthermore, the powerful antihypertensive effect of the CWL was similar to that observed for the drug Captopril. Phenolic extracts obtained from grape seeds (GSPE) have shown similar antihypertensive effects, with a maximum drop in BP at 6 h post-administration [15,17]. Similarly, Valls et al. observed a significant enhancement of endothelial function 5 h after administration to volunteers of a phenolic-enriched olive oil. In addition, this effect correlated with an increase of phenolic-derived metabolites in blood 2 h after its intake [48]. Notably, the BP-lowering effect produced by CWL (approximately 30 mmHg) could be a promising result since small reductions in BP may have an important impact on cardiovascular events in the hypertensive population [49]. In this sense, a reduction in 5 mmHg for DBP and 10 mmHg for SBP produces a significant reduction in the risk of suffering or worsening CVD [50,51]. The effect on BP of CWL was also tested in normotensive rats in order to rule out hypotensive effects. CWL did not modify the BP of these animals (Figure 5). This indicates that the antihypertensive effect of these CWL is specific to the hypertensive condition.

In order to understand the different BP-lowering effects exhibited by the tested WL in rats, the phenolic profile of these three samples was studied. Phenolic compounds have been widely investigated due to their large number of beneficial properties, such as their cardioprotective effect [52], in which antihypertensive activity is included [53]. Specifically, a meta-analysis focused on grape phenolic compounds showed that their daily consumption reduced SBP by 1.48 mmHg when compared with the control group [54]. Thus, the different BP-lowering effects exhibited by the three WL would lie within the phenolic composition. According to this, results revealed that CWL contained twice the amount of total phenolic compounds when compared to Mazuela or Garnacha WL. These results highlight the importance of phenolic compounds for the antihypertensive activity of the WL. Specifically, it can be observed a higher concentration of flavanol and anthocyanin families in CWL compared to Mazuela or Garnacha WL (Table 2). Numerous studies have shown that the intake of flavanol-rich foods such as cocoa, red grapes, and red wine can be associated with improved vascular function and can repair and reduce BP in both hypertensive and pre-hypertensive individuals [55]. It has also been reported that flavanol-rich extracts from grape seed or cocoa showed antihypertensive effect after their acute [15,17,30,46] and chronic administration [16] to hypertensive rats. Furthermore, the flavanol monomers epicatechin and catechin have shown antihypertensive effect in both rats and humans administered at low concentrations [56–58]. The high levels of catechin, epicatechin, and procyanidins (Table 3) present in CWL suggest that these compounds could be in part responsible for the BP-lowering effect of this variety of WL. In addition, the

polyphenol family of anthocyanins has also shown cardioprotective and antihypertensive properties [59,60]. Their circulating metabolites have also been directly related to vascular benefits [61]. Moreover, malvidin-3-glucoside, a compound from the anthocyanins family, has been reported as a potent vasodilator [62]. In our study, the anthocyanin content in CWL was higher than in the other WL. The main differences were in the levels of malvidin-3-glucoside, malvidin-(6-acetyl)-3-glucoside, and malvidin-(6-coumaroyl)-3-glucoside (Table 4). Therefore, anthocyanin family and specifically these compounds could be also involved in the BP-lowering effect of CWL.

Finally, the ACEi and antihypertensive activities of a CWL coming from a different harvest were also studied. No significant differences in the ACEi activity or in the decrease of BP produced by the different CWL were observed (Figure 6), indicating good reproducibility of the CWL beneficial effects.

5. Conclusions

This study shows that WL from red grapes present a potent ACEi activity and that CWL, WL coming from the grape variety Cabernet, also exhibited a potent BP-lowering effect, specific to a hypertensive condition. It is noteworthy that in this study CWL were administered at 5 mL/kg bw. This dose corresponds to an intake of 73 mL/day in humans, using a translation of animal to human doses [63] and estimating the daily intake for an adult human with body weight 70 kg and body height 175 cm. Although experimental results obtained in animals cannot be directly translatable to humans, the fact that only 73 mL of CWL exhibit antihypertensive effects opens the door to the valorization of CWL by their BP-lowering properties. Nevertheless, the quantity of CWL necessary to decrease arterial BP in humans should be definitively established when clinical trials are conducted. CWL antihypertensive activity has been related to their highest content in anthocyanins and flavanols. In addition, these beneficial effects were reproducible in CWL from different vintages. These findings open the door to the use of CWL to alleviate hypertension. At the same time, this study would also allow the wine industry to revalue by-products as WL and, therefore, reduce their associated environmental problems. However, HTN is a chronic pathology that requires chronic treatment; thus, chronic studies are necessary to evaluate of antihypertensive effect of long-term administration of CWL.

6. Patents

Patent application "Wine lees, derivatives thereof and their uses": application number EP20382358.8 and PCT/EP2021/053051.

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Institutional Review Board Statement: The animal protocols followed in this study were conducted in accordance with the European Communities Council Directive (86/609/EEC) and approved by the Animal Ethics Review Committee for Animal Experimentation of the Universitat Rovira i Virgili and further approved by Generalitat de Catalunya (permission number 10780).

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Oparil, S.; Acelajado, M.C.; Bakris, G.L.; Berlowitz, D.R.; Cífková, R.; Dominiczak, A.F.; Grassi, G.; Jordans, J.; Poulter, N.R.; Rodgers, A.; et al. Hypertension. *Nat. Rev. Dis. Prim.* **2018**, *4*, 18014. [CrossRef]
2. Forouzanfar, M.H.; Liu, P.; Roth, G.A.; Ng, M.; Biryukov, S.; Marczak, L.; Alexander, L.; Estep, K.; Abate, K.H.; Akinyemiju, T.F.; et al. Global burden of hypertension and systolic blood pressure of at least 110 to 115 mmHg, 1990–2015. *JAMA J. Am. Med. Assoc.* **2017**, *317*, 165–182. [CrossRef]
3. Hypertension. Available online: https://www.who.int/news-room/fact-sheets/detail/hypertension (accessed on 22 May 2020).
4. Hedayati, S.S.; Elsayed, E.F.; Reilly, R.F. Non-pharmacological aspects of blood pressure management: What are the data? *Kidney Int.* **2011**, *79*, 1061–1070. [CrossRef] [PubMed]
5. Snauwaert, E.; Vande, W.J.; De Bruyne, P. Therapeutic efficacy and safety of ACE inhibitors in the hypertensive paediatric population: A review. *Arch. Dis. Child.* **2017**, *102*, 63–71. [CrossRef] [PubMed]
6. Sparks, M.A.; Crowley, S.D.; Gurley, S.B.; Mirotsou, M.; Coffman, T.M. Classical Renin-Angiotensin System in Kidney Physiology. *Compr. Physiol.* **2014**, *4*, 1201–1228. [PubMed]
7. Brown, N.J.; Vaughan, D.E. Angiotensin-Converting Enzyme Inhibitors. *Circulation* **1998**, *97*, 1411–1420. [CrossRef]
8. Margalef, M.; Bravo, F.I.; Muguerza, B.; Arola-Arnal, A. Natural Angiotensin Converting Enzyme (ACE) Inhibitors with Antihypertensive Properties. In *Natural Products Targeting Clinically Relevant Enzymes*; Wiley-VCH Verlag GmbH & Co. KGaA.: Weinheim, Germany, 2017; pp. 45–67.
9. Ben-Othman, S.; Jõudu, I.; Bhat, R. Bioactives from Agri-Food Wastes: Present Insights and Future Challenges. *Molecules* **2020**, *25*, 510. [CrossRef] [PubMed]
10. Trigo, J.P.; Alexandre, E.M.C.; Saraiva, J.A.; Pintado, M.E. High value-added compounds from fruit and vegetable by-products –Characterization, bioactivities, and application in the development of novel food products. *Crit. Rev. Food Sci. Nutr.* **2019**, *60*, 1388–1416. [CrossRef]
11. Mak, T.M.; Xiong, X.; Tsang, D.C.; Yu, I.K.; Poon, C.S. Sustainable food waste management towards circular bioeconomy: Policy review, limitations and opportunities. *Bioresour. Technol.* **2020**, *297*, 122497. [CrossRef]
12. Musee, N.; Lorenzen, L.; Aldrich, C. Cellar waste minimization in the wine industry: A systems approach. *J. Clean. Prod.* **2007**, *15*, 417–431. [CrossRef]
13. Vázquez-Garza, E.; Bernal-Ramírez, J.; Jerjes-Sánchez, C.; Lozano, O.; Acuña-Morín, E.; Vanoye-Tamez, M.; Ramos-González, M.R.; Chapoy-Villanueva, H.; Pérez-Plata, L.; Sánchez-Trujillo, L.; et al. Resveratrol Prevents Right Ventricle Remodeling and Dysfunction in Monocrotaline-Induced Pulmonary Arterial Hypertension with a Limited Improvement in the Lung Vasculature. *Oxid. Med. Cell. Longev.* **2020**, *2020*, 1841527. [CrossRef]
14. Jia, X.; Zhang, R.; Guo, J.; Yue, H.; Liu, Q.; Guo, L.; Zhang, Q. Resveratrol Supplementation Prevents Hypertension in Hypertensive Pregnant Rats by Increasing Sodium Excretion and Serum Nitric Oxide Level. *Int. J. Hypertens.* **2020**, *2020*, 4154010. [CrossRef]
15. Pons, Z.; Guerrero, L.; Margalef, M.; Arola, L.; Arola-Arnal, A.; Muguerza, B. Effect of low molecular grape seed proanthocyanidins on blood pressure and lipid homeostasis in cafeteria diet-fed rats. *J. Physiol. Biochem.* **2014**, *70*, 629–637. [CrossRef]
16. Pons, Z.; Margalef, M.; Bravo, F.I.; Arola-Arnal, A.; Muguerza, B. Chronic administration of grape-seed polyphenols attenuates the development of hypertension and improves other cardiometabolic risk factors associated with the metabolic syndrome in cafeteria diet-fed rats. *Br. J. Nutr.* **2017**, *117*, 200–208. [CrossRef]
17. Quiñones, M.; Guerrero, L.; Suárez, M.; Pons, Z.; Aleixandre, A.; Arola, L.; Muguerza, B. Low-molecular procyanidin rich grape seed extract exerts antihypertensive effect in males spontaneously hypertensive rats. *Food Res. Int.* **2013**, *51*, 587–595. [CrossRef]
18. Pérez-Serradilla, J.; De Castro, M.L. Role of lees in wine production: A review. *Food Chem.* **2008**, *111*, 447–456. [CrossRef] [PubMed]
19. Romero-Díez, R.; Rodríguez-Rojo, S.; Cocero, M.J.; Duarte, C.M.M.; Matias, A.A.; Bronze, M.R. Phenolic characterization of aging wine lees: Correlation with antioxidant activities. *Food Chem.* **2018**, *259*, 188–195. [CrossRef] [PubMed]
20. Jara-Palacios, M.J. Wine Lees as a Source of Antioxidant Compounds. *Antioxidants* **2019**, *8*, 45. [CrossRef]
21. Landeka Jurčević, I.; Dora, M.; Guberović, I.; Petras, M.; Rimac Brnčić, S.; Đikić, D. Wine Lees Polyphenols as a Novel Functional Bioactive Compound in the Protection against Oxidative Stress and Hyperlipidemia. *Food Technol. Biotechnol.* **2017**, *55*, 109. [CrossRef] [PubMed]
22. De Iseppi, A.; Lomolino, G.; Marangon, M.; Curioni, A. Current and future strategies for wine yeast lees valorization. *Food Res. Int.* **2020**, *137*, 109352. [CrossRef]

23. Romero-Díez, R.; Matos, M.; Rodrigues, L.; Bronze, M.R.; Rodríguez-Rojo, S.; Cocero, M.; Matias, A. Microwave and ultrasound pre-treatments to enhance anthocyanins extraction from different wine lees. *Food Chem.* **2019**, *272*, 258–266. [CrossRef] [PubMed]
24. Matos, M.S.; Romero-Díez, R.; Álvarez, A.; Bronze, M.R.; Rodríguez-Rojo, S.; Mato, R.B.; Cocero, M.J.; Matias, A.A. Polyphenol-Rich Extracts Obtained from Winemaking Waste Streams as Natural Ingredients with Cosmeceutical Potential. *Antioxidants* **2019**, *8*, 355. [CrossRef] [PubMed]
25. De La Torre, M.P.D.; Priego-Capote, F.; De Castro, M.D.L. Characterization and Comparison of Wine Lees by Liquid Chromatography–Mass Spectrometry in High-Resolution Mode. *J. Agric. Food Chem.* **2015**, *63*, 1116–1125. [CrossRef]
26. Barcia, M.T.; Pertuzatti, P.B.; Rodrigues, D.; Gómez-Alonso, S.; Hermosín-Gutíerrez, I.; Godoy, H.T. Occurrence of low molecular weight phenolics in Vitis vinifera red grape cultivars and their winemaking by-products from São Paulo (Brazil). *Food Res. Int.* **2014**, *62*, 500–513. [CrossRef]
27. Mas-Capdevila, A.; Iglesias-Carres, L.; Arola-Arnal, A.; Suarez, M.; Muguerza, B.; Bravo, F.I. Long-term administration of protein hydrolysate from chicken feet induces antihypertensive effect and confers vasoprotective pattern in diet-induced hypertensive rats. *J. Funct. Foods* **2019**, *55*, 28–35. [CrossRef]
28. Iglesias-Carres, L.; Mas-Capdevila, A.; Sancho-Pardo, L.; Bravo, F.I.; Mulero, M.; Muguerza, B.; Arola-Arnal, A. Optimized extraction by response surface methodology used for the characterization and quantification of phenolic compounds in whole red grapes (*Vitis vinifera*). *Nutrients* **2018**, *10*, 1931. [CrossRef]
29. Laitila, J.E.; Suvanto, J.; Salminen, J.P. Liquid chromatography–tandem mass spectrometry reveals detailed chromatographic fingerprints of anthocyanins and anthocyanin adducts in red wine. *Food Chem.* **2019**, *294*, 138–151. [CrossRef]
30. Quinones, M.; Miguel, M.; Muguerza, B.; Aleixandre, A. Effect of a cocoa polyphenol extract in spontaneously hypertensive rats. *Food Funct.* **2011**, *2*, 649–653. [CrossRef]
31. Costa, G.N.S.; Tonon, R.V.; Mellinger-Silva, C.; Galdeano, M.C.; Iacomini, M.; A Santiago, M.C.P.; Almeida, E.L.; Freitas, S.P. Grape seed pomace as a valuable source of antioxidant fibers. *J. Sci. Food Agric.* **2019**, *99*, 4593–4601. [CrossRef] [PubMed]
32. Mattos, G.N.; Tonon, R.V.; Furtado, A.A.; Cabral, L.M. Grape by-product extracts against microbial proliferation and lipid oxidation: A review. *J. Sci. Food Agric.* **2017**, *97*, 1055–1064. [CrossRef]
33. Wang, H.; Xue, Y.; Zhang, H.; Huang, Y.; Yang, G.; Du, M.; Zhu, M.-J. Dietary grape seed extract ameliorates symptoms of inflammatory bowel disease in IL10-deficient mice. *Mol. Nutr. Food Res.* **2013**, *57*, 2253–2257. [CrossRef]
34. Hernández-Salinas, R.; Decap, V.; Leguina, A.; Cáceres, P.; Perez, D.; Urquiaga, I.; Iturriaga, R.; Velarde, V. Antioxidant and antihyperglycemic role of wine grape powder in rats fed with a high fructose diet. *Biol. Res.* **2015**, *48*, 53. [CrossRef] [PubMed]
35. Diebolt, M.; Bucher, B.; Andriantsitohaina, R. Wine Polyphenols Decrease Blood Pressure, Improve NO Vasodilatation, and Induce Gene Expression. *Hypertension* **2001**, *38*, 159–165. [CrossRef] [PubMed]
36. De Moura, R.S.; Viana, F.S.C.; Souza, M.A.V.; Kovary, K.; Guedes, D.C.; Oliveira, E.P.B.; Rubenich, L.M.S.; Carvalho, L.C.R.M.; Oliveira, R.M.; Tano, T.; et al. Antihypertensive, vasodilator and antioxidant effects of a vinifera grape skin extract. *J. Pharm. Pharmacol.* **2002**, *54*, 1515–1520. [CrossRef]
37. Lin, Y.S.; Lu, Y.L.; Wang, G.J.; Chen, L.G.; Wen, C.L.; Hou, W.C. Ethanolic extracts and isolated compounds from small-leaf grape (Vitis thunbergii var. taiwaniana) with antihypertensive activities. *J. Agric. Food Chem.* **2012**, *60*, 7435–7441. [CrossRef]
38. Gupta, M.; Dey, S.; Marbaniang, D.; Pal, P.; Ray, S.; Mazumder, B. Grape seed extract: Having a potential health benefits. *J. Food Sci. Technol.* **2019**, *57*, 1205–1215. [CrossRef] [PubMed]
39. Tzakos, A.G.; Naqvi, N.; Comporozos, K.; Pierattelli, R.; Theodorou, V.; Husain, A.; Gerothanassis, I.P. The molecular basis for the selection of captopril cis and trans conformations by angiotensin I converting enzyme. *Bioorganic Med. Chem. Lett.* **2006**, *16*, 5084–5087. [CrossRef] [PubMed]
40. Mattivi, F.; Guzzon, R.; Vrhovsek, U.; Stefanini, A.M.; Velasco, R. Metabolite Profiling of Grape: Flavonols and Anthocyanins. *J. Agric. Food Chem.* **2006**, *54*, 7692–7702. [CrossRef] [PubMed]
41. Alcaide-Hidalgo, J.M.; Martínez-Rodríguez, A.J.; Martin-Álvarez, P.J.; Pueyo, E. Influence of the elaboration process on the peptide fraction with angiotensin I-converting enzyme inhibitor activity in sparkling wines and red wines aged on lees. *Food Chem.* **2008**, *111*, 965–969. [CrossRef]
42. Pozo-Bayón, M.A.; Alcaide, J.M.; Polo, M.C.; Pueyo, E. Angiotensin I-converting enzyme inhibitory compounds in white and red wines. *Food Chem.* **2007**, *100*, 43–47. [CrossRef]
43. Fuglsang, A.; Nilsson, D.; Nyborg, N.C.B. Cardiovascular effects of fermented milk containing angiotensin-converting enzyme inhibitors evaluated in permanently catheterized, spontaneously hypertensive rats. *Appl. Environ. Microbiol.* **2002**, *68*, 3566–3569. [CrossRef]
44. Muguerza, B.; Ramos, M.; Sánchez, E.; Manso, M.; Miguel, M.; Aleixandre, A.; Delgado, M.; Recio, I. Antihypertensive activity of milk fermented by Enterococcus faecalis strains isolated from raw milk. *Int. Dairy J.* **2006**, *16*, 61–69. [CrossRef]
45. Iglesias-Carres, L.; Mas-Capdevila, A.; Bravo, F.I.; Aragonès, G.; Arola-Arnal, A.; Muguerza, B. A comparative study on the bioavailability of phenolic compounds from organic and nonorganic red grapes. *Food Chem.* **2019**, *299*, 125092. [CrossRef] [PubMed]
46. Pons, Z.; Margalef, M.; Bravo, F.I.; Arola, A.; Muguerza, B. Acute administration of single oral dose of grape seed polyphenols restores blood pressure in a rat model of metabolic syndrome: Role of nitric oxide and prostacyclin. *Eur. J. Nutr.* **2016**, *55*, 749–758. [CrossRef]

47. Mas-Capdevila, A.; Pons, Z.; Aleixandre, A.; Bravo, F.I.; Muguerza, B. Dose-Related Antihypertensive Properties and the Corresponding Mechanisms of a Chicken Foot Hydrolysate in Hypertensive Rats. *Nutrients* **2018**, *10*, 1295. [CrossRef]
48. Valls, R.M.; Farràs, M.; Suárez, M.; Fernández-Castillejo, S.; Fitó, M.; Konstantinidou, V.; Fuentes, F.; Lopes-Miranda, J.; Giralt, M.; Covas, M.I.; et al. Effects of functional olive oil enriched with its own phenolic compounds on endothelial function in hyper-tensive patients. A randomised controlled trial. *Food Chem.* **2015**, *167*, 30–35. [CrossRef] [PubMed]
49. Turnbull, F. Blood Pressure Lowering Treatment Trialists' Collaboration. Effects of different blood-pressure-lowering regimens on major cardiovascular events: Results of prospectively-designed overviews of randomised trials. *Lancet* **2003**, *362*, 1527–1535. [PubMed]
50. Ettehad, D.; Emdin, C.A.; Kiran, A.; Anderson, S.G.; Callender, T.; Emberson, J.; Chalmers, J.; Rodgers, A.; Rahimi, K. Blood pressure lowering for prevention of cardiovascular disease and death: A systematic review and meta-analysis. *Lancet* **2016**, *387*, 957–967. [CrossRef]
51. Zanchetti, A. Hypertension: Lower or higher blood-pressure targets for high-risk patients? *Nat. Rev. Cardiol.* **2016**, *13*, 637–638. [CrossRef] [PubMed]
52. Dell'Agli, M.; Buscialà, A.; Bosisio, E. Vascular effects of wine polyphenols. *Cardiovasc. Res.* **2004**, *63*, 593–602. [CrossRef]
53. Durazzo, A.; Lucarini, M.; Souto, E.B.; Cicala, C.; Caiazzo, E.; Izzo, A.A.; Novellino, E.; Santini, A. Polyphenols: A concise overview on the chemistry, occurrence, and human health. *Phytother. Res.* **2019**, *33*, 2221–2243. [CrossRef]
54. Li, S.-H.; Zhao, P.; Tian, H.-B.; Chen, L.-H.; Cui, L.-Q. Effect of Grape Polyphenols on Blood Pressure: A Meta-Analysis of Randomized Controlled Trials. *PLoS ONE* **2015**, *10*, e0137665. [CrossRef] [PubMed]
55. Al-Dashti, Y.A.; Holt, R.R.; Stebbins, C.L.; Keen, C.L.; Hackman, R.M. Dietary Flavanols: A Review of Select Effects on Vascular Function, Blood Pressure, and Exercise Performance. *J. Am. Coll. Nutr.* **2018**, *37*, 553–567. [CrossRef]
56. Alañón, M.; Castle, S.; Serra, G.; Lévèques, A.; Poquet, L.; Actis-Goretta, L.; Spencer, J. Acute study of dose-dependent effects of (−)-epicatechin on vascular function in healthy male volunteers: A randomized controlled trial. *Clin. Nutr.* **2020**, *39*, 746–754. [CrossRef]
57. Rodriguez-Mateos, A.; Weber, T.; Skene, S.S.; Ottaviani, J.I.; Crozier, A.; Kelm, M.; Schroeter, H.; Heiss, C. Assessing the respective contributions of dietary flavanol monomers and procyanidins in mediating cardiovascular effects in humans: Randomized, controlled, double-masked intervention trial. *Am. J. Clin. Nutr.* **2018**, *108*, 1229–1237. [CrossRef]
58. Quiñones, M.; Margalef, M.; Arola-Arnal, A.; Muguerza, B.; Miguel, M.; Aleixandre, A. The blood pressure effect and related plasma levels of flavan-3-ols in spontaneously hypertensive rats. *Food Funct.* **2015**, *6*, 3479–3489. [CrossRef]
59. Jennings, A.; Welch, A.A.; Fairweather-Tait, S.J.; Kay, C.; Minihane, A.-M.; Chowienczyk, P.; Jiang, B.; Cecelja, M.; Spector, T.; MacGregor, A.; et al. Higher anthocyanin intake is associated with lower arterial stiffness and central blood pressure in women. *Am. J. Clin. Nutr.* **2012**, *96*, 781–788. [CrossRef] [PubMed]
60. Zhu, Y.; Bo, Y.; Wang, X.; Lu, W.; Wang, X.; Han, Z.; Qiu, C. The Effect of Anthocyanins on Blood Pressure. *Med.* **2016**, *95*, e3380. [CrossRef]
61. Rodriguez-Mateos, A.; Istas, G.; Boschek, L.; Feliciano, R.P.; Mills, E.C.; Boby, C.; Gomez-Alonso, S.; Milenkovic, D.; Heiss, C. Circulating Anthocyanin Metabolites Mediate Vascular Benefits of Blueberries: Insights from Randomized Controlled Trials, Metabolomics, and Nutrigenomics. *J. Gerontol. Ser. A Boil. Sci. Med Sci.* **2019**, *74*, 967–976. [CrossRef] [PubMed]
62. Calfío, C.; Huidobro-Toro, J.P. Potent Vasodilator and Cellular Antioxidant Activity of Endemic Patagonian Calafate Berries (Berberis microphylla) with Nutraceutical Potential. *Molecules* **2019**, *24*, 2700. [CrossRef]
63. Reagan-Shaw, S.; Nihal, M.; Ahmad, N. Dose translation from animal to human studies revisited. *FASEB J.* **2007**, *22*, 659–661. [CrossRef] [PubMed]

Manuscript 2:

Objective:

To release non-soluble phenolic compounds from WL by using an enzyme-assisted extraction to maximize the phenolic yield and their functional properties

Enzyme-assisted extraction to obtain phenolic-enriched wine lees with enhanced bioactivity in hypertensive rats

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Enzyme-Assisted Extraction to Obtain Phenolic-Enriched Wine Lees with Enhanced Bioactivity in Hypertensive Rats

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Abstract: The antihypertensive effect of the soluble fraction of wine lees (WL) from Cabernet variety grapes was recently reported by our group. This blood pressure (BP)-lowering effect was attributed to the presence of flavanols and anthocyanins. In this context, phenolic-enriched wine lees (PWL) could potentially exhibit a stronger bioactivity. Therefore, the aim of this study was to obtain a soluble fraction of WL with increased phenolic content and evaluate its functionality. The PWL were obtained using an enzyme-assisted extraction based on the hydrolysis of WL proteins with Flavourzyme®. They contained 57.20% more total phenolic compounds than WL, with anthocyanins and flavanols being the largest families present. In addition, PWL also showed greater angiotensin-converting enzyme inhibitory and antioxidant activities. Finally, the antihypertensive activity of the PWL was evaluated in spontaneously hypertensive rats. A single dose of 5 mL/kg body weight of PWL showed a greater BP-lowering effect than the one shown by WL. Moreover, this antihypertensive effect was more prolonged than the one produced by the antihypertensive drug Captopril. These results demonstrate that enzymatic protein hydrolysis is a useful method to maximize the extraction of phenolic compounds from WL and to obtain extracts with enhanced functionalities.

Keywords: hypertension; phenolic compounds; hydrolysate; spontaneously hypertensive rats; bioactive compounds; enzymatic hydrolysis; UHPLC; grape by-products

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1. Introduction

Nowadays, it is known that some dietary components play an important role in the prevention of hypertension (HTN) [1]. Thus, an increase of fruit and vegetable intake has been evidenced to reduce the risk of cardiovascular disease (CVD) in hypertensive subjects [2,3]. Their health benefits are attributed to the presence and synergetic effect of different bioactive compounds including fiber, vitamins or phenolic compounds [4]. In the last two decades, phenolic compounds have been widely studied for their beneficial health effects, including antihypertensive activity [5–7].

There are different mechanisms related to the blood pressure (BP)-lowering effect of phenolic compounds. Some of these have been referred to their antioxidant capacity [6,8]. In fact, it has been reported for anthocyanin, flavanol or flavonol rich extracts that the oxidative stress improvement is one of the mechanisms responsible for their antihypertensive properties [9–11]. In addition, phenolic compounds can also act on HTN via the inhibition of angiotensin-converting enzyme (ACE) [12]. This enzyme is key in the BP regulation produced by the renin-angiotensin-aldosterone system (RAAS). In fact, ACE inhibition is usually used in HTN treatment [13,14]. Although synthetic ACE inhibitors such as Enalapril or Captopril are very efficient in reducing BP, in some cases they can cause

unwanted side effects [15,16]. Thus, new antihypertensive compounds, acting on different HTN targets, are being highly investigated, mainly from natural sources. In this regard, the ACE inhibitory (ACEi) activity of different phenolic compounds has been demonstrated in in vitro studies [17]. Phenolics do not reach the potency of drugs commonly used in the treatment of HTN as Captopril but they may be considered as naturally functional compounds since their dietary intake can be as high as 1 g/day [17].

Agri-food by-products have recently emerged as a new source of bioactive compounds [18]. In this regard, winery by-products (seeds, skin, pomace, stems, or lees) have been used to obtain extracts rich in phenolic compounds with antioxidant and/or antihypertensive properties [10,11,19–21]. However, these phenolic compounds can be in the food matrix in free form or covalently bound to soluble or insoluble substances [22]. For example, tannins, phenolic compounds present in winery by-products, form complexes with proteins [23]. Thus, extraction methods must be used to release these bioactive compounds [22]. However, some of these methods produce residual substances and more eco-friendly alternatives are being developed. In this regard, enzyme-assisted extraction has emerged as a green technique that brakes or softens the cell wall to release bioactive compounds [24]. Agri-food by-products contain macromolecules that can be susceptible to hydrolysis such as proteins, cellulose, lignin or hemicellulose [25]. In fact, this reaction has already been used to release phenolic compounds from the cell wall matrix [22]. Specifically, protein hydrolysis has been evidenced to be a good method to release proteins and phenolic compounds [26,27].

Wine lees (WL), a winery by-product, account for 25% of the waste from the wine-making process [28]. The Council Regulation (EEC) No. 337/79 defined this by-product as "the residue that forms at the bottom of recipients containing wine, after fermentation, during storage or after authorized treatments, as well as the residue obtained following the filtration or centrifugation of this product" [29]. WL can be classified according to the stage of the wine making process, as first-fermentation WL, second-fermentation WL, and aging WL (after wine alcoholic fermentation, malolactic fermentation or aging in wood barrels, respectively) [30]. WL can be separated into two phases after centrifugation or filtration of WL, the solid and the liquid phases. The proportion of both phases can vary depending on the type of WL or the procedure to obtain the WL, since water can be added to remove WL from wine tanks. The solid phase is mainly composed of yeasts, insoluble carbohydrates, phenolic compounds, proteins, lignin, tartrates, and other materials such as grape skins [31]. Regarding the soluble phase, this is composed of organic acids, ethanol, and soluble phenolic compounds [19,31]. A previous study from our group evidenced the antihypertensive effect of the soluble fraction of WL from grapes of the Cabernet variety [19]. Phenolic analysis revealed that this fraction was rich in flavanols and anthocyanins. However, since only a centrifugation was performed and no particular extraction method was applied, phenolic compounds from the non-soluble fraction of WL were not extracted. In this regard, the presence of phenolic compounds in the solid fraction, bound to yeast cell walls, has been reported [32]. Enzymatic-assisted extraction using Glucanex® and Mannaway® (β-1, 3 glucanase and β-1,4-mannanase activities, respectively) has been efficiently used in WL as treatment previous to a solid–liquid extraction to release anthocyanins or glycocompounds from the non-soluble fraction [33,34]. Considering this evidence, the aim of this study was to increase the release of WL phenolic compounds by protein hydrolysis of WL to maximize the phenolic yield, improving the valorization of this by-product. The antioxidant, ACEi and antihypertensive activities of the phenolic-enriched WL (PWL) were also evaluated.

2. Materials and Methods

2.1. Chemicals and Reagents

Flavourzyme® 1000 L (EC 3.4.11.1, 500 leucine amino peptidase units (LAPU)/g from *Aspergillus oryzae*) was kindly provided by Novozymes (Bagsværd, Denmark). ACE (EC 3.4.15.1), 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH), picrylsulfonic acid and acetonitrile for HPLC were purchased from Sigma-Aldrich (Madrid, Spain). Captopril

(PubChem CID: 44093) and resveratrol were provided by Santa Cruz Biotechnology (Dallas, TX, USA) and Carl Roth (Karlsruhe, Germany), respectively. Folin–Ciocalteu reagent, coumaric acid, quercetin, gallic acid, catechin, and epicatechin were purchased from Fluka/Sigma-Aldrich. 4-Hydroxybenzoic acid, caffeic acid, procyanidin dimer B2, vanillic acid, and malvidin-3-*O*-glucoside were purchased from Extrasynthése (Lyon, France). Ferulic acid, cyanidin-3-*O*-rutinoside, and peonidin-3-*O*-rutinoside were purchased from PhytoLab (Vestenbergsgreuth, Germany). Analytical grade reagents were always used.

2.2. Preparation of the Wine Lees Hydrolysate

WL were kindly provided by the cellar Grandes Vinos y Viñedos S.A (Cariñena PDO area, Cariñena, Spain). They were collected after racking the red wine (first-fermentation WL), which was made from grapes of Cabernet variety. The preparation of the new PWL was as follows: 10 mL of WL were mixed with the commercial enzyme solution Flavourzyme® (enzyme/substrate ratio, 80 LAPU/g protein). Hydrolysis was carried out at 25 °C for 2 h at pH 4.0 and 250 rpm in a MaxQ Orbital Shaker Thermo Scientific (Thermo Fisher Scientific, Waltham, MA, USA). These enzymatic conditions were set according to a previous study focus on selecting the most appropriate enzymatic preparation and choosing process conditions easily applicable on an industrial scale (data not shown). Reaction was finished adding 1 M HCl to decrease the solution pH to 3. The final volume of the hydrolysate was 11.25 mL. Subsequently, the hydrolysate was centrifuged at 3000× *g* for 15 min at 4 °C to eliminate non-soluble particles and the supernatant (PWL) was collected for analysis. In addition, no-hydrolysis control WL (0 WL) was also subjected to the same procedure but replacing the enzyme volume by Milli-Q water.

2.3. Characterization of Wine Lees

Initial WL consisted of 28.0 ± 6.6% (*w*/*v*) solid, determined by AOAC official method [35], 27.14 ± 1.13% ((*w*/(*w*), protein, determined applying the factor 6.25 to the total nitrogen content measured by the Kjeldahl method [35] and 100.50 ± 1.36 mg GA/g total phenolic compound contents, determined by the Folin–Ciocalteu method according to Iglesias-Carres et al. [36]. Gallic acid (GA) was used as the calibration curve (40–400 mg/L) and mg GA equivalents per mL of WL (mg GAE/mL) were used to express the results. Protein and total polyphenol contents were expressed per gram of dry weight. The analysis was done in triplicate.

The percentage of solid (%, (*w*/(*v*) and total protein contents (%, (*w*/*w*) of both 0 WL and PWL were determined according the AOAC official methods [35] and total phenolic compounds (mg GAE/g of dry weight), were determined by the Folin–Ciocalteu method [36], as already mentioned. The determination of the total amino acid content was carried out following the method described by Mas-Capdevila et al. [37]. Briefly, proteins in the samples suffered both acidic and basic hydrolysis under heat conditions for 1.5 h using increasing temperatures up to 150 °C. Identification and quantification of the amino acids in the samples were performed using high-performance liquid chromatography coupled to electrospray ionization and quadrupole time-of-flight mass spectrometry (UHPLC-Q-TOF/MS). All analyses were done at least in duplicate.

The hydrolysis degree of the PWL was determined by the TNBS (2,4,6-trinitrobenzenes ulfonic acid) method according to Aldler–Nissen through the determination of free α-amino acid groups [38]. Total hydrolysis was performed adding 6 N HCl to the sample, incubating it for 24 h at 110 °C. Leucine was used as a calibration curve. The protein content used to calculate the hydrolysis degree was the one obtained by the Kjeldahl method. The analysis was done in triplicate.

All the analyses were carried out using the samples directly without any extraction method.

2.4. Identification and Quantification of the Phenolic Profile

The phenolic profiles of 0 WL and PWL were also analyzed. Separation, identification, and quantification of anthocyanin and non-anthocyanin phenolic compounds were performed following the method described by López–Fernández–Sobrino et al. [19]. 0 WL and PWL were diluted with water:methanol (50:50, *v:v*) and directly injected in a 1290 UHPLC Infinity II series coupled to a Q-TOF/MS 6550 (Agilent Technologies, Palo Alto, CA, USA). Both positive and negative ionization ([M-H]– or [M-H]+) were used to identify parental ions and fragmentation patterns as shown by López–Fernández–Sobrino et al. [19]. Commercial standards were used to construct a calibration curve to carry out the quantitative analysis of coumaric acid, procyanidin dimer B2, quercetin, catechin, epicatechin, 4-hydroxybenzoic acid, gallic acid, malvidin-3-*O*-glucoside, caffeic acid, vanillic acid, ferulic acid, cyanidin-3-*O*-rutinoside, peonidin-3-*O*-rutinoside, and resveratrol. For the rest of the compounds, the analysis was semi-quantitative using the calibration curve of the commercial standard having the most similar structure to the analyzed compound.

2.5. Antioxidant Activity

DPPH assay was used to determine the antioxidant activity of both samples following the method described by Shen et al. 2010 [39] with some modifications. An aliquot of 500 µL of sample was mixed with 200 µL of DPPH 0.5 mM (in ethanol). After vortexing, the samples were kept in the dark at room temperature for 30 min. Absorbance was measured at 517 nm. Ascorbic acid was used as a positive control, and samples were diluted in ethanol at different concentrations (0–400 µg/mL). A non-lineal fit was performed on the experimental data to calculate the EC_{50}, that is the quantity necessary to reduce 50% of radical scavenging. DPPH radical scavenging activity was expressed as percentage of activity (%) or EC_{50} (µg of dry weight/mL of dissolution). Data are presented as the mean value of three determinations ± SD.

2.6. ACE Inhibitory Activity

ACEi activity was determined according to López–Fernández–Sobrino et al. [19]. The method uses the fluorescence compound *o*-Abz-Gly-*p*-Phe(NO_2)-Pro-OH as ACE substrate. In the presence of an ACE inhibitor, a partial or total loss of fluorescence is produced during the reaction depending on the ACE inhibitory potential of the studied compound. Specifically, in this study, fluorescence was measured at 30 min (37 °C), using 360 nm and 400 nm as excitation and emission wavelengths respectively. ACEi activity was expressed as percentage of inhibition (%) or IC_{50} (µg of dry weight/mL of dissolution). The comparison of the percentage of ACEi activity of both WL was carried out using the same volume of the samples, 0.16 µL. IC_{50} was calculated by constructing a dose-response curve. A linear approximation regression was used. Data are presented as the mean value of three determinations ± SD. Captopril, a synthetic ACE inhibitor, was used to validate the methodology.

2.7. Antihypertensive Effect

Male spontaneously hypertensive rats (SHR) (17–20 weeks old, weighing 350–400 g) were purchased from Charles River Laboratories España S.A. (Barcelona, Spain). Rats were singly housed in a temperature-controlled animal quarter (22 °C) with a 12 h light/dark period. During the experiment, they had free access to standard chow (Panlab A04, Panlab, Barcelona, Spain) and to water.

After a 10-day adaptation period and two weeks of a training period, animals were given a single dose of the different tested compounds between 9 and 10 a.m. by oral gavage. A dose of 5 mL/kg body weight (bw) of PWL and 0 WL was administered to SHR. Captopril (50 mg/kg bw, a known antihypertensive drug) and tap water were used as controls (positive and negative, respectively).

BP was measured in the SHR before and after (2, 4, 6, 8, 24, 48, and 72 h) administration of treatments, using the tail-cuff method and the LE 5002 system (Letica, Hospitalet,

Barcelona, Spain) according to Quiñones et al. [40]. Prior to BP measurements and to facilitate the detection of the tail artery pulsations, SHR were kept at 38 °C for 10 min. Changes in systolic and diastolic BP (SBP and DBP, respectively) were expressed as the differences in these variables before and after the administration for each treatment. Data are shown as the mean values ± standard error of mean (SEM) for a minimum of six experiments. BP measurements were recorded by the same person in a peaceful environment to minimize stress-induced variations.

Experimental in vivo studies were carried out following the European Communities Council Directive (86/609/EEC). In addition, the protocol was reviewed and approved by the Animal Ethics Review Committee for Animal Experimentation of the Universitat Rovira i Virgili and further approved by Generalitat de Catalunya (permission number 10780).

2.8. Statistical Analysis

Differences between 0 WL and the PWL in their amino acid content, total phenolic content, phenolic profile, antioxidant activity, and ACEi activity were analyzed by Student's T-test. Two-way analysis of variance (two-way ANOVA) and Tukey test as post hoc were used to detect differences between treatments in BP over the evaluated time. One-way ANOVA was used to analyze the BP treatment differences at a specific time point. Differences between compounds were considered significant when $p < 0.05$. GraphPad Prism 7.04 for Windows (GraphPad Software, San Diego, CA, USA) was used to perform the statistical analysis. Grubbs' test was used to detect outliers.

3. Results

3.1. Wine Lees Composition

Samples were characterized according to their humidity, total protein, total amino acid, total phenolic content, and hydrolysis degree. Solid contents of 0 WL and PWL were 2.49 ± 0.05% and 2.78 ± 0.16%, respectively. Total protein contents of the 0 WL and PWL were 26.08 ± 0.60 and 27.50 ± 0.71%, respectively. Total amino acid compositions of the 0 WL and PWL are shown in Table 1. The amino acid content of the 0 WL was 24.83 mg/g of dry product, with Pro being the major amino acid found. Regarding PWL, total amino acid concentration was 1.7 times higher (41.20 mg/g of dry product) than the one shown by the WL without hydrolysis. An increase in the content of Pro, Leu, Ile and Val (1.4, 6.6, 5.6 and 5.5 times higher, respectively) was observed in the hydrolysate compared to the 0 WL. In addition, PWL also contained Trp, while this amino acid residue was not found in the 0 WL. The hydrolysis degree of the PWL was 7.61 ± 0.65%.

Total phenolic content of PWL, measured by the Folin–Ciocalteu method, was 160.06 ± 0.32 mg GAE/g, significantly higher than the one observed by 0 WL (148.03 ± 0.48 mg GAE/g).

3.2. Determination of the Phenolic Profile in Wine Lees Samples

Phenolic profiles of the 0 WL and PWL were determined. As example, Figure 1 shows the PWL phenolic profile of non-anthocyanin (Figure 1A) and anthocyanin (Figure 1B) analyzed by UHPLC-(ESI)-Q-TOF-MS. The total amount of phenolic compounds in the PWL was significantly higher than the one shown by 0 WL (15.59 mg/g and 24.50 mg/g, respectively) (Figure 2A). This increase was observed in all phenolic families following this order: anthocyanins > flavonols > flavanols > phenolic acids > stilbenes (Figure 2A).

Table 1. Total amino acid content of 0 WL and PWL by UHPLC-Q-TOF/MS.

Amino Acids	0 WL (μg/g)	PWL (μg/g)
Alanine	706.3 ± 62.8	854.5 ± 70.1
Arginine	110.5 ± 12.5	195.2 ± 19.9
Asparagine	273.6 ± 59.5	417.6 ± 91.0
Aspartate	307.9 ± 37.4	702.9 ± 78.3
Glutamate	1086.1 ± 265.5	1278.4 ± 325.8
Glutamine	N.D	N.D
Glycine	345.5 ± 65.2	442.1 ± 68.8
Histidine	75.6 ± 9.3	135.4 ± 7.2
Isoleucine	358.8 ± 54.7	2014.6 ± 256.2 **
Leucine	435.8 ± 74.4	2857.6 ± 445.7 **
Lysine	143.7 ± 28.2	238.4 ± 46.6
Phenylalanine	64.9 ± 11.7	189.9 ± 34.8
Proline	19,828.7 ± 1230.8	28,623.8 ± 1761.1 **
Serine	517.0 ± 188.6	1091.9 ± 348.0
Threonine	326.2 ± 87.7	720.5 ± 181.0
Tryptohan	N.D	52.9 ± 18.6 *
Tyrosine	46.8 ± 7.2	152.8 ± 19.9
Valine	181.7 ± 29.4	1187.0 ± 172.4 *
Methionine	25.0 ± 7.6	46.8 ± 13.1
Cystine	N.D	N.D
Hydroxiproline	N.D	N.D
TOTAL	24,834.1	41,202.3

N.D: no detected. Data are expressed as mean (μg/g of dry sample) ± SD. Statistical differences by Student's T-test between control wine lees (0 WL) and phenolic-enriched wine lees (PWL) are indicated as (*) when $p < 0.05$ and (**) when $p < 0.01$.

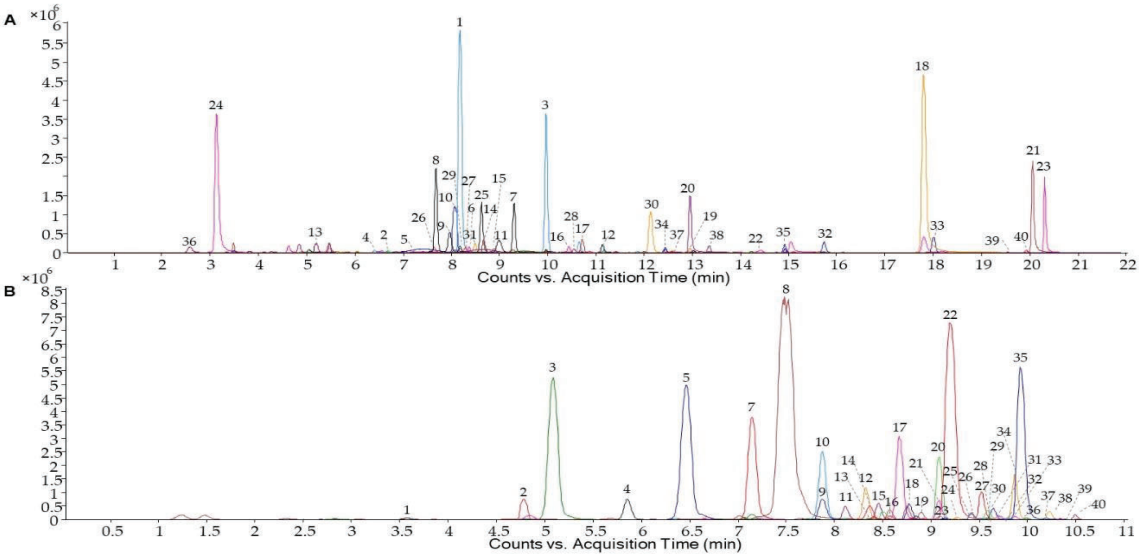

Figure 1. Phenolic profile of wine lees hydrolysate. Extracted ion chromatograms (EIC) of non-anthocyanin (**A**) and anthocyanin (**B**) phenolic compounds analyzed by UHPLC-(ESI-)-Q-TOF-MS and UHPLC-(ESI+)-Q-TOF-MS, respectively. Identified individual compounds are numbered according to Tables 2 and 3.

Table 2. Non-anthocyanin characterization by UHPLC-(ESI-)-Q-TOF-MS in 0 WL and PWL.

	Compound	R.T. (min)	[M-H]-	Fragment (*m*/*z*)	0 WL (μg/g)	PWL (μg/g)
	Flavanols					
1	Catechin	8.17	289.0718		2681.20 ± 19.20	3289.60 ± 20.80 *
2	Catechin gallate [a]	6.66	441.0827	289.07209	18.00 ± 0.40	16.40 ± 0.40 **
3	Epicatechin	9.96	289.0718		1035.60 ± 6.00	1242.00 ± 6.80 *
4	(Epi)catechin *O*-glucoside iso1 [b]	6.55	451.1246	289.0721	20.80 ± 0.00	22.80 ± 0.00 *
5	(Epi)catechin *O*-glucoside iso2 [b]	7.41	451.1246	289.0721	10.80 ± 0.00	16.40 ± 0.00 *
6	(Epi)catechin *O*-glucoside iso3 [b]	8.37	451.1246	289.0721	62.80 ± 1.20	70.80 ± 1.20 *
7	Procyanidin dimer B2	9.30	577.1387	289.0733	514.40 ± 0.40	634.00 ± 0.40 **
8	Procyanidin dimer iso1 [c]	7.68	577.1387	289.0733	1020.80 ± 6.00	1178.00 ± 6.40 *
9	Procyanidin dimer iso2 [c]	7.97	577.1387	289.0733	276.80 ± 2.00	334.40 ± 2.00 *
10	Procyanidin dimer iso3 [c]	8.18	577.1387	289.0733	85.20 ± 0.80	86.80 ± 0.40
11	Procyanidin dimer iso4 [c]	8.99	577.1387	289.0733	211.20 ± 0.00	262.80 ± 0.00 *
12	Procyanidin dimer iso5 [c]	11.14	577.1387	289.0733	70.40 ± 0.80	106.00 ± 1.20
13	Procyanidin trimer iso1 [c]	5.46	865.2016	577.1369	206.00 ± 4.00	251.60 ± 4.40
14	Procyanidin trimer iso2 [c]	8.67	865.2016	577.1369	184.00 ± 10.40	274.00 ± 13.60 *
15	Procyanidin trimer iso3 [c]	8.89	865.2016	577.1369	72.40 ± 0.40	84.00 ± 0.40 *
16	Procyanidin trimer iso4 [c]	10.55	865.2016	577.1369	68.80 ± 3.60	89.60 ± 0.40 *
17	Procyanidin trimer iso5 [c]	10.71	865.2016	577.1369	177.60 ± 3.60	265.60 ± 4.40 *
	Flavonols					
18	Quercetin	17.80	301.0372		888.40 ± 4.80	1954.40 ± 9.20 **
19	Quercetin-3-*O*-glucoside [d]	13.00	463.0904	301.0361	19.20 ± 0.40	48.40 ± 1.20 **
20	Quercetin-3-*O*-glucuronide [d]	12.95	477.0702	301.0369	115.20 ± 0.80	255.20 ± 1.20 **
21	Kaempferol [d]	20.07	285.0405		319.60 ± 1.60	632.00 ± 2.40 **
22	Kaempferol-3-*O*-glucuronide [d]	14.22	461.0763	285.0412	27.60 ± 0.40	66.00 ± 0.80 **
23	Isorhamnetin [d]	20.31	315.0531		203.20 ± 2.40	481.60 ± 5.20 **

Table 2. *Cont.*

	Compound	R.T. (min)	[M-H]-	Fragment (*m*/*z*)	0 WL (μg/g)	PWL (μg/g)
	Phenolic acids					
24	Gallic acid	3.13	169.0193		2734.80 ± 93.60	3496.80 ± 106.40
25	Caffeic acid	8.63	179.0401		70.80 ± 0.80	97.20 ± 1.20 *
26	Caffeic acid *O*-glucoside iso1 [e]	7.64	341.0878	179.0350	22.40 ± 0.80	23.20 ± 0.80
27	Caffeic acid *O*-glucoside iso2 [e]	8.29	341.0878	179.0350	22.40 ± 1.20	19.60 ± 0.80
28	p-Coumaric acid	10.65	163.0439		28.00 ± 0.40	117.60 ± 1.20 **
29	4-Hydroxybenzoic acid	8.17	137.0243		58.40 ± 2.00	66.40 ± 2.00
30	Ferulic acid	12.00	193.0506		13.20 ± 0.40	18.80 ± 0.40 *
31	Vanillic acid	8.51	167.0350		76.40 ± 2.40	90.40 ± 2.80
	Stilbenes					
32	trans-Resveratrol [f]	15.73	227.0714		120.40 ± 0.80	164.00 ± 0.80 **
33	Resveratrol iso1 [f]	18.00	227.0714		66.00 ± 0.40	152.40 ± 0.80 **
34	Resveratrol *O*-glucoside iso1 [f]	12.44	389.1242	227.0721	30.00 ± 0.40	56.40 ± 0.80 **
35	Resveratrol *O*-glucoside iso2 [f]	14.92	389.1242	227.0721	88.00 ± 1.60	138.00 ± 2.40 **
36	Piceatannol [f]	2.59	243.0663	203.0727	124.40 ± 2.00	136.80 ± 1.60 *
37	Piceatannol 3-*O*-glucoside iso1 [f]	12.89	405.1208	243.0670	5.60 ± 0.00	9.60 ± 0.40 *
38	Piceatannol 3-*O*-glucoside iso2 [f]	13.15	405.1208	243.0670	2.40 ± 0.00	4.80 ± 0.00 *
39	Viniferin-iso1 [f]	19.53	453.1344	116.9291	4.40 ± 0.00	6.40 ± 0.00 *
40	Viniferin-iso2 [f]	19.92	453.1344	116.9291	20.80 ± 0.40	29.20 ± 0.80 *

Abbreviations: Retention time (R.T.); Control WL (0 WL); Phenolic-enriched wine lees (PWL). [a] Quantified using catechin calibration curve. [b] Quantified using epicatechin calibration curve. [c] Quantified using procyanidin dimer B2 calibration curve. [d] Quantified using quercetin calibration curve. [e] Quantified the caffeic acid calibration curve. [f] Quantified using resveratrol calibration curve. Statistical differences by Student's *t*-test between 0 WL and PWL are indicated (*) when $p < 0.05$ and (**) when $p < 0.01$.

Table 3. Anthocyanin characterization by UHPLC-(ESI+)-Q-TOF-MS in 0 WL and PWL.

	Anthocyanins	R.T. (min)	[M-H]+	Fragment (*m*/*z*)	0 WL (μg/g)	PWL (μg/g)
1	Gallocatechin-malvidin-3-glucoside dimer [a]	3.58	797.2035		1.60 ± 0.04	4.46 ± 0.11 *
2	Malvidin-3-glucoside-(epi)catechin [a]	4.84	781.1974		16.67 ± 0.13	19.27 ± 0.14 *
3	Delphinidin-3-glucoside [b]	5.06	465.1028	303.0511	184.88 ± 1.82	573.34 ± 5.64 **
4	Cyanidin-3-glucoside [b]	5.85	449.1078	287.0531	21.72 ± 0.81	48.11 ± 1.79 *
5	Petunidin-3-glucoside [c]	6.47	479.1184	317.0669	195.11 ± 2.26	513.73 ± 5.96 **
6	Petunidin-3-glucoside-pyruvic acid [c]	7.05	547.1082	385.0547	1.61 ± 0.04	3.54 ± 0.09 *
7	Peonidin-3-glucoside [c]	7.14	463.1235	301.0717	151.78 ± 2.50	312.22 ± 5.14 *
8	Malvidin-3-glucoside [a]	7.48	493.1341	331.0843	1780.76 ± 20.01	3334.75 ± 37.47 *
9	Peonidin-3-glucoside-pyruvic acid [c]	7.81	531.1133	369.0607	0.93 ± 0.03	1.90 ± 0.06 *
10	Delphinidin-(6-acetyl)-3-glucoside [b]	7.87	507.1133	303.0496	47.82 ± 0.90	151.91 ± 2.85 *
11	Visitin A (malvidin-3-glucoside-pyruvic acid) [a]	8.11	561.1239	399.0730	14.80 ± 0.13	31.02 ± 0.27 **
12	Visitin B (malvidin-3-glucoside-acetaldehyde) [a]	8.32	517.1341	355.0826	24.08 ± 0.59	70.03 ± 1.73 *
13	Malvidin-3-glucoside-ethyl-(epi)catechin [a]	8.40	809.2287		4.62 ± 0.03	8.91 ± 0.05 *
14	Cyanidin-(6-acetyl)-3-glucoside [b]	8.45	491.1184	491.1189	15.21 ± 0.23	33.29 ± 0.50 *
15	Acetylvisitin A [a]	8.50	603.1344	399.0718	13.66 ± 0.44	19.65 ± 0.63 *
16	Malvidin-3-glucoside-ethyl-(epi)catechin [a]	8.57	809.2287		17.54 ± 0.24	31.82 ± 0.43 *
17	Petunidin-(6-acetyl)-3-glucoside [c]	8.66	521.1378	317.0667	55.81 ± 1.87	150.18 ± 5.04 **
18	Malvidin-3-glucoside-ethyl-(epi)catechin [a]	8.75	809.2287		22.92 ± 0.73	42.10 ± 1.34 **
19	Acetylvisitin B [a]	8.77	559.1446	355.0813	14.50 ± 0.44	36.86 ± 1.12 *
20	Peonidin-(6-acetyl)-3-glucoside [c]	9.08	505.1341	301.0714	55.47 ± 1.24	124.75 ± 2.80 *
21	Delphinidin-(6-coumaroyl)-3-glucoside [b]	9.08	611.1395	303.0508	14.72 ± 0.27	48.48 ± 0.89 *
22	Malvidin-(6-acetyl)-3-glucoside [a]	9.13	535.1446	331.0836	727.35 ± 0.84	1503.53 ± 1.74 **
23	Coumaroylvisitin A [a]	9.29	707.1607	399.0718	2.88 ± 0.07	6.18 ± 0.15 *
24	Malvidin-(6-caffeoyl)-3-glucoside [a]	9.41	655.1657	331.0808	5.99 ± 0.27	13.98 ± 0.63 *
25	Cyanidin-(6-coumaroyl)-3-glucoside [b]	9.42	595.1446	287.0560	5.01 ± 0.16	13.77 ± 0.43 *
26	Catechin-ethyl-malvidin-3-acetylglucoside dimer [a]	9.43	851.2511		10.30 ± 0.31	19.05 ± 0.56 **
27	Petunidin-(6-coumaroyl)-3-glucoside [c]	9.52	625.1552	317.0662	21.11 ± 0.36	63.19 ± 1.08 *
28	Pinotin A (malvidin-3-glucoside-vinylcatechol) [a]	9.53	625.1552	463.0998	23.80 ± 0.51	71.25 ± 1.52 *
29	Malvidin-glucoside-vinyl-catechin [a]	9.56	805.1974		1.46 ± 0.03	4.23 ± 0.09 *
30	Coumaroylvisitin B [a]	9.58	663.1708	355.0822	8.53 ± 0.28	23.20 ± 0.76 *
31	Malvidin-3-glucoside-vinylguaiacol [a]	9.63	639.1708	331.0823	10.07 ± 0.20	27.49 ± 0.54 *
32	Catechin-ethyl-malvidin-3-coumaroylglucoside dimer [a]	9.70	955.2785		5.95 ± 0.11	13.77 ± 0.25 *
33	Catechin-ethyl-malvidin-3-acetylglucoside dimer [a]	9.81	851.2511		1.93 ± 0.06	4.27 ± 0.14 *
34	Peonidin-(6-coumaroyl)-3-glucoside [c]	9.87	609.1603	301.0716	38.72 ± 1.08	85.15 ± 2.37 *
35	Malvidin-(6-coumaroyl)-3-glucoside [a]	9.92	639.1708	331.0823	274.63 ± 0.60	768.26 ± 1.69 **
36	Malvidin-glucoside-vinyl-catechin [a]	9.99	805.1974		1.58 ± 0.02	3.74 ± 0.05 **
37	Acetyl-pinotin A [a]	10.19	667.1657		0.08 ± 0.00	0.26 ± 0.00 *
38	Malvidin 3-*O*-glucoside 4-vinylphenol (Pigment A) [a]	10.22	609.1603	447.1079	7.40 ± 0.08	19.53 ± 0.21 **
39	Catechin-ethyl-malvidin-3-coumaroylglucoside dimer [a]	10.33	955.2785		1.14 ± 0.01	3.06 ± 0.02 *
40	Malvidin acetyl 3-*O*-glucoside 4-vinylphenol (Acetyl-pigment A) [a]	10.50	651.1708	447.1076	4.46 ± 0.17	10.42 ± 0.39 *

Abbreviations: Retention time (R.T.); Control WL (0 WL); Phenolic-enriched wine lees (PWL). [a] Quantified using calibration curve of malvidin-3-*O*-glucoside. [b] Quantified using calibration curve of cyanidin-3-*O*-rutinoside. [c] Quantified using calibration curve of peonidin-3-*O*-rutinoside. Statistical differences by Student *t*-test between 0 WLE and PWL are indicated (*) when $p < 0.05$ and (**) when $p < 0.01$.

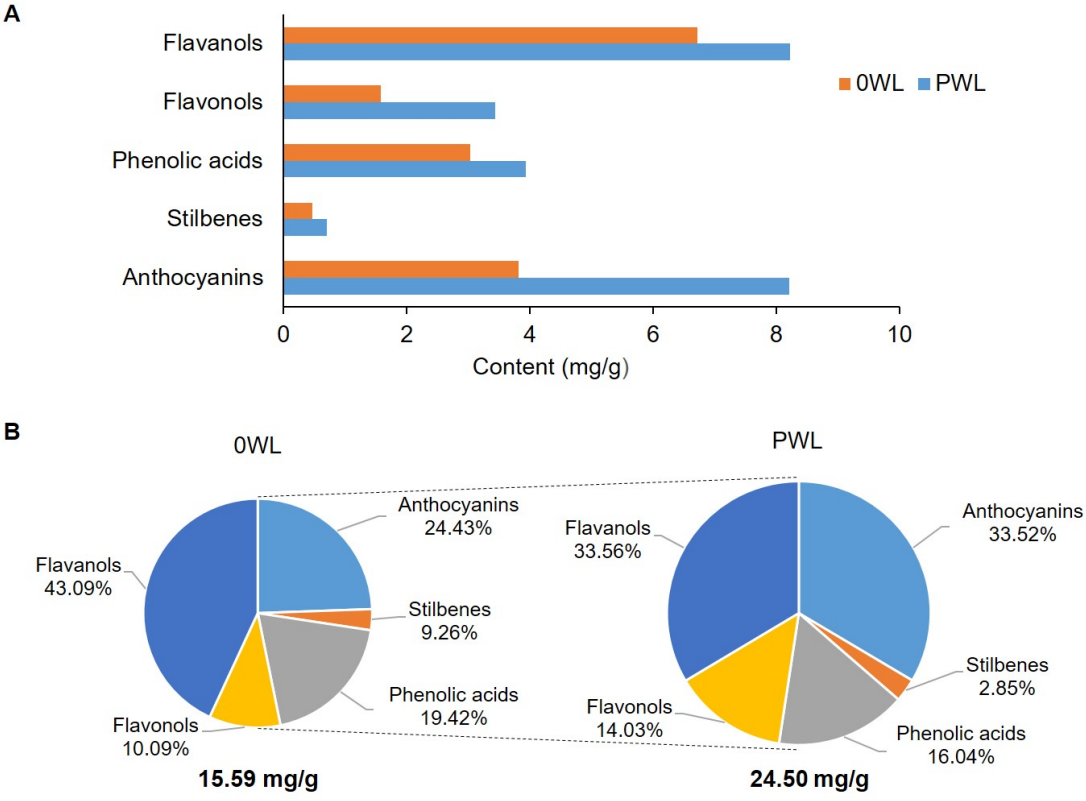

Figure 2. Flavanols, flavonols, phenolic acids, stilbenes, and anthocyanins content analyzed by UHPLC-(ESI)-Q-TOF-MS of control wine lees (0 WL) and phenolic-enriched wine lees (PWL) (**A**). Total phenolic compounds and percentages of the different families of phenolic compounds in 0 WL and PWL (**B**).

Flavanols were the main family of phenolic compounds in the 0 WL, representing 43.1 % of total phenolic content, followed by anthocyanins (24.4 %, Figure 2B). However, in the PWL, flavanols and anthocyanins were found in the same proportion (33.6 % and 33.5 % of the total phenolic compounds, respectively; Figure 2B). Tables 2 and 3 show the individual phenolic compounds content in both 0 WL and PWL. A total amount of 40 non-anthocyanins and 40 anthocyanins were identified by UHPLC-ESI-Q-TOF-MS, respectively. The major components in PWL were gallic acid (3.5 mg/g), catechin (3.3 mg/g), malvidin-3-glucoside (3.3 mg/g), procyanidin dimers (2.6 mg/g), quercetin (2.0 mg/g), malvidin-(6-acetyl)-3-glucoside (1.5 mg/g), and epicatechin (1.2 mg/g). Protein hydrolysis released large amounts of anthocyanins, especially delphinidin-3-glucoside, petunidin-3-glucoside, malvidin-(6-acetyl)-3-glucoside, malvidin-(6-coumaroyl)-3-glucoside, and malvidin-3-glucoside. Within the family of flavanols, catechin, epicatechin, and procyanidin dimer B2 were those that showed the greatest increase after hydrolysis. All the identified compounds of the flavonol family suffered an increase between 97.7–152.1% in their content after hydrolysis (Table 2), quercetin being the major flavonol. Regarding phenolic acids, the main increase after the hydrolysis was observed in p-coumaric acid. While, resveratrol and its derivatives were the stilbenes that presented the highest release after hydrolysis of WL.

3.3. Antioxidant and ACEi Activities of the Wine Lees

Figure 3A shows an example of a dose–response curve of both wine lees used to determine their antioxidant activity as DPPH radical scavenging activity (%). EC_{50} values were 12.89 ± 0.72 μg/mL and 8.14 ± 0.81 μg/mL in the 0 WL and PWL, respectively (Figure 3B). ACEi activity of the samples was also evaluated. The PWL showed a higher ACEi activity than the 0 WL (53.6 and 35.7 %, respectively at 0.59 mg/mL) (Figure 3C). According to these percentages, the IC_{50} value was lower in the hydrolysate (0.63 ± 0.02 mg/mL) in comparison with the control 0 WL (0.74 ± 0.06 mg/mL) (Figure 3D).

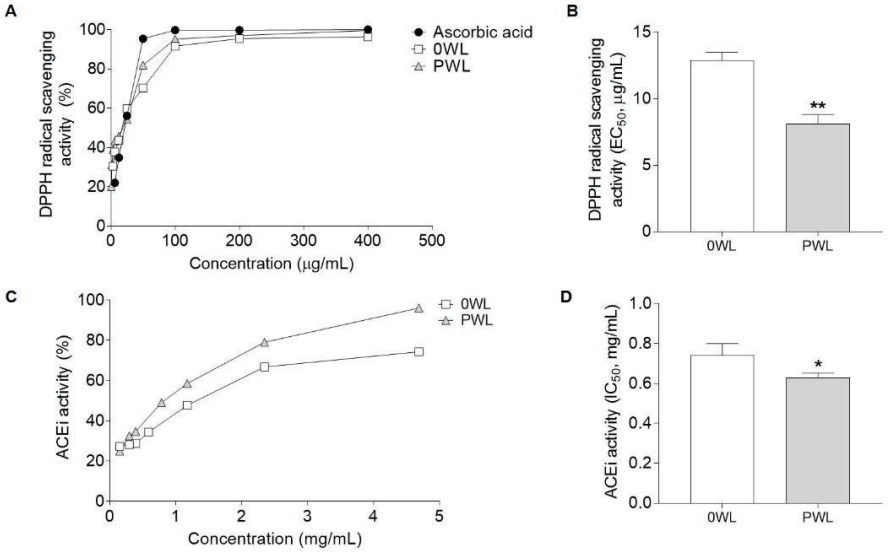

Figure 3. Dose–response curves of DPPH radical scavenging activity (%, **A**) and angiotensin-converting enzyme inhibitory (ACEi) activity (%, **C**) for control wine lees (0 WL) and phenolic-enriched wine lees (PWL). (**B**) shows DPPH radical scavenging activity as EC_{50} and (**D**) show ACEi activity as IC_{50}. Values are the average of three replicates ± SD. Statistical differences by Student's T-test between 0 WL and PWL are indicated (*) when $p < 0.05$ and (**) when $p < 0.01$.

3.4. Antihypertensive Activity of the Wine Lees

The antihypertensive effect of both 0 WL and PWL was evaluated in SHR at 5 mL/kg bw. This dose is equivalent to the doses of 18.50 and 20.01 mg GAE/kg bw and 1.95 and 3.06 mg/kg bw of phenolic compounds determined by UHPLC-(ESI)-Q-TOF-MS for 0 WL and PWL, respectively.

Water and Captopril (50 mg/kg bw) were used as negative and positive controls, respectively. Prior to oral treatment administration, animals presented SBP and DBP values characteristic of the hypertensive condition (205.6 ± 3.6 mmHg and 164.7 ± 10.0 mmHg, respectively). As shown in Figure 4, the administration of water to animals did not significantly change either SBP or DBP during the experiment. However, Captopril produced a decrease in SBP in SHR after 2 h of its administration, its maximum being at 6 h post-administration (−41.3 ± 3.2 mm Hg) (Figure 4A). The same pattern of SBP drop occurred after administration of 0 WL. A maximum SBP reduction of −32.5 ± 2.3 mmHg was observed at 6 h post-administration. Regarding the effect of the PWL, the maximum SBP decrease was observed at 4–6 h post-administration (−32.3 ± 1.5 and −35.6 ± mmHg, respectively). Notably, the BP lowering effect of PWL was greater than the antihypertensive effect shown by 0 WL. Interestingly, no significant differences were found between the antihypertensive effect of Captopril and PWL (two-way ANOVA). In addition, the duration of the effect was different. The antihypertensive effect produced by PWL was more

prolonged than the one showed by 0 WL or Captopril. Basal SBP levels were recovered at 24, 48, and 72 h post-administration for 0 WL, Captopril and PWL, respectively.

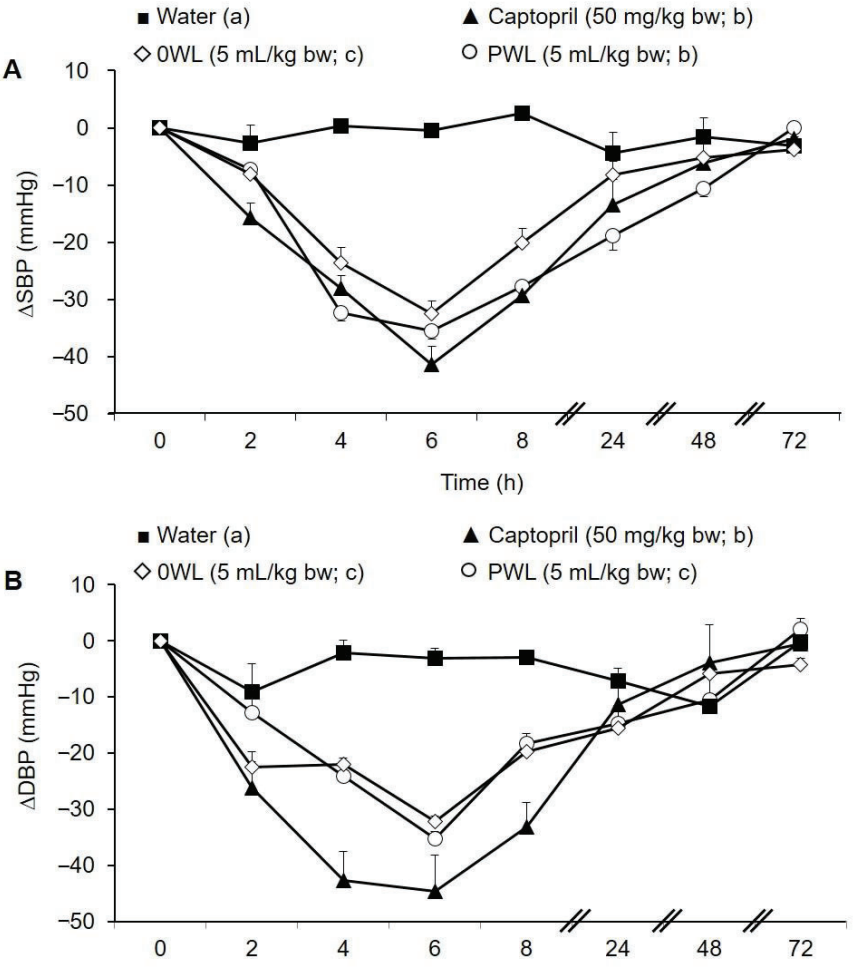

Figure 4. Changes in systolic (**A**) and diastolic (**B**) blood pressure (SBP and DBP, respectively) in spontaneously hypertensive rats after the administration of the following: water (■), Captopril (50 mg/kg bw; ▲), control wine lees (5 mL/kg bw; 0 WL ◊), and phenolic-enriched wine lees (5 mL/kg bw; PWL ○). Data are expressed as the mean ± SEM (n = 6). Different letters in the legend represent significant differences ($p < 0.05$). p was estimated by two-way ANOVA.

Regarding DBP values, Captopril administration also produced a significant reduction, reaching a maximum decrease 4 h post-administration (−42.7 ± 5.2 mmHg) (Figure 4B). The antihypertensive effect was observed up to 24 h after Captopril administration. 0 WL and the hydrolysate also produced a significant antihypertensive effect from 2 h post-administration, reaching the maximum DBP values at 6 h (−32.2 ± 5.2 and −35.2 ± 1.2 mmHg, respectively). No differences in DBP were found between the two WL samples under study.

4. Discussion

Winery by-products such as seeds, pomace or skin, have shown to be rich in phenolic compounds [41]. In fact, these by-products of the winemaking process have been used to obtain extracts, rich in phenolic compounds with beneficial properties such as antioxidant

or antihypertensive activities [9–11,19,21]. However, some of the phenolic compounds are located inside the cells (and in some cases bound to different compounds such as proteins) making it difficult to remove them from the food matrix and therefore decreasing their full potential [42]. Recently, our group demonstrated the antihypertensive effect of a soluble fraction of WL from Cabernet variety after its acute administration (5 mL/kg bw) to SHR [19]. This effect was attributed to their high content of phenolic compounds. Nevertheless, this fraction did not contain the non-soluble phenolic compounds since no method of extraction was applied.

Different methodologies have been described to facilitate the release of phenolic compounds from the matrix [43]. Enzyme-assisted extraction has emerged as a more eco-friendly alternative to conventional solvent-based extraction methods [24]. In addition, enzymatic hydrolysis is a more efficient extraction method that increases the extractability of phenolic compounds from the food matrix (including those non-extractable as proanthocyanidins) using neither organic solvents nor any other toxic chemicals. Moreover, it is a cost-efficient method to convert by-products into new and safe food ingredients or products with enhanced nutritional value and functionality [25]. This technique is based on the use of enzymes to break down different components of the cell to enhance the release of phenolic compounds [26].

Therefore, since WL with a higher content in phenolic compounds could potentially exhibit a stronger functionality, enzyme-assisted extraction was used to release the phenolic compounds present in the non-soluble fraction of WL. The enzymatic preparation Flavourzyme® was used because it is widely used for protein hydrolysis in industrial and research applications [44]. To the best of our knowledge, it is the first time that this method has been used in WL to extract phenolic compounds. The results showed changes in the amino acid composition of PWL with respect to 0 WL, indicating that protein hydrolysis occurred. This protein hydrolysis was expected since Flavourzyme® is an enzymatic preparation with peptidase activity. The amino acid residues Pro, Ile, Leu, Val, and Trp were those that increased their content in the WL hydrolysate (PWL) more than the 0 WL control. In addition, the hydrolysis of WL also produced an increase in the phenol content quantified by the Folin–Ciocalteu method and by UHPLC-(ESI-)-Q-TOF-MS, which increased 57.20%.

The specific locations of phenolic compounds, their type of bonding and possible physical entrapment in WL is largely unknown at present. Methods such as ultrasound [45,46] and microwave [47] assisted extraction have been used to enhance the extraction of active compounds from many vegetable matrixes, including WL. In this study, enzyme-assisted extraction using Flavourzyme® was demonstrated to be a useful technique to release phenolic compounds from WL. In concordance with these results, Senevirathne et al. showed that the hydrolysis of blueberries, with different enzymes, including the enzymatic preparation Flavourzyme®, released phenolic compounds [48]. In the process of wine maceration, the phenolic compounds from grapes are transferred to wine. However, a high proportion of these compounds remains in winery by-products such as WL. The presence of phenolic compounds in WL is due to the great adsorption capacity of the yeast cell wall used in the winemaking process [49]. The phenolic profile present in the WL depends on the type of grapes and other factors involved in the winemaking [50]. The WL used in this study were obtained from grapes of the variety Cabernet, and their functionality was linked to the high amount of anthocyanins and flavanols present in these lees [19]. In this study, the hydrolysis of WL caused an increase in content of all the phenolic compound families. However, anthocyanins and flavonols were the categories of polyphenols whose concentration increased more in the PWL compared to WL, doubling both their content. Regarding the phenolic profile, PWL were especially rich in flavanols (33.56%) and anthocyanin (33.52%), the flavanols being catechin and epicatechin and the anthocyanins delphinidin-3-glucoside, petunidin-3-glucoside, and malvidin-(6-acetyl)-3-glucoside, the most increased by the hydrolysis process. Thus, the application of this enzymatic-assisted

extraction method to WL produced PWL rich in gallic acid, catechin, malvidin-3-glucoside, procyanidin dimers, quercetin, malvidin-(6-acetyl)-3-glucoside, and epicatechin.

Phenolic compounds exhibit numerous beneficial effects. Among them, their radical scavenging capacity stands out, giving them antioxidant properties [5]. Thus, since the hydrolysis causes a release of phenolic compounds, PWL should show a higher antioxidant capacity than 0 WL. The results in this study showed that both samples presented antioxidant activity although, as expected, the hydrolysate presented a more potent antioxidant capacity. This improvement in the antioxidant effect could be linked to the higher content of anthocyanins, since a good correlation between anthocyanin compounds and antioxidant activity has been reported for different WL extracts [51]. Both DPPH radical scavenging activities are higher than previously reported for other winery by-products, i.e., a grape stem phenolic extract [20,52]. Along with other beneficial effects, the antioxidant properties of phenolic compounds have also been related to the improvement of HTN [53,54]. In fact, one of the underlying mechanisms involved in the endothelial damage is oxidative stress, which increases the contractibility of the vascular smooth muscle and promotes its proliferation [55]. Furthermore, free radicals in the endothelium can scavenge nitric oxide (NO), avoiding NO-dependent vasodilation and stimulating the production of pro-inflammatory agents and endothelium-derived vasoconstrictor factors [56].

The RAAS is another key factor in the maintenance of arterial BP. One of the main components of this system is the ACE. In fact, the inhibition of this enzyme is usually used to select antihypertensive compounds. ACE inhibitors such as Enalapril or Captopril are usually used as treatment against HTN [57]. ACE catalyzes the conversion of angiotensin I into the potent vasoconstrictor angiotensin II [58]. The ACEi properties of the soluble fraction of the WL have been previously reported [19]. Since the ACEi activity of phenolic compounds has also been reported [17], the hydrolysate PWL, as expected, exerted a more potent ACEi activity than its control counterpart 0 WL.

Subsequently, the antihypertensive effect of the hydrolysate was evaluated *in vivo*, using SHR as a hypertensive animal model. This is the one well-established of the most used experimental models of HTN, very similar to the HTN found in humans [59]. The administration of 5 mL/kg bw of the hydrolysate produced a potent antihypertensive effect, reaching the maximum SBP decrease between 4 and 8 h post-administration (-32.29 ± 1.46 mmHg). In addition, this effect was long lasting, remaining until 48 h post administration. A similar time response of the SBP-lowering effect has been shown by hydrolysates obtained from other food by-products such as garlic protein hydrolysates, which exhibited the maximum effect between 4–6 h post-administration [60]. It is noteworthy that, since it lasted longer, the antihypertensive effect of the PWL was more potent than the one shown by the commercially available drug Captopril or the 0 WL. Considering that HTN is a chronic pathology that needs lifetime treatment, the use of strategies with long lasting antihypertensive effects is always desired. Therefore, the antihypertensive properties of PWL seem more favorable.

It is known that phenolic compounds have numerous effects on health, including antihypertensive effects [61]. The improvement of the antihypertensive effect of the WL using enzymatic hydrolysis is linked to the release of phenolic compounds. In this sense, intake of compounds rich in anthocyanins has shown to be associated with a lower arterial stiffness and with a reduction in BP [62–64]. More specifically, malvidin-3-glucoside, present in high amounts in the PWL, has shown to be a potent vasodilator [65]. Furthermore, compounds rich in flavanols such as catechin, epicatechin or procyanidins, have been shown to have antihypertensive effect in rats and humans [9,66,67]. In our previous study, we also related the WL bioactivity to the content of flavanols and anthocyanins [19], which is in line with the present results. Moreover, PWL showed an increment in flavonols, especially in quercetin. It is known that flavonols, such as quercetin, improve vascular endothelial function and reduce BP [68,69]. In this regard, several studies have reported BP- lowering effects of quercetin in several animal models of HTN (10 mg/kg/day for 4 or 5 weeks) and in hypertensive patients (730 mg/day for 28 days) [70,71]. In addition, PWL

also presented high levels of gallic acid. Kang et al. showed that the administration of 40 mg/kg bw of this phenolic acid to SHR produced an antihypertensive effect similar to the observed by Captopril [72].

Taken together, the data obtained in this study show that the hydrolysis of WL is a good strategy to release phenolic compounds, specifically anthocyanins and flavonols, and the results strongly suggest that these phenolic compounds enhance the antihypertensive effect of WL. Moreover, taking into account that WL also contain ethanol, which can reach values similar to wine of at least 8.5% [73], an improvement of WL antihypertensive activity using dealcoholized WL might be attained.

5. Conclusions

The phenolic extraction from the solid fraction of WL via enzymatic hydrolysis is a useful method to obtain phenolic-enriched WL with enhanced antioxidant, ACEi and antihypertensive properties. As a result, a WL fraction rich in flavanols, anthocyanins, phenolic acids, and flavonols was obtained. The phenolic composition is considered of special relevance since multiple beneficial effects have been linked to these phenolic families, including antihypertensive properties. Therefore, this study opens the door to the wine industry for the commercial use of PWL due to its high content of phenolic compounds. Moreover, the enzyme-assisted extraction of phenolic compounds also modified the amino acid content of WL, indicating that protein hydrolysis was taking place. Therefore, the release of other molecules with antihypertensive properties, different to phenolic compounds, such as bioactive peptides should not be ruled out. Moreover, as in addition to phenolic compounds, WL contains ethanol, it would be of interest to investigate the BP-lowering effect of the dealcoholized WL samples. Finally, further studies would be needed to confirm the long-term effect of PWL in hypertensive and normotensive rats.

6. Patents

Patent application "Wine lees, derivatives thereof and their uses": application number EP20382358.8 and and PCT/EP2021/053051.

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References

1. Li, B.; Li, F.; Wang, L.; Zhang, D. Fruit and Vegetables Consumption and Risk of Hypertension: A Meta-Analysis. *J. Clin. Hypertens* **2016**, *18*, 468–476. [CrossRef]
2. Adebawo, O.; Salau, B.; Ezima, E.; Oyefuga, O.; Ajani, E.; Idowu, G.; Famodu, A.; Osilesi, O. Fruits and Vegetables Moderate Lipid Cardiovascular Risk Factor in Hypertensive Patients. *Lipids Health Dis.* **2006**, *5*, 1–4. [CrossRef] [PubMed]
3. Alissa, E.M.; Ferns, G.A. Dietary Fruits and Vegetables and Cardiovascular Diseases Risk. *Crit. Rev. Food Sci. Nutr.* **2017**, *57*, 1950–1962. [CrossRef]
4. Liu, R.H. Health-Promoting Components of Fruits and Vegetables in the Diet. *Adv. Nutr.* **2013**, *4*, 384S. [CrossRef] [PubMed]
5. Ruskovska, T.; Maksimova, V.; Milenkovic, D. Polyphenols in Human Nutrition: From the In Vitro Antioxidant Capacity to the Beneficial Effects on Cardiometabolic Health and Related Inter-Individual Variability—An Overview and Perspective. *Br. J. Nutr.* **2020**, *123*, 241–254. [CrossRef]
6. Yahfoufi, N.; Alsadi, N.; Jambi, M.; Matar, C. The Immunomodulatory and Anti-Inflammatory Role of Polyphenols. *Nutrients* **2018**, *10*, 1618. [CrossRef] [PubMed]
7. Spagnuolo, C.; Russo, M.; Bilotto, S.; Tedesco, I.; Laratta, B.; Russo, G.L. Dietary Polyphenols in Cancer Prevention: The Example of the Flavonoid Quercetin in Leukemia. *Ann. N. Y. Acad. Sci.* **2012**, *1259*, 95–103. [CrossRef]
8. Ansari, M.Y.; Ahmad, N.; Haqqi, T.M. Oxidative Stress and Inflammation in Osteoarthritis Pathogenesis: Role of Polyphenols. *Biomed. Pharmacother.* **2020**, *129*, 110452. [CrossRef]
9. Pons, Z.; Margalef, M.; Bravo, F.I.; Arola-Arnal, A.; Muguerza, B. Grape Seed Flavanols Decrease Blood Pressure via Sirt-1 and Confer a Vasoprotective Pattern in Rats. *J. Funct. Foods* **2016**, *24*, 164–172. [CrossRef]
10. Rasines-Perea, Z.; Ky, I.; Cros, G.; Crozier, A.; Teissedre, P.-L. Grape Pomace: Antioxidant Activity, Potential Effect against Hypertension and Metabolites Characterization after Intake. *Diseases* **2018**, *6*, 60. [CrossRef] [PubMed]
11. De Costa, G.F.; Ognibene, D.T.; Da Costa, C.A.; Teixeira, M.T.; Da Silva Cristino Cordeiro, V.; De Bem, G.F.; Moura, A.S.; De Castro Resende, A.; De Moura, R.S. Vitis Vinifera L Grape Skin Extract Prevents Development of Hypretension and Altered Lipid Profile in Sponteneously Hypertensive Tats: Role of Oxidative Stress. *Prev. Nutr. Food Sci.* **2020**, *25*, 25–31. [CrossRef]
12. Guerrero, L.; Castillo, J.; Quiñones, M.; Garcia-Vallvé, S.; Arola, L.; Pujadas, G.; Muguerza, B. Inhibition of Angiotensin-Converting Enzyme Activity by Flavonoids: Structure-Activity Relationship Studies. *PLoS ONE* **2012**, *7*, e49493. [CrossRef]
13. Foëx, P.; Sear, J. Hypertension: Pathophysiology and Treatment. *Contin. Educ. Anaesth. Crit. Care Pain* **2004**, *4*, 71–75. [CrossRef]
14. Sparks, M.A.; Crowley, S.D.; Gurley, S.B.; Mirotsou, M.; Coffman, T.M. Classical Renin-Angiotensin System in Kidney Physiology. *Compr. Physiol.* **2014**, *4*, 1201–1228. [PubMed]
15. Snauwaert, E.; Vande Walle, J.; De Bruyne, P. Therapeutic Efficacy and Safety of ACE Inhibitors in the Hypertensive Paediatric Population: A Review. *Arch. Dis. Child.* **2017**, *102*, 63–71. [CrossRef] [PubMed]
16. Alderman, C.P. Adverse Effects of the Angiotensin-Converting Enzyme Inhibitors. *Ann. Pharm.* **1996**, *30*, 55–61. [CrossRef] [PubMed]
17. Margalef, M.; Bravo, F.I.; Arola-Arnal, A.; Muguerza, B. Natural Angiotensin Converting Enzyme (ACE) Inhibitors with Antihypertensive Properties. In *Natural Products Targeting Clinically Relevant Enzymes*; Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, Germany, 2017; pp. 45–67.
18. Ferreira-Santos, P.; Zanuso, E.; Genisheva, Z.; Rocha, C.M.R.; Teixeira, J.A. Green and Sustainable Valorization of Bioactive Phenolic Compounds from Pinus By-Products. *Molecules* **2020**, *25*, 2931. [CrossRef]
19. López-Fernández-Sobrino, R.; Soliz-Rueda, J.R.; Margalef, M.; Arola-Arnal, A.; Suárez, M.; Bravo, F.I.; Muguerza, B. ACE Inhibitory and Antihypertensive Activities of Wine Lees and Relationship among Bioactivity and Phenolic Profile. *Nutrients* **2021**, *13*, 679. [CrossRef]
20. Queiroz, M.; Oppolzer, D.; Gouvinhas, I.; Silva, A.M.; Barros, A.I.R.N.A.; Domínguez-Perles, R. New Grape Stems' Isolated Phenolic Compounds Modulate Reactive Oxygen Species, Glutathione, and Lipid Peroxidation In Vitro: Combined Formulations with Vitamins C and E. *Fitoterapia* **2017**, *120*, 146–157. [CrossRef]
21. Quiñones, M.; Guerrero, L.; Suarez, M.; Pons, Z.; Aleixandre, A.; Arola, L.; Muguerza, B. Low-Molecular Procyanidin Rich Grape Seed Extract Exerts Antihypertensive Effect in Males Spontaneously Hypertensive Rats. *Food Res. Int.* **2013**, *51*, 587–595. [CrossRef]
22. Shahidi, F.; Yeo, J.D. Insoluble-Bound Phenolics in Food. *Molecules* **2016**, *21*, 1216. [CrossRef]
23. Ozdal, T.; Capanoglu, E.; Altay, F. A Review on Protein-Phenolic Interactions and Associated Changes. *Food Res. Int.* **2013**, *51*, 954–970. [CrossRef]
24. Panja, P. Green Extraction Methods of Food Polyphenols from Vegetable Materials. *Curr. Opin. Food Sci.* **2018**, *23*, 173–182. [CrossRef]
25. Radenkovs, V.; Juhnevica-Radenkova, K.; Górnaś, P.; Seglina, D. Non-Waste Technology through the Enzymatic Hydrolysis of Agro-Industrial by-Products. *Trends Food Sci. Technol.* **2018**, *77*, 64–76. [CrossRef]
26. Landbo, A.K.; Meyer, A.S. Enzyme-Assisted Extraction of Antioxidative Phenols from Black Currant Juice Press Residues (Ribes Nigrum). *J. Agric. Food Chem.* **2001**, *49*, 3169–3177. [CrossRef] [PubMed]
27. Ribeiro, B.D.; Barreto, D.W.; Coelho, M.A.Z. Enzyme-Enhanced Extraction of Phenolic Compounds and Proteins from Flaxseed Meal. *ISRN Biotechnol.* **2013**, *2013*, 1–6. [CrossRef]

28. De Iseppi, A.; Lomolino, G.; Marangon, M.; Curioni, A. Current and Future Strategies for Wine Yeast Lees Valorization. *Food Res. Int.* **2020**, *137*, 109352. [CrossRef]
29. Union, C. of the E. Council Regulation (EEC). *No 337/79 of 5 February 1979 on the Common Organization of the Market in Wine*; EEC: Brussels, Belgium, 1979; pp. 1–47.
30. Jara-Palacios, M.J. Wine Lees as a Source of Antioxidant Compounds. *Antioxidants* **2019**, *8*, 45. [CrossRef]
31. Pérez-Bibbins, B.; Torrado-Agrasar, A.; Salgado, J.M.; Oliveira, R.P.d.S.; Domínguez, J.M. Potential of Lees from Wine, Beer and Cider Manufacturing as a Source of Economic Nutrients: An Overview. *Waste Manag.* **2015**, *40*, 72–81. [CrossRef]
32. Ye, Z.; Harrison, R.; Cheng, V.; Bekhit, A. Wine Making By-Products. In *Valorization of Wine Making By-Products*; CRC Press: Boca Raton, FL, USA, 2015; pp. 73–116.
33. Romero-Díez, R.; Rodrigues, L.; Rodríguez-Rojo, S.; Cocero, M.; Matias, A. Pretreatment Effect on Anthocyanin Extraction Kinetics from Different Wine Lees. In Proceedings of the 13th International Conference Renewable Resources, Wroclaw, Poland, 7–9 June 2017.
34. De Iseppi, A.; Marangon, M.; Vincenzi, S.; Lomolino, G.; Curioni, A.; Divol, B. A Novel Approach for the Valorization of Wine Lees as a Source of Compounds Able to Modify Wine Properties. *LWT* **2021**, *136*, 110274. [CrossRef]
35. Cunniff, P. Official Method 969.33 of AOAC International. In *Official Methods of Analysis of AOAC Internationa*; Association of Official Analytical Chemists: Arlington, VA, USA, 1995.
36. Iglesias-Carres, L.; Mas-Capdevila, A.; Sancho-Pardo, L.; Bravo, F.I.; Mulero, M.; Muguerza, B.; Arola-Arnal, A. Optimized Extraction by Response Surface Methodology Used for the Characterization and Quantification of Phenolic Compounds in Whole Red Grapes (Vitis Vinifera). *Nutrients* **2018**, *10*, 1931. [CrossRef] [PubMed]
37. Mas-Capdevila, A.; Iglesias-Carres, L.; Arola-Arnal, A.; Suarez, M.; Muguerza, B.; Bravo, F.I. Long-Term Administration of Protein Hydrolysate from Chicken Feet Induces Antihypertensive Effect and Confers Vasoprotective Pattern in Diet-Induced Hypertensive Rats. *J. Funct. Foods* **2019**, *55*, 28–35. [CrossRef]
38. Adler-Nissen, J. Determination of the Degree of Hydrolysis of Food Protein Hydrolysates by Trinitrobenzenesulfonic Acid. *J. Agric. Food Chem.* **1979**, *27*, 1256–1262. [CrossRef]
39. Shen, Q.; Zhang, B.; Xu, R.; Wang, Y.; Ding, X.; Li, P. Antioxidant Activity In Vitro of the Selenium-Contained Protein from the Se-Enriched Bifidobacterium Animalis 01. *Anaerobe* **2010**, *16*, 380–386. [CrossRef] [PubMed]
40. Quiñones, M.; Miguel, M.; Muguerza, B.; Aleixandre, A. Effect of a Cocoa Polyphenol Extract in Spontaneously Hypertensive Rats. *Food Funct.* **2011**, *2*, 649–653. [CrossRef] [PubMed]
41. Teixeira, A.; Baenas, N.; Dominguez-Perles, R.; Barros, A.; Rosa, E.; Moreno, D.; Garcia-Viguera, C. Natural Bioactive Compounds from Winery By-Products as Health Promoters: A Review. *Int. J. Mol. Sci.* **2014**, *15*, 15638–15678. [CrossRef]
42. Manach, C.; Scalbert, A.; Morand, C.; Rémésy, C.; Jiménez, L. Polyphenols: Food Sources and Bioavailability. *Am. J. Clin. Nutr.* **2004**, *79*, 727–747. [CrossRef]
43. Hogervorst, J.C.; Miljić, U.; Puškaš, V. Extraction of Bioactive Compounds from Grape Processing By-Products. In *Handbook of Grape Processing By-Products*; Elsevier: Amsterdam, The Netherlands, 2017; pp. 105–135.
44. Merz, M.; Eisele, T.; Berends, P.; Appel, D.; Rabe, S.; Blank, I.; Stressler, T.; Fischer, L. Flavourzyme, an Enzyme Preparation with Industrial Relevance: Automated Nine-Step Purification and Partial Characterization of Eight Enzymes. *J. Agric. Food Chem.* **2015**, *63*, 5682–5693. [CrossRef]
45. Tao, Y.; Wu, D.; Zhang, Q.A.; Sun, D.W. Ultrasound-Assisted Extraction of Phenolics from Wine Lees: Modeling, Optimization and Stability of Extracts during Storage. *Ultrason. Sonochem.* **2014**, *21*, 706–715. [CrossRef]
46. Barcia, M.T.; Pertuzatti, P.B.; Rodrigues, D.; Gómez-Alonso, S.; Hermosín-Gutiérrez, I.; Godoy, H.T. Occurrence of Low Molecular Weight Phenolics in Vitis Vinifera Red Grape Cultivars and Their Winemaking By-Products from São Paulo (Brazil). *Food Res. Int.* **2014**, *62*, 500–513. [CrossRef]
47. Pérez-Serradilla, J.A.; Luque de Castro, M.D. Microwave-Assisted Extraction of Phenolic Compounds from Wine Lees and Spray-Drying of the Extract. *Food Chem.* **2011**, *124*, 1652–1659. [CrossRef]
48. Senevirathne, M.; Kim, S.-H.; Jeon, Y.-J. Protective Effect of Enzymatic Hydrolysates from Highbush Blueberry (*Vaccinium Corymbosum L.*) against Hydrogen Peroxide-Induced Oxidative Damage in Chinese Hamster Lung Fibroblast Cell Line. *Nutr. Res. Pr.* **2010**, *4*, 183. [CrossRef]
49. Morata, A.; Gómez-Cordovés, M.C.; Colomo, B.; Suárez, J.A. Cell Wall Anthocyanin Adsorption by Different Saccharomyces Strains during the Fermentation of Vitis Vinifera L. Cv Graciano Grapes. *Eur. Food Res. Technol.* **2005**, *220*, 341–346. [CrossRef]
50. Mena, P.; Ascacio-Valdés, J.A.; Gironés-Vilaplana, A.; Del Rio, D.; Moreno, D.A.; García-Viguera, C. Assessment of Pomegranate Wine Lees as a Valuable Source for the Recovery of (Poly)Phenolic Compounds. *Food Chem.* **2014**, *145*, 327–334. [CrossRef]
51. Romero-Díez, R.; Rodríguez-Rojo, S.; Cocero, M.J.; Duarte, C.M.M.; Matias, A.A.; Bronze, M.R. Phenolic Characterization of Aging Wine Lees: Correlation with Antioxidant Activities. *Food Chem.* **2018**, *259*, 188–195. [CrossRef]
52. Bei, Q.; Chen, G.; Liu, Y.; Zhang, Y.; Wu, Z. Improving Phenolic Compositions and Bioactivity of Oats by Enzymatic Hydrolysis and Microbial Fermentation. *J. Funct. Foods* **2018**, *47*, 512–520. [CrossRef]
53. Sorriento, D.; De Luca, N.; Trimarco, B.; Iaccarino, G. The Antioxidant Therapy: New Insights in the Treatment of Hypertension. *Front. Physiol.* **2018**, *9*, 258. [CrossRef]

54. Komes, D.; Belščak-Cvitanović, A.; Horžić, D.; Rusak, G.; Likić, S.; Berendika, M. Phenolic Composition and Antioxidant Properties of Some Traditionally Used Medicinal Plants Affected by the Extraction Time and Hydrolysis. *Phytochem. Anal.* **2011**, *22*, 172–180. [CrossRef] [PubMed]
55. Taniyama, Y.; Griendling, K.K. Reactive Oxygen Species in the Vasculature: Molecular and Cellular Mechanisms. *Hypertension* **2003**, *39*, 788–801. [CrossRef] [PubMed]
56. Campia, U.; Tesauro, M.; Cardillo, C. Human Obesity and Endothelium-Dependent Responsiveness. *Br. J. Pharmacol.* **2012**, *165*, 561–573. [CrossRef]
57. Tzakos, A.G.; Naqvi, N.; Comporozos, K.; Pierattelli, R.; Theodorou, V.; Husain, A.; Gerothanassis, I.P. The Molecular Basis for the Selection of Captopril Cis and Trans Conformations by Angiotensin I Converting Enzyme. *Bioorganic Med. Chem. Lett.* **2006**, *16*, 5084–5087. [CrossRef] [PubMed]
58. Griendling, K.K.; Murphy, T.J.; Alexander, R.W. Molecular Biology of the Renin-Angiotensin System. *Circulation* **1993**, *87*, 1816–1828. [CrossRef] [PubMed]
59. Pinto, Y.M.; Paul, M.; Ganten, D. Lessons from Rat Models of Hypertension: From Goldblatt to Genetic Engineering. *Cardiovasc. Res.* **1998**, *39*, 77–88. [CrossRef]
60. Gao, X.; Xue, Z.; Ma, Q.; Guo, Q.; Xing, L.; Santhanam, R.K.; Zhang, M.; Chen, H. Antioxidant and Antihypertensive Effects of Garlic Protein and Its Hydrolysates and the Related Mechanism. *J. Food Biochem.* **2020**, *44*, e13126. [CrossRef]
61. Godos, J.; Vitale, M.; Micek, A.; Ray, S.; Martini, D.; Del Rio, D.; Riccardi, G.; Galvano, F.; Grosso, G. Dietary Polyphenol Intake, Blood Pressure, and Hypertension: A Systematic Review and Meta-Analysis of Observational Studies. *Antioxidants* **2019**, *8*, 152. [CrossRef]
62. Jennings, A.; Welch, A.A.; Fairweather-Tait, S.J.; Kay, C.; Minihane, A.-M.; Chowienczyk, P.; Jiang, B.; Cecelja, M.; Spector, T.; Macgregor, A.; et al. Higher Anthocyanin Intake Is Associated with Lower Arterial Stiffness and Central Blood Pressure in Women. *Am. J. Clin. Nutr.* **2012**, *96*, 781–788. [CrossRef] [PubMed]
63. Kent, K.; Charlton, K.E.; Jenner, A.; Roodenrys, S. Acute Reduction in Blood Pressure Following Consumption of Anthocyanin-Rich Cherry Juice May Be Dose-Interval Dependant: A Pilot Cross-over Study. *Int. J. Food Sci. Nutr.* **2016**, *67*, 47–52. [CrossRef]
64. Zhu, Y.; Bo, Y.; Wang, X.; Lu, W.; Wang, X.; Han, Z.; Qiu, C. The Effect of Anthocyanins on Blood Pressure. *Medicine* **2016**, *95*, e3380. [CrossRef] [PubMed]
65. Calfío, C.; Huidobro-Toro, J.P. Potent Vasodilator and Cellular Antioxidant Activity of Endemic Patagonian Calafate Berries (Berberis Microphylla) with Nutraceutical Potential. *Molecules* **2019**, *24*, 2700. [CrossRef]
66. Alañón, M.E.; Castle, S.M.; Serra, G.; Lévèques, A.; Poquet, L.; Actis-Goretta, L.; Spencer, J.P.E. Acute Study of Dose-Dependent Effects of (−)-Epicatechin on Vascular Function in Healthy Male Volunteers: A Randomized Controlled Trial. *Clin. Nutr.* **2020**, *39*, 746–754. [CrossRef] [PubMed]
67. Rodriguez-Mateos, A.; Weber, T.; Skene, S.S.; Ottaviani, J.I.; Crozier, A.; Kelm, M.; Schroeter, H.; Heiss, C. Assessing the Respective Contributions of Dietary Flavanol Monomers and Procyanidins in Mediating Cardiovascular Effects in Humans: Randomized, Controlled, Double-Masked Intervention Trial. *Am. J. Clin. Nutr.* **2018**, *108*, 1229–1237. [CrossRef] [PubMed]
68. Lin, X.; Han, T.; Fan, Y.; Wu, S.; Wang, F.; Wang, C. Quercetin Improves Vascular Endothelial Function through Promotion of Autophagy in Hypertensive Rats. *Life Sci.* **2020**, *258*, 118106. [CrossRef] [PubMed]
69. Marunaka, Y.; Marunaka, R.; Sun, H.; Yamamoto, T.; Kanamura, N.; Inui, T.; Taruno, A. Actions of Quercetin, a Polyphenol, on Blood Pressure. *Molecules* **2017**, *22*, 209. [CrossRef] [PubMed]
70. Edwards, R.L.; Lyon, T.; Litwin, S.E.; Rabovsky, A.; Symons, J.D.; Jalili, T. Quercetin Reduces Blood Pressure in Hypertensive Subjects. *J. Nutr.* **2007**, *137*, 2405–2411. [CrossRef]
71. Perez-Vizcaino, F.; Duarte, J.; Jimenez, R.; Santos-Buelga, C.; Osuna, A. Antihypertensive Effects of the Flavonoid Quercetin. *Pharm. Rep.* **2009**, *61*, 67–75. [CrossRef]
72. Kang, N.; Lee, J.-H.; Lee, W.; Ko, J.-Y.; Kim, E.-A.; Kim, J.-S.; Heu, M.-S.; Kim, G.H.; Jeon, Y.-J. Gallic Acid Isolated from Spirogyra Sp. Improves Cardiovascular Disease through a Vasorelaxant and Antihypertensive Effect. *Environ. Toxicol. Pharm.* **2015**, *39*, 764–772. [CrossRef] [PubMed]
73. OIV. *Código Prácticas Enológicas*; OIV: Paris, France, 2016; Volume 33.

Manuscript 3:

Objective:

To identify the ACE inhibitors and/or antihypertensive peptides released during the enzyme-assisted extraction process carried out in WL.

Identification of novel antihypertensive peptides from wine lees hydrolysate

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Keywords: hypertension, protein hydrolysates, ACE inhibitory peptides, mass spectrometry, spontaneously hypertensive rats

Abbreviations: ACE, angiotensin-converting enzyme; ACEI, angiotensin-converting enzyme inhibitory; CVD, cardiovascular disease; BP, blood pressure; RP-HPLC, reverse phase high performance liquid chromatography; HPLC-MS, high performance liquid chromatography- mass spectrometry; SBP, systolic blood pressure; DBP, diastolic blood pressure; SHR, spontaneously hypertensive rats; PWL, phenol-enriched wine lees

ABSTRACT

Enzymatic-assisted extraction using Flavourzyme has been demonstrated to be a useful methodology to obtain wine lees enriched in phenolic compounds and with enhanced antihypertensive activity. However, in addition of phenolic compounds, this enzymatic hydrolysis of WL can also release other bioactive compounds such as peptides, which could also be involved in the antihypertensive effect. In this study, we investigate the presence of antihypertensive peptides in the WL hydrolysate. Peptides were separated into fractions by ultrafiltration and RP-HPLC according to their molecular size and hydrophobicity, respectively. Next, peptide identification by nano-LC-(Orbitrap)MS/MS was performed in the fractions with the highest ACEi activities. Six peptides were identified, three of them showing ACEi (IC_{50}) values lower than 20 μM. The antihypertensive effect of all the identified peptides was evaluated in spontaneously hypertensive rats. Peptides FKTTDQQTRTTVA, NPKLVTIV, TVTNPARIA, LDSPSEGRAPG and LDSPSEGRAPGAD exhibited antihypertensive activity when were administered at an oral dose of 10 mg/kg bw, confirming that they could contribute to the blood pressure-lowering effect of the WL hydrolysate. In addition, these peptides could be used as functional ingredients with antihypertensive effects, although it would be necessary to perform clinical studies to demonstrate their efficiency in humans.

1. Introduction

Hypertension (HTN) is one of the main risk factor for cardiovascular diseases (CVD), having a high prevalence in the global population (*Hypertension*, 2019; Mills et al., 2016). The control of CVD risk factors and the reduction of the incidence of these diseases have become a major global goal (*WHO | Strategic Priorities*, 2016). In this regard, blood pressure (BP) reduction is one of the strategies used to achieve these objectives (Unger et al., 2020). One of the main mechanisms in regulating BP and vascular tone is the renin-angiotensin-aldosterone system, in which the angiotensin-converting enzyme (ACE) is a key player. ACE is considered an important target for the treatment of HTN since the inhibition of its activity leads to BP reduction (Foëx & Sear, 2004; Sparks et al., 2014). Although synthetic ACE inhibitors are effective for HTN, it can exert different adverse side effects in some patients (Alderman, 1996; Snauwaert et al., 2017). Thus, natural ACE inhibitors with antihypertensive properties are investigated as alternative to synthetic drugs. Moreover, these compounds could be especially useful for pre-hypertensive subjects, since this population is not usually clinically treated (Margalef et al., 2017).

Peptides from dietary proteins exert a wide variety of functional activities such as ACE inhibitory (ACEi), anti-inflammatory or antihypertensive activities (Hernández-Ledesma et al., 2011; Majumder & Wu, 2014; Margalef et al., 2017; Yoshikawa, 2015). Bioactive peptides can be released from the native protein during food processing (fermentation or curing) or gastrointestinal (GI) digestion (Toldrá et al., 2018). However, most of them are usually obtained by an intentionally and controlled hydrolysis of food-derived proteins using specific proteases or microorganisms (Toldrá et al., 2018). In the last decade, agri-food industry by-products have emerged as an alternative protein source (Margalef et al., 2017). In this regard, different by-products generated during the processing of foods from vegetal and animal origin, have been successfully used to obtain peptides with a wide range of bioactivities such as ACEi or

antihypertensive (Kuba et al., 2005; Shobako et al., 2018; Toldrá et al., 2020; Yathisha et al., 2019). This has led to the valorisation of these by-products allowing for their recycling and making agri-food industries more environmentally friendly (Darewicz et al., 2014; Mora et al., 2014; Musee et al., 2007).

Wine making is one of the most important agricultural activities in the world (258 mhL of wine estimated for 2020 harvest) (OIV, 2020). This activity produces tons of by-products annually, including pomace (pulp, stems, seeds, skins), lees, organic acids, CO_2 and water (Maicas & Mateo, 2020). Wine lees (WL) represent between 2–6% of the produced wine volume and between 14-25% of all winery by-products (Dimou et al., 2015, 2016). According to EEC regulation No. 337/79, wine lees (WL) "is the residue formed at the bottom of recipients containing wine, after fermentation, during storage or after authorized treatments, as well as the residue obtained following the filtration or centrifugation of this product". WL contain yeast cells, insoluble carbohydrates, organic acids, ethanol, phenolic compounds, proteins, inorganic salts, lignin, pulp and other parts of the grape (Jara-Palacios, 2019). In a recent study, our group reported that acute administration of the soluble fraction of WL from grapes of Cabernet variety exerted a potent antihypertensive effect in spontaneously hypertensive rats (SHR) (López-Fernández-Sobrino, Soliz-Rueda, et al., 2021). Moreover, in other study, enzymatic-assisted extraction was demonstrated to be a useful methodology to maximize the release of phenolic compounds from WL and to obtain extracts with enhanced functionalities. Specifically, WL hydrolysis was performed with Flavourzyme®, which is a preparation widely used for protein hydrolysis in industrial and research applications (Merz et al., 2015). After the hydrolysis, in addition to phenolic compounds, a higher total amino acid residues content was also observed in hydrolyzed WL compared to control WL, indicating peptides or amino acid release. Therefore, it cannot be ruled out the involvement of some of these peptides in the antihypertensive effect of this hydrolysate. In this regard,

different studies have reported generation of ACEi and antihypertensive peptides from different protein sources after using Flavourzyme® (He et al., 2013; Lee & Hur, 2017).

Therefore, considering all these evidence, the aim of this study was to investigate the presence of antihypertensive peptides in the WL hydrolysate.

2. Materials and methods

2.1. Chemicals and reagents

Flavourzyme® 1000 L (EC 3.4.11.1, 500 LAPU/g from *Aspergillus oryzae*) was kindly provided by Novozymes (Bagsværd, Denmark). Angiotensin converting enzyme (ACE, EC 3.4.15.1) and the HPLC grade solvents acetonitrile and trifluoroacetic acid were purchased from Sigma-Aldrich (Madrid, Spain). *O*-aminobenzoylglicil-*p*-nitrofenilalanilprolina (o-Abz-Gly-p-Phe(NO_2)-Pro-OH) and Captopril (PubChem CID: 44093) were provided by Bachem Feinchemikalien (Bubendorf, Switzerland) and Santa Cruz Biotechnology (Dallas, TX, USA), respectively. The peptides (FKTTDQQTRTTVA, NPKLVTIV, TVTNPARIA, PAGELHP, LDSPSEGRAPG, LDSPSEGRAPGAD, purity grade ≥ 95 %) were synthetized by Caslo Laboratory ApS (Caslo, Kongens Lyngby, Denmark). All other chemical solvents used were of analytical grade.

2.2. Wine lees hydrolysate

WL were kindly provided by the cellar Grandes Vinos y Viñedos S.A, located in the Cariñena P.O.D area (Spain). They were collected after racking the red wine, which was made from grapes of Cabernet variety. WL hydrolysate was carried out following the method described by López-Fernández-Sobrino et al.(López-Fernández-Sobrino, Margalef, et al., 2021). Briefly, enzymatic hydrolysis of WL was carried out with Flavourzyme® (enzyme/substrate ratio, 80 LAPU/g protein) for 2 h at 25 ºC, pH 4.0 and 150 rpm in a MaxQ Orbital Shaker (Thermo Fisher Scientific, Waltham, MA, USA). Hydrolysis was stopped by lowering the pH of the solution to 3. Then, solution was centrifuged at 3,000 × *g* for 15 min at 4 ºC

and supernatant was collected and kept at -20 ºC for further analysis. The soluble fraction of WL was also obtained by centrifugation in the same conditions as a control (control WL).

Total protein content, measured by Kjeldahl method using 6.25 as factor, hydrolysis degree, determined by TNBS method, and ACEi activity of hydrolyzed WL were 0.69 ± 0.01 % (w/w), 7.61 ± 0.65 % and 0.63 ± 0.02 mg of dry weight/mL (IC_{50} value), respectively (López-Fernández-Sobrino, Margalef, et al., 2021). Table S1 shows the amino acid profile of the hydrolysate (adapted from López-Fernández-Sobrino, Margalef, et al., 2021b). Amino acids were characterised by using high-performance liquid chromatography coupled to electrospray ionization and quadrupole time-of-flight mass spectrometry (UHPLC-Q-TOF/MS). All analyses were done at least in duplicate.

2.3. Isolation and identification of ACEi peptides from wine lees and the hydrolysate

2.3.1. Step I: Peptides separation based on their molecular weight and hydrophobicity

First, the <3 kDa peptide fractions of control WL and hydrolysed WL were obtained by ultrafiltration using a hydrophilic membrane with cut-off value of 3 kDa (Centripep, Amicon, Inc., Beverly, MA, USA). The obtained fractions were freeze-dried and kept at -20 ºC until use.

Regarding WL hydrolysate, peptides in the <3 kDa fraction (dissolved in Milli-Q water) were separated by a semipreparative reversed-phase liquid chromatography (RP-HPLC), using the same equipment and methodology described by Bravo et al., 2019 with some modifications. Specifically, in the current study, peptide separation was performed in gradient mode as follows: initial conditions 0 % B; 0-23.5 % B in 39.2 min; 23.5-90 % B in 9.8 min; 90-0 % B in 1 min. A 10 min post-run was required for column re-equilibration. Peptides were collected in different subfractions, which were freeze-dried and kept at -

20 ºC until use. ACEi activity was determined in all subfractions (methodology described in 2.4 section) and the most active fractions were selected for peptide identification as it is described in section 2.3.2.

Non hydrolyzed WL was used as control and peptide identification was directly carried out using the <3KDa fraction as further peptide separation was not necessary.

2.3.2. Step II: Peptide identification by nanoLC–LTQ-Orbitap HPLC-MS

Before mass spectrometry analysis, peptides in the <3kDa fraction of the control WL and in the RP-HPLC subfractions of the hydrolyzed WL (reconstituted in Milli-Q water) were purified by using an HLB SPE (Waters, USA) starting from 500 µL of sample, and eluted in 75:25 (v:v) acetonitrile: water with 0.1 % formic acid. The eluates were dried in a speedvac and resuspended in 50 µL 0.1% formic acid. Then, peptides were separated and analyzed by a nanoLC-(Orbitrap) MS/MS (LTQ-Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific, San José, CA, USA). Peptides were separated by a 120 min acetonitrile gradient (A = water, 0.1% formic acid; B = acetonitrile, 0.1% formic acid) in a C18 reversed phase (RP) nano-column (75 µm I.D.; 15 cm length; 3 µm particle diameter, Nikkyo Technos Co. LTD, Japan) coupled to a trap nano-column (100 µm I.D.; 2 cm length; 5 µm particle diameter, Thermo Fisher Scientific). The flow rate during elution gradient was 300 nL/min.

For real time ionization and peptide fragmentation, an enhanced FT-resolution spectrum (resolution = 30,000 FHMW) followed by a data dependent FT-MS/MS scan from most intense ten parent ions with a charge state rejection of one using a HCD fragmentation with a normalized collision energy of 35 % and dynamic exclusion of 0.5 min.

For protein identification analysis, tandem mass spectra were extracted, and charge state was deconvoluted by Proteome Discoverer version 1.4.0.288 (Thermo Fisher Scientific). All MS and MS/MS samples were analyzed using

Mascot (Version 2.5) search engine with three different nodes: 1) the proteome of *Vitis vinifera* (29907 entries) from uniprot database, 2) the Swiss-Prot database limited from fungi taxonomy (33712 entries) and 3) the common contaminants for proteomic applications (247 entries). All these three searches assumed no enzyme digestion and an error of 20 mmu for fragment ion mass and 10 ppm for precursor ions. Oxidation of methionine and acetylation of N-termini were specified as variable modifications, Visual verification of fragmentation spectra was done for the identified peptides and only those found in both replicates were considered. Identified peptides were chemical synthetized for further studies.

2.4. Determination of ACEi activity

ACEi activity was measured by a fluorescence technique according to López-Fernández-Sobrino et al. (López-Fernández-Sobrino, Soliz-Rueda, et al., 2021). Specifically, λ_{ex} 360 nm, λ_{em} 400 nm and fluorescence measurements at 60 min (37ºC) were used to determine ACEi activity, expressed initially as percentage of inhibition (%). Fractions or peptides reconstituted in Milli-Q water, were tested at 0.11 mg of protein/mL and 0.83 mg of peptide/mL, respectively (concentration in the well). IC_{50} were also calculated and expressed in µg/mL for RP-HPLC subfractions and in µM for the synthetic peptides. Data are represented as a mean value of three determinations ± SD.

For ACEi activity, protein content of HPLC subfractions were determined by the bicinchoninic acid method (ThermoFisher Scientific) following the manufacturer's instructions in a microplate format. A calibration standard curve was elaborated with seroalbumin bovine. Determination of the protein content was performed at least in duplicate. The results are expressed as the mean ± SD.

2.5. Determination of antihypertensive activity

Male SHR (17–20 weeks old, weighing 350–400 g) were obtained from Charles River Laboratories España S.A. (Barcelona, Spain). Rats were singly housed in a temperature-controlled animal quarter (22 ºC) with a 12 h light/dark period. They were fed a standard diet based on chow Panlab A04 (Panlab, Barcelona, Spain) and had free access to water.

After a 10-day adaptation period, animals were given a single dose of the synthetic peptides dissolved in a volume of 1.5 mL in tap water by oral gavage between 9 and 10 a.m. Synthesized peptides (FKTTDQQTRTTVA, NPKLVTIV, TVTNPARIA, PAGELHP, LDSPSEGRAPG, LDSPSEGRAPGAD) were administered to SHR at 10 mg/kg bw. Tap water and Captopril (50 mg/kg bw, a known antihypertensive drug) were used as negative and positive controls, respectively.

Systolic and diastolic blood pressure (SBP and DBP, respectively) were measured using the tail-cuff method before and after 2, 4, 6, 8, 24, 48 and 72 h of treatments administration (synthesized peptides, Captopril or water), according to Quiñones et al., 2011. ΔSBP and ΔDBP were calculated as the difference between the mean values of SBP or DBP after and before treatment administration for each rat. Data were expressed as the mean values ± standard error of the mean (SEM) for a minimum of six experiments.

The animal protocol followed in this study was conducted in accordance with the European Communities Council Directive(86/609/EEC) and approved by the Animal Ethics Review Committee for Animal Experimentation of the Universitat Rovira i Virgili and by Generalitat de Catalunya (permission number 10522).

2.6. *In silico* simulated peptides digestion

In silico simulated peptide digestion was carried out using the program ExPASy PeptideCutter, available at http://web.expasy.org/peptide_cutter. PeptideCutter was used to predict the hydrolysis of peptide sequences

identified in hydrolyzed WL using the known enzymatic cleavage sites. Pepsin, chymotrypsin, trypsin, lipase, and colipase were used to this purpose.

Moreover, BIOPEP data base was used to identify reported ACEi and antihypertensive activities of the peptide-derived fragments obtained in the simulated GI digestion of peptides (Minkiewicz et al., 2019).

2.7. Statistical analysis

Differences between treatments were analysed by two-way analysis of variance (two-way ANOVA) and Tukey test as post hoc. All the analyses were performed using GraphPad Prism 7.04 for Windows (GraphPad Software, San Diego, California). Outliers were determined by using Grubbs' test. Differences between groups were considered significant when $p< 0.05$.

3. Results

3.1. Identification of ACEi peptides

WL hydrolysate was firstly subjected to ultrafiltration through a 3 kDa cut-off membrane to isolate the low molecular weight peptides. Peptides in the <3 kDa fraction were separated using a semipreparative RP-HPLC (conditions explained in section 2.3.1). The obtained chromatogram showed a large number of peaks (**Figure 1A**), indicating that the <3 kDa fraction is comprised of a complex mixture of compounds. This fraction was divided into 9 RP-HPLC subfractions (named from F.1 to F.9, **Figure 1A**), which were collected and lyophilized, and their ACEi activities were measured (**Figure 1B**). Five subfractions showed an ACEi activity higher than 50 % (F.4, F.6, F.7, F.8 and F.9) at 0.11 mg/mL. ACEi activity of these selected fractions was also determined and expressed as IC_{50} values (µg of protein/mL) (**Figure 1C**). The observed range of IC_{50} values were between 107 and 10 µg protein/mL, being the most active subfraction the F8 (12.3 µg of protein/mL).

These active fractions were further analyzed by nanoLC–LTQ-Orbitap HPLC-MS. **Table 1** shows the six amino acid sequences identified in those fractions,

namely from P1 to P6. As an example, **Figures 2A and 2B** show the MS/MS spectrum of the matched ions at m/z 543.267 and 636.298, corresponding to the sequences LDSPSEGRAPG (P5) and LDSPSEGRAPGAD (P6), respectively. These six peptides were chemical synthetized and their ACEi activity was evaluated at a concentration of 0.83 mg/mL. ACEi activities ranged from 2 to 95 % (**Table 1**). Peptides TVTNPARIA, PAGELHP and LDSPSEGRAPG stood out for their high ACEi activity. Indeed, they showed an ACEi activity higher than 70 % at the evaluated peptide concentration and with IC_{50} value lower than 20 µM. The amino acid sequence PAGELHP was the most active peptide with an IC_{50} value of 0.5 µM.

In addition, peptides in the <3kDa fraction of the control WL was also analyzed by nanoLC–LTQ-Orbitap HPLC-MS. No peptides were identified.

3.2. Antihypertensive activity of the synthetic peptides

Antihypertensive effects of the six synthesized peptides were evaluated in SHR (**Figures 3 and 4**). Before starting the study, SBP and DBP of animals were 198.9 ± 2.9 mmHg and 154.2 ± 3.8 mmHg, respectively, confirming hypertensive conditions. As expected, BP of SHR receiving water did not significantly vary throughout the study. Administration of Captopril (50 mg/kg bw) led to an important decrease in SBP of SHR 2 h after its administration (**Figure 3**), reaching the maximum decrease at 6-8 h post-administration (-44.0 ± 4.4 and 44.7 ± 2.9 mmHg, respectively). Its effect lasted until 24 h after its administration (-22.0 ± 5.7 mmHg of SBP).

Regarding peptides, treatment with peptides P1, P2, P3, P5 and P6 produced a decrease in SBP of more than 10 mmHg (**Figure 3**). P1 and P6 were the most effective peptides (**Figures 3A and 3F**). Specifically, their administration reduced SBP from 2 h to 8 h post-administration, reaching the maximum values at 6 h post-administration (-27.6 ± 2.2 and -24.3 ± 3.6 mmHg for P1 and P6, respectively). P2, P3 and P5 also exerted an antihypertensive effect (**Figures 3B, 3C, 3E**). The maximum effect was also at 6 h post-administration, decreasing

SBP by -16.2 ± 2.0 to -19.0 ± 4.8 mmHg. In the case of the peptide P4, its administration to the animals did not affect BP levels.

Regarding DBP, as expected, no differences were found in rats administered water. Captopril produced the maxim BP drop by -47.2 ± 3.9 mmHg at 4h post-administration (**Figure 4**). All peptides produced a decrease in DBP, except P4 that no presented differences with water-administered group. P1, P5 and P6 were the peptides that showed the greatest antihypertensive effects, reducing DBP by -32.0 ± 7.5, -27.0 ± 7.6 and -33.6 ± 3.6 mmHg at 6 h after peptide administration.

3.3. *In silico* simulated gastrointestinal digestion of peptides

The six peptides identified in hydrolyzed WL were subjected to an *in silico* study to simulate their GI digestion. All the peptides were susceptible to be hydrolyzed by gastric and/or intestinal proteases. **Table 2** shows the prediction of amino acid sequences released after FKTTDQQTRTTVA, NPKLVTIV, TVTNPARIA, PAGELHP, LDSPSEGRAPG or LDSPSEGRAPGAD gastric and duodenal digestion based on the *in silico* digestion approach. None of the obtained amino acid sequences have been already reported in the data base BIOPEP to show antihypertensive or ACEi activities. Only the amino acid sequences FK, IA and HP, presenting in the extreme C-terminal of P1, P3, P4, respectively, were previously reported to show an ACEi activity (**Table 2**).

4. Discussion

Bioactive peptides are small protein fragments encrypted in the protein, which can exert a biological activity when they are released from the native protein (Margalef et al., 2017). They can regulate important body functions through their myriad activities, including antihypertensive, antimicrobial, antithrombotic, immunomodulatory, opioid, antioxidant, and mineral binding functions (Chakrabarti et al., 2018). Antihypertensive effect is one of the most important properties attributed to bioactive peptides, which is frequently mediated by inhibition of ACE. In the last years, the study of bioactive peptides

is increasing due to the great variety of foods in which they are found and their use in the revaluation of by-products (Chakrabarti et al., 2018). In a previous study, our group demonstrated that enzymatic protein hydrolysis of WL was a useful methodology to maximize the extraction of phenolic compounds and to obtain extracts with enhanced bioactivities, including ACEi and antihypertensive properties. Moreover, since an increase in the amino acid content was also observed after the hydrolysis of WL, the potential involvement of bioactive peptides in BP-lowering effect of the hydrolysate was not ruled out (López-Fernández-Sobrino, Margalef, et al., 2021). Therefore, in the present study, we investigated the presence of antihypertensive peptides in the WL hydrolysate.

The biological activity of the peptides present in different protein hydrolysates is related to their composition, their amino acid sizes and sequence and their configuration (Möller et al., 2008). In order to the identify potential bioactive peptides, WL hydrolysate was firstly ultrafiltered to obtain the smaller fraction of 3 kDa since peptides with the highest ACEi activity have been usually reported to be short in length (between 2-11 residues) (Margalef et al., 2017). In fact, fraction < 3kDa is usually used to identify ACEi peptides in protein hydrolysates (Bravo et al., 2019; Malomo et al., 2015; Quirós et al., 2007). This fraction was further fractioned using RP-HPLC to separate the peptides according to their sizes and hydrophilicities. Subsequently, ACEi properties of the fractions were determined to select those most active for peptides identification by mass spectrometry. Six amino acid sequences were identified (FKTTDQQTRTTVA, NPKLVTIV, TVTNPARIA, PAGELHP, LDSPSEGRAPG and LDSPSEGRAPGAD), which were released during WL protein hydrolysis since no peptides were detected in non-hydrolyzed WL. The identified peptides showed a wide range of ACEi activities from 2.4 and 95.4 % at the same concentration (0.83 mg/mL). It is known that ACEi activity is dependent on the amount and type of amino acid composition of peptides. Short amino acid sequences between 2-12 residues are usually related with the best ACEi activities (Auwal

et al., 2019; Daskaya-Dikmen et al., 2017). However, amino acid composition seems to be more important than peptide length for ACE inhibition, in di and tripeptides overall. In this regard, the amino acid sequence, concretely the three last amino acid positions in the C-terminal extreme, plays an important role in ACE competitive inhibition (Murray & FitzGerald, 2007). Peptides containing Pro and hydrophobic amino acids (Tyr, Trp or Phe) in that extreme have been shown to exert the highest ACEi activity. In fact, the presence of Pro at the last and second last positions is very common in ACE inhibitory peptides. Moreover, Gly, Val, Leu and Ile at the first position of N-terminal extreme or the presence of positively charge amino acids (Arg or Lys) in the N-terminal amino acid sequence have been linked with high ACE inhibitions (Aluko, 2015; Asoodeh et al., 2016). In our study, peptides P3, P4 and P5 (TVTNPARIA, PAGELHP and LDSPSEGRAPG) showed the greatest ACEi activities. The bioactivity of these peptides could be due to the fact that they are small peptides, between 7-11 amino acids and they contained Pro and other hydrophobic amino acids at the C-terminal position and/or, in the case of P5 Leu at the N-terminal position for. To the best of our knowledge, none of the six identified peptides have been previously identified to have any bioactivity (search carried out in the BIOPEP database as of March 2021, http://www.uwm.edu.pl/biochemia/index.php/pl/biopep). In addition, this is the first time that ACEi peptides are identified in WL. Alcaide-Hidalgo et al. reported that ACEi activity showed by red wines aged with lees could partially be mediated by peptides released during the wine ageing. However, peptides were not characterized (Alcaide-Hidalgo et al., 2008). Moreover, the results found in our study are relevant since 3 peptides with a great potential to inhibit ACE were identified. IC_{50} values showed by peptides P3, P4 and P5 were between 0.5 and 18 µM. These values were similar to those presented by other peptides, recognized as good ACE inhibitors, such as the peptide RDGGYCC founded in virgin olive oil with 0.84 µM (Alcaide-Hidalgo et al., 2020) and the peptides IPP, VPP and LHLPLP with 5, 9 and 5.5 µM, respectively obtained from

fermented milk (Iwai et al., 2008; Quirós et al., 2007) or much higher than the peptide AVFQHNCQE from a chicken foot hydrolysate (44.8 μM) (Bravo et al., 2019). All these reported peptides showed antihypertensive effects in SHR after their acute administration. Thus, P3, P4 and P5 could be considered good ACE inhibitors and potentially could exert antihypertensive effects.

However, since *in vitro* ACE inhibition and antihypertensive properties are not necessarily directly related (Margalef et al., 2017), it was important to evaluate the *in vivo* efficacy of these peptides. In this regard, when the peptides are orally consumed they can be hydrolyzed during the GI digestion due to the action of the enzymes pepsin, trypsin, α-chymotrypsin, elastases, and carboxypeptidase A and B or other intracellular peptidases. Therefore, peptide bioactivity could get lost or also be increased depending on the bioactivity showed by the generated peptide-derived fragments. In addition, the length of the peptides is very important to be absorbed for intestinal cells. Small peptides (< 6 amino acids) can pass through enterocytes, decreasing their absorption efficiency as the peptides are longer. Furthermore, their charge and hydrophobicity play an important role in their absorption by enterocytes (Fan et al., 2019). Considering all these facts, it is the great relevance to evaluate the bioactivity of these peptides *in vivo*.

All six identified peptides were synthetized and their antihypertensive effects were evaluated in SHR at an acute and oral dose of 10 mg/kg bw. All peptides showed an antihypertensive effect in both SBP and DBP, except for P4 (PAGELHP) that did not show this bioactivity. Peptides P2, P3 and P5 produced a reduction in SBP between -16 or -19 mmHg with the maximum BP-reduction at 6 h post-administration. P1 and P6 showed the highest effect, with a SBP reduction to -27.6 and -24.3 mmHg, respectively and with similar results in DBP after 6 h post-administration. Similar BP- lowering effects have been found in purified peptides obtain from dietary food (Alcaide-Hidalgo et al., 2020; Bravo et al., 2019; Shobako et al., 2018). Thus, AVFQHNCQE peptide administration to SHR at the same concentration used in this study (10 mg/kg bw) showed a

maximum BP reduction at 6 h post-administration by -25.07 ± 4.21 mmHg and −17.65 ± 3.24 mmHg in SBP and DBP, respectively, similar to the peptides obtained from WL hydrolysate (Bravo et al., 2019). It is well known that a reduction by 10 mmHg and 5 mmHg in SBP and DBP respectively, leads to healthy effects including the reduction of cardiovascular diseases by 25% and stroke by 36 % (Law et al., 2009). Thus, these results show the potential of the peptides P1, P2, P3, P5 and P6 to manage hypertension. It should be noted that the peptide P4, which showed the higher ACEi activity, did not have antihypertensive effect. This fact was also observed for other peptides from a chicken foot hydrolysate with high ACEi activities such as VGKPGARAPMY, LSGPVKF and AVKILP, that did not showed antihypertensive effects *in vivo* (Bravo et al., 2019). In addition, the peptide P6, with the lowest ACEi activity, showed the best antihypertensive effect. This could be indicative that other peptides could be generated during GI digestion.

Digestion *in silico* studies have shown to be a good tool to predict the possible protein hydrolysis produced by GI enzymes (Haines et al., 2019; Mas-Capdevila et al., 2020). All the peptides identified in this study were susceptible to GI hydrolysis by trypsin, chymotrypsin or pepsin, generating other amino acid sequences which could be responsible of the bioactivity of these peptides identified in hydrolyzed WL. This fact could explain for example why P6 did not have a good ACEi activity but exhibited antihypertensive effect. Moreover, it was observed that the peptide LDSPSEGRAPGAD produced a higher decrease in BP compared to the peptide LDSPSEGRAPG, which have a similar sequence except for missing two amino acids at its C-terminal (AD). Digestion of the largest peptide (LDSPSEGRAPGAD) did not produce the smallest peptide but similar peptide-derived fragments were generated after *in silico* digestion in both. Therefore, the greatest antihypertensive effect is probably due to the terminal amino acids AD in the peptide LDSPSEGRAPGAD. However, the *in silico* study is a predictive model that does not take into account the tertiary structure of proteins; therefore, some of the predicted amino acid sequences may not be

generated by an *in vivo* GI digestion. The sequences obtained after *in silico* digestion were also analysed using the BIOPEP database to consult if these amino acid sequences have been already reported as ACE inhibitors and/or antihypertensive sequences. None of the peptides or their predicted GI-derived amino acid sequences were found in this database having those activities except for the dipeptides IA and HP. These two peptides had been reported as ACE inhibitors. Therefore, further studies are required to demonstrate which amino acid sequences could be responsible of the antihypertensive activity of the peptides identified in this WL hydrolysate.

5. Conclusions

The hydrolysis of WL under specific conditions produced the release of peptides with antihypertensive effect. BP-lowering effects of the novel peptides FKTTDQQTRTTVA, NPKLVTIV, TVTNPARIA, LDSPSEGRAPG, LDSPSEGRAPGAD were shown in SHR. For the first time, it was demonstrated that WL can be source of ACE inhibitory and antihypertensive peptides. These results confirm the potential of these compounds to be used as functional ingredient or nutraceuticals to treat HTN.

6. Patents

Patent application “Wine lees, derivatives thereof and their uses”: application number EP20382358.8 and PCT/EP2021/053051.

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Conflicts of Interest:

The authors declare no conflict of interest.

Bibliography

Alcaide-Hidalgo, J. M., Martínez-Rodríguez, A. J., Martín-Álvarez, P. J., & Pueyo, E. (2008). Influence of the elaboration process on the peptide fraction with angiotensin I-converting enzyme inhibitor activity in sparkling wines and red wines aged on lees. *Food Chemistry*, *111*(4), 965–969. https://doi.org/10.1016/j.foodchem.2008.05.015

Alcaide-Hidalgo, J. M., Romero, M., Duarte, J., & López-Huertas, E. (2020). Antihypertensive Effects of Virgin Olive Oil (Unfiltered) Low Molecular Weight Peptides with ACE Inhibitory Activity in Spontaneously Hypertensive Rats. *Nutrients*, *12*(1), 271. https://doi.org/10.3390/nu12010271

Alderman, C. P. (1996). Adverse Effects of the Angiotensin-Converting Enzyme Inhibitors. *Annals of Pharmacotherapy*, *30*(1), 55–61. https://doi.org/10.1177/106002809603000110

Aluko, R. E. (2015). Antihypertensive Peptides from Food Proteins. *Annual Review of Food Science and Technology*, *6*(1), 235–262.

Asoodeh, A., Homayouni-Tabrizi, M., Shabestarian, H., Emtenani, S., & Emtenani, S. (2016). Biochemical characterization of a novel antioxidant and angiotensin I-converting enzyme inhibitory peptide from Struthio camelus egg white protein hydrolysis. *Journal of Food and Drug Analysis*, *24*(2), 332–342. https://doi.org/10.1016/j.jfda.2015.11.010

Auwal, S. M., Zainal Abidin, N., Zarei, M., Tan, C. P., & Saari, N. (2019). Identification, structure-activity relationship and in silico molecular docking analyses of five novel angiotensin I-converting enzyme (ACE)-inhibitory peptides from stone fish (Actinopyga lecanora) hydrolysates. *PLOS ONE*, *14*(5), e0197644. https://doi.org/10.1371/journal.pone.0197644

Bravo, F. I., Mas-Capdevila, A., Margalef, M., Arola-Arnal, A., & Muguerza, B. (2019). Novel Antihypertensive Peptides Derived from Chicken Foot Proteins. *Molecular Nutrition & Food Research*, *63*(12), 1801176. https://doi.org/10.1002/mnfr.201801176

Chakrabarti, S., Guha, S., & Majumder, K. (2018). Food-derived bioactive peptides in human health: Challenges and opportunities. In *Nutrients* (Vol. 10, Issue 11). MDPI AG. https://doi.org/10.3390/nu10111738

Darewicz, M., Borawska, J., Vegarud, G., Minkiewicz, P., & Iwaniak, A. (2014). Angiotensin I-Converting Enzyme (ACE) Inhibitory Activity and ACE Inhibitory Peptides of Salmon (Salmo salar) Protein Hydrolysates Obtained by Human and Porcine Gastrointestinal Enzymes. *International Journal of Molecular Sciences*, *15*(8), 14077–14101. https://doi.org/10.3390/ijms150814077

Daskaya-Dikmen, C., Yucetepe, A., Karbancioglu-Guler, F., Daskaya, H., & Ozcelik, B. (2017). Angiotensin-I-Converting Enzyme (ACE)-Inhibitory Peptides from Plants. *Nutrients*, *9*(4), 316. https://doi.org/10.3390/nu9040316

Dimou, C., Kopsahelis, N., Papadaki, A., Papanikolaou, S., Kookos, I. K., Mandala, I., & Koutinas, A. A. (2015). Wine lees valorization: Biorefinery development including production of a generic fermentation feedstock employed for poly(3-hydroxybutyrate) synthesis. *Food Research International*, *73*, 81–87. https://doi.org/10.1016/j.foodres.2015.02.020

Dimou, C., Vlysidis, A., Kopsahelis, N., Papanikolaou, S., Koutinas, A. A., & Kookos, I. K. (2016). Techno-economic evaluation of wine lees refining for the production of value-added products. *Biochemical Engineering Journal*, *116*, 157–165.

https://doi.org/10.1016/j.bej.2016.09.004

Fan, H., Liao, W., & Wu, J. (2019). Molecular interactions, bioavailability, and cellular mechanisms of angiotensin-converting enzyme inhibitory peptides. *Journal of Food Biochemistry*, *43*(1), e12572. https://doi.org/10.1111/jfbc.12572

Foëx, P., & Sear, J. (2004). Hypertension: pathophysiology and treatment. *Continuing Education in Anaesthesia Critical Care & Pain*, *4*(3), 71–75. https://doi.org/10.1093/BJACEACCP/MKH020

Haines, S. R., McCann, M. J., Grosvenor, A. J., Thomas, A., Noble, A., & Clerens, S. (2019). ACE inhibitory peptides in standard and fermented deer velvet: An in silico and in vitro investigation. *BMC Complementary and Alternative Medicine*, *19*(1). https://doi.org/10.1186/s12906-019-2758-3

He, R., Alashi, A., Malomo, S. A., Girgih, A. T., Chao, D., Ju, X., & Aluko, R. E. (2013). Antihypertensive and free radical scavenging properties of enzymatic rapeseed protein hydrolysates. *Food Chemistry*, *141*(1), 153–159. https://doi.org/10.1016/j.foodchem.2013.02.087

Hernández-Ledesma, B., Del Mar Contreras, M., & Recio, I. (2011). Antihypertensive peptides: Production, bioavailability and incorporation into foods. *Advances in Colloid and Interface Science*, *165*(1), 23–35. https://doi.org/10.1016/j.cis.2010.11.001

Hypertension. (2019). https://www.who.int/news-room/fact-sheets/detail/hypertension

Iwai, K., Saiga-Egusa, A., Hayakawa, T., Shimizu, M., Takahata, Y., & Morimatsu, F. (2008). An Angiotensin I-converting Enzyme (ACE)-inhibitory Peptide Derived from Chicken Collagen Hydrolysate Lowers Blood Pressure in Spontaneously Hypertensive Rats. *JOURNAL OF THE JAPANESE SOCIETY FOR FOOD SCIENCE AND TECHNOLOGY-NIPPON SHOKUHIN KAGAKU KOGAKU KAISHI*, *55*(12), 602–605.

Jara-Palacios, M. J. (2019). Wine lees as a source of antioxidant compounds. In *Antioxidants* (Vol. 8, Issue 2). MDPI AG. https://doi.org/10.3390/antiox8020045

Kuba, M., Tana, C., Tawata, S., & Yasuda, M. (2005). Production of angiotensin I-converting enzyme inhibitory peptides from soybean protein with Monascus purpureus acid proteinase. *Process Biochemistry*, *40*(6), 2191–2196. https://doi.org/10.1016/j.procbio.2004.08.010

Law, M. R., Morris, J. K., & Wald, N. J. (2009). Use of blood pressure lowering drugs in the prevention of cardiovascular disease: Meta-analysis of 147 randomised trials in the context of expectations from prospective epidemiological studies. *BMJ (Online)*, *338*(7705), 1245. https://doi.org/10.1136/bmj.b1665

Lee, S. Y., & Hur, S. J. (2017). Antihypertensive peptides from animal products, marine organisms, and plants. *Food Chemistry*, *228*, 506–517. https://doi.org/10.1016/j.foodchem.2017.02.039

López-Fernández-Sobrino, R., Margalef, M., Torres-Fuentes, C., Ávila-Román, J., Aragonès, G., Muguerza, B., & Bravo, F. I. (2021). Enzyme-Assisted Extraction to Obtain Phenolic-Enriched Wine Lees with Enhanced Bioactivity in Hypertensive Rats. *Antioxidants*, *10*(4), 517. https://doi.org/10.3390/antiox10040517

López-Fernández-Sobrino, R., Soliz-Rueda, J. R., Margalef, M., Arola-Arnal, A., Suárez, M., Bravo, F. I., & Muguerza, B. (2021). ACE Inhibitory and Antihypertensive Activities of Wine Lees and Relationship among Bioactivity and Phenolic Profile. *Nutrients*, *13*(2), 679. https://doi.org/10.3390/nu13020679

Maicas, S., & Mateo, J. J. (2020). Sustainability of wine production. *Sustainability (Switzerland)*, *12*(2), 559. https://doi.org/10.3390/su12020559

Majumder, K., & Wu, J. (2014). Molecular Targets of Antihypertensive Peptides:

Understanding the Mechanisms of Action Based on the Pathophysiology of Hypertension. *International Journal of Molecular Sciences*, *16*(1), 256–283. https://doi.org/10.3390/ijms16010256

Malomo, S. A., Onuh, J. O., Girgih, A. T., & Aluko, R. E. (2015). Structural and antihypertensive properties of enzymatic hemp seed protein hydrolysates. *Nutrients*, *7*(9), 7616–7632. https://doi.org/10.3390/nu7095358

Margalef, M., Bravo, F. I., Arola-Arnal, A., & Muguerza, B. (2017). Natural Angiotensin Converting Enzyme (ACE) Inhibitors with Antihypertensive Properties. In *Natural Products Targeting Clinically Relevant Enzymes* (pp. 45–67). Wiley-VCH Verlag GmbH & Co. KGaA. https://doi.org/10.1002/9783527805921.ch3

Mas-Capdevila, A., Iglesias-Carres, L., Arola-Arnal, A., Aragonès, G., Muguerza, B., & Bravo, F. I. (2020). Implication of opioid receptors in the antihypertensive effect of a novel chicken foot-derived peptide. *Biomolecules*, *10*(7), 1–12. https://doi.org/10.3390/biom10070992

Merz, M., Eisele, T., Berends, P., Appel, D., Rabe, S., Blank, I., Stressler, T., & Fischer, L. (2015). Flavourzyme, an Enzyme Preparation with Industrial Relevance: Automated Nine-Step Purification and Partial Characterization of Eight Enzymes. *Journal of Agricultural and Food Chemistry*, *63*(23), 5682–5693. https://doi.org/10.1021/acs.jafc.5b01665

Mills, K. T., Bundy, J. D., Kelly, T. N., Reed, J. E., Kearney, P. M., Reynolds, K., Chen, J., & He, J. (2016). Global disparities of hypertension prevalence and control. *Circulation*, *134*(6), 441–450. https://doi.org/10.1161/CIRCULATIONAHA.115.018912

Minkiewicz, P., Iwaniak, A., & Darewicz, M. (2019). BIOPEP-UWM database of bioactive peptides: Current opportunities. *International Journal of Molecular Sciences*, *20*(23), 5978. https://doi.org/10.3390/ijms20235978

Möller, N. P., Scholz-Ahrens, K. E., Roos, N., & Schrezenmeir, J. (2008). Bioactive peptides and proteins from foods: Indication for health effects. In *European Journal of Nutrition* (Vol. 47, Issue 4, pp. 171–182). Springer. https://doi.org/10.1007/s00394-008-0710-2

Mora, L., Reig, M., & Toldrá, F. (2014). Bioactive peptides generated from meat industry by-products. *Food Research International*, *65*, 344–349. https://doi.org/10.1016/j.foodres.2014.09.014

Murray, B., & FitzGerald, R. (2007). Angiotensin Converting Enzyme Inhibitory Peptides Derived from Food Proteins: Biochemistry, Bioactivity and Production. *Current Pharmaceutical Design*, *13*(8), 773–791. https://doi.org/10.2174/138161207780363068

Musee, N., Lorenzen, L., & Aldrich, C. (2007). Cellar waste minimization in the wine industry: a systems approach. *Journal of Cleaner Production*, *15*(5), 417–431. https://doi.org/10.1016/j.jclepro.2005.11.004

OIV. (2020). 2020 Wine prodution - OIV First Estimates. *International Organisation of Vine and Wine*, 1–8.

Quiñones, M., Miguel, M., Muguerza, B., & Aleixandre, A. (2011). Effect of a cocoa polyphenol extract in spontaneously hypertensive rats. *Food & Function*, *2*(11), 649. https://doi.org/10.1039/c1fo10119f

Quirós, A., Ramos, M., Muguerza, B., Delgado, M. A., Miguel, M., Aleixandre, A., & Recio, I. (2007). Identification of novel antihypertensive peptides in milk fermented with Enterococcus faecalis. *International Dairy Journal*, *17*(1), 33–41. https://doi.org/10.1016/j.idairyj.2005.12.011

Shobako, N., Ogawa, Y., Ishikado, A., Harada, K., Kobayashi, E., Suido, H., Kusakari, T.,

Maeda, M., Suwa, M., Matsumoto, M., Kanamoto, R., & Ohinata, K. (2018). A Novel Antihypertensive Peptide Identified in Thermolysin-Digested Rice Bran. *Molecular Nutrition & Food Research*, *62*(4), 1700732. https://doi.org/10.1002/mnfr.201700732

Snauwaert, E., Vande Walle, J., & De Bruyne, P. (2017). Therapeutic efficacy and safety of ACE inhibitors in the hypertensive paediatric population: A review. In *Archives of Disease in Childhood* (Vol. 102, Issue 1, pp. 63–71). BMJ Publishing Group. https://doi.org/10.1136/archdischild-2016-310582

Sparks, M. A., Crowley, S. D., Gurley, S. B., Mirotsou, M., & Coffman, T. M. (2014). Classical renin-angiotensin system in kidney physiology. *Comprehensive Physiology*, *4*(3), 1201–1228. https://doi.org/10.1002/cphy.c130040

Toldrá, F., Gallego, M., Reig, M., Aristoy, M. C., & Mora, L. (2020). Bioactive peptides generated in the processing of dry-cured ham. *Food Chemistry*, *321*, 126689. https://doi.org/10.1016/j.foodchem.2020.126689

Toldrá, F., Reig, M., Aristoy, M. C., & Mora, L. (2018). Generation of bioactive peptides during food processing. *Food Chemistry*, *267*, 395–404. https://doi.org/10.1016/j.foodchem.2017.06.119

Unger, T., Borghi, C., Charchar, F., Khan, N. A., Poulter, N. R., Prabhakaran, D., Ramirez, A., Schlaich, M., Stergiou, G. S., Tomaszewski, M., Wainford, R. D., Williams, B., & Schutte, A. E. (2020). 2020 International Society of Hypertension Global Hypertension Practice Guidelines. *Hypertension*, *75*(6), 1334–1357. https://doi.org/10.1161/HYPERTENSIONAHA.120.15026

WHO | Strategic priorities. (2016). https://www.who.int/cardiovascular_diseases/priorities/en/

Yathisha, U. G., Bhat, I., Karunasagar, I., & Mamatha, B. S. (2019). Antihypertensive activity of fish protein hydrolysates and its peptides. In *Critical Reviews in Food Science and Nutrition* (Vol. 59, Issue 15, pp. 2363–2374). Taylor and Francis Inc. https://doi.org/10.1080/10408398.2018.1452182

Yoshikawa, M. (2015). Bioactive peptides derived from natural proteins with respect to diversity of their receptors and physiological effects. *Peptides*, *72*, 208–225. https://doi.org/10.1016/j.peptides.2015.07.013

TABLES

Table 1. Amino acid sequences identified by reverse phase liquid chromatography, percentage of purity and angiotensin-converting enzyme inhibitory (ACEi) activity of the peptides identified in the wine lees hydrolysate.

	Sequence[a]	Theoretical M.W.	MH+ (Da)	m/z (Da)	Charge	Purity grade (%)	ACEi activity	
							%[b]	IC_{50} (µM)[c]
P1	FKTTDQQTRTTVA	1496.65	1496.77	748.89	2	97.8	27.0 ± 5.6	N.D
P2	NPKLVTIV	883.11	883.56	413.75	2	99.2	48.5 ± 9.1	N.D
P3	TVTNPARIA	942.09	942.53	471.77	2	96.4	76.4 ± 9.2	14.5
P4	PAGELHP	719.80	720.37	360.68	2	99.4	72.3 ± 11.5	0.5
P5	LDSPSEGRAPG	1085.15	1085.52	543.26	2	96.9	95.4 ± 2.2	17.9
P6	LDSPSEGRAPGAD	1271.32	1271.59	636.29	2	98.5	2.4 ± 0.9	N.D

[a] Amino acids are designated using their one letter codes.
[b] ACEi activity determined at 0.83 mg/mL.
[c] Peptide concentration needed to inhibit 50 % of the original ACE activity N.D= no determined

Table 2. *In silico* simulated digestion of the peptides P1-P6 identified in the wine lees hydrolysate.

Peptide	Original Sequence	Enzyme	Digestion Stage	Final Sequence	ACE inhibitor
P1	FKTTDQQTRTTVA	Trypsin	Duodenal	TTDQQTRTTVA	
	FKTTDQQTRTTVA	Trypsin	Duodenal	FK	FK-
	TTDQQTRTTVA	Trypsin	Duodenal	TTDQQTR	
	TTDQQTRTTVA	Trypsin	Duodenal	TTVA	
P2	NPKLVTIV	Pepsin	Gastric	NPKL	-KL
	NPKLVTIV	Pepsin	Gastric	VTIV	
	NPKLVTIV	Chymotrypsin	Duodenal	NPKL	-KL
	NPKLVTIV	Chymotrypsin	Duodenal	VTIV	
	NPKLVTIV	Trypsin	Duodenal	NPK	
	NPKLVTIV	Trypsin	Duodenal	LVTIV	
	NPKL	Trypsin	Duodenal	NPK	
	LVTIV	Chymotrypsin	Duodenal	VTIV	
P3	TVTNPARIA	Trypsin	Duodenal	TVTNPAR	-AR
	TVTNPARIA	Trypsin	Duodenal	IA	IA
P4	PAGELHP	Pepsin	Gastric	PAGE	-GE
	PAGELHP	Pepsin	Gastric	LHP	LHP-, -HP
	PAGELHP	Chymotrypsin	Duodenal	PAGEL	
	PAGELHP	Chymotrypsin	Duodenal	HP	HP
	LHP	Chymotrypsin	Duodenal	HP	HP
P5	LDSPSEGRAPG	Pepsin	Gastric	DSPSEGRAPG	
	LDSPSEGRAPG	Chymotrypsin	Duodenal	DSPSEGRAPG	
	LDSPSEGRAPG	Trypsin	Duodenal	LDSPSEGR	-GR
	LDSPSEGRAPG	Trypsin	Duodenal	APG	APG-, -APG
	DSPSEGRAPG	Trypsin	Duodenal	DSPSEGR	-GR
	DSPSEGRAPG	Trypsin	Duodenal	APG	APG-, -APG
	LDSPSEGR	Chymotrypsin	Duodenal	DSPSEGR	-GR

	LDSPSEGRAPGAD	Pepsin	Gastric	DSPSEGRAPGAD	
	LDSPSEGRAPGAD	Chymotrypsin	Duodenal	DSPSEGRAPGAD	
P6	LDSPSEGRAPGAD	Trypsin	Duodenal	LDSPSEGR	-GR
	LDSPSEGRAPGAD	Trypsin	Duodenal	APGAD	APG-
	DSPSEGRAPGAD	Trypsin	Duodenal	DSPSEGR	-GR
	DSPSEGRAPGAD	Trypsin	Duodenal	APGAD	APG-
	LDSPSEGR	Chymotrypsin	Duodenal	DSPSEGR	-GR

S 1. Characterization of free amino acid content in WL hydrolysate by UHPLC-QToF/MS.

Amino acids	**WL hydrolysate (µg/mL)**
Alanine	18.88 ± 1.55
Arginine	4.31 ± 0.44
Asparagine	9.23 ± 2.01
Aspartate	15.53 ± 1.73
Glutamate	28.25 ± 7.20
Glutamine	-
Glycine	9.77 ± 1.52
Histidine	2.99 ± 0.16
Isoleucine	44.52 ± 5.86
Leucine	63.15 ± 9.85
Lysine	5.27 ± 1.03
Phenylalanine	4.20 ± 0.77
Proline	632.58 ± 38.92
Serine	24.13 ± 7.69
Threonine	15.92 ± 3.98
Tryptohan	1.17 ± 0.41
Tyrosine	3.37 ± 0.44
Valine	26.23 ± 3.81
Methionine	1.03 ± 0.29
Cystine	-
Hydroxiproline	-
TOTAL	910.56

FIGURES

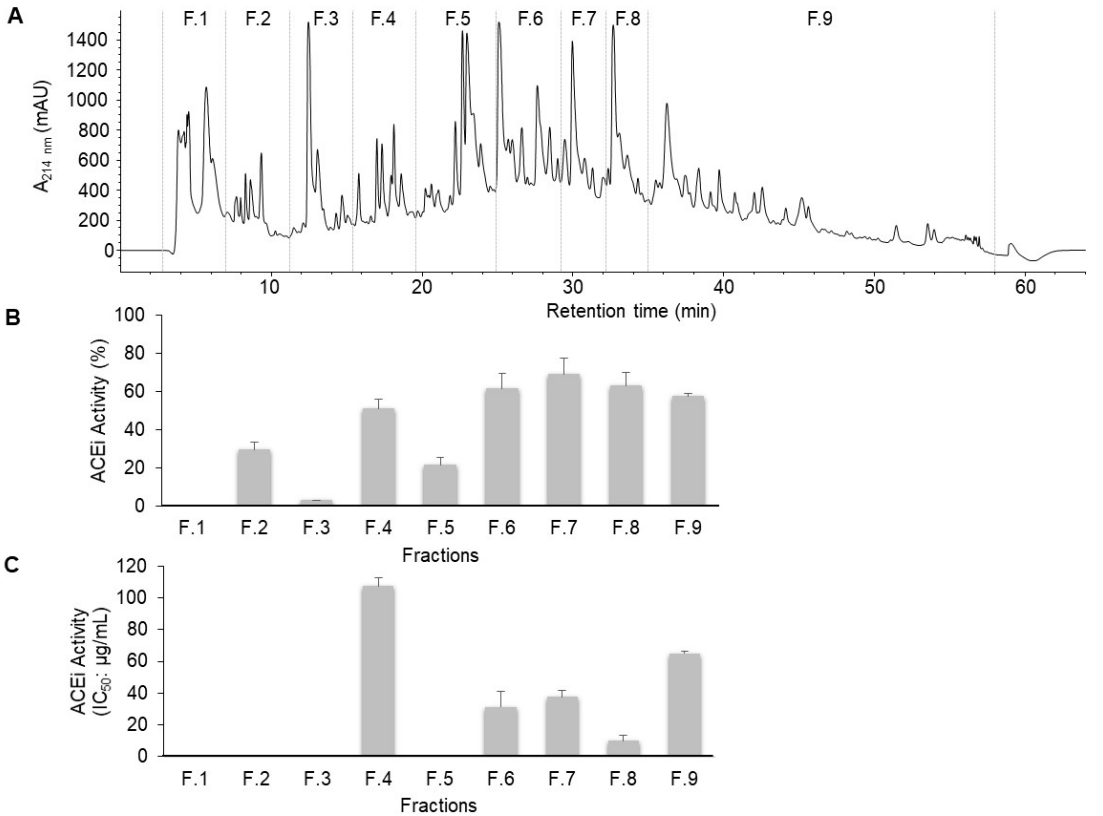

Figure 1. A) Fractionation by RP-HPLC at a semipreparative scale for the<3 kDa fraction obtained from the wine lees hydrolysate. The collected fractions were termed with F. followed by a number (F.1–F.9). Angiotensin-converting enzyme inhibitory (ACEi) activity expressed as B) percentage of inhibition and C) IC_{50} (μgmL^{-1}) of the collected fractions from semipreparative RP-HPLC system. Data are expressed as the mean ± SD (n ≥ 2). Protein contents of fractions were estimated using the bicinchoninic acid assay.

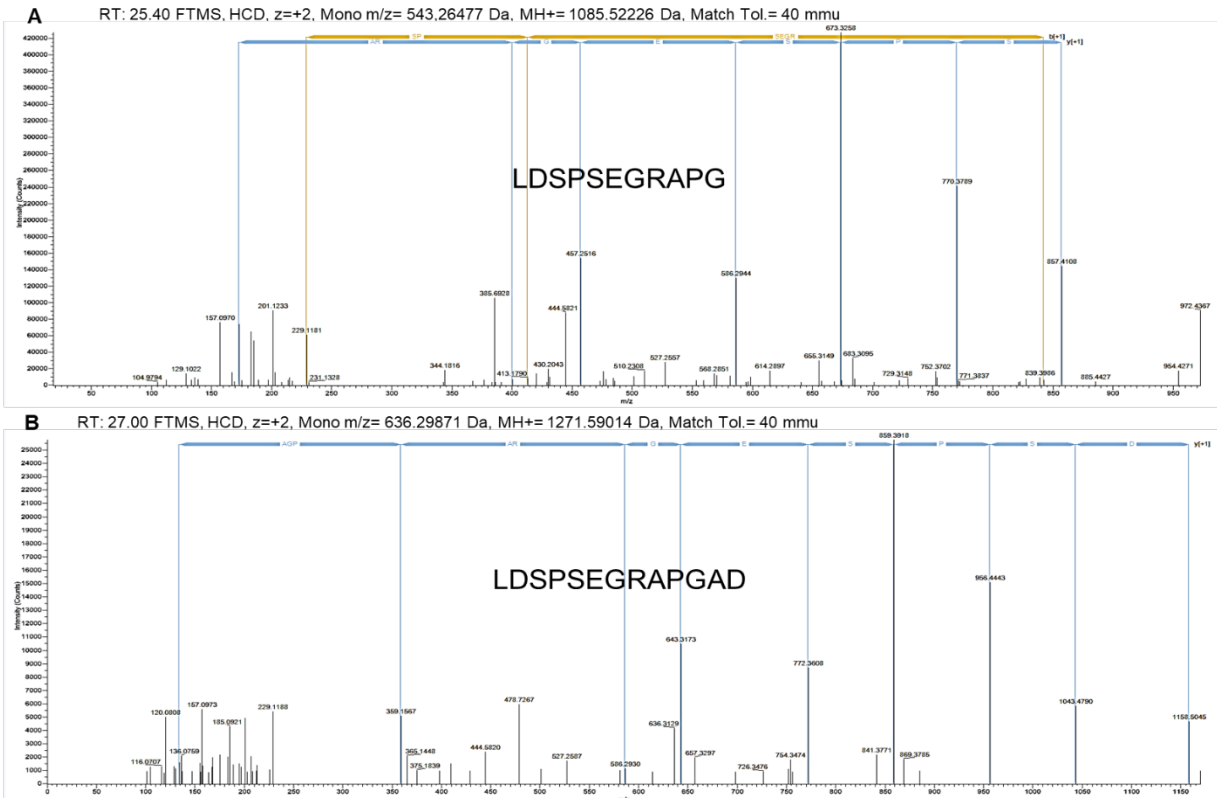

Figure 2. MS/MS spectrum of the doubled charged ions m/z 543,2 (A) and 636.2 (B). Following sequence interpretation and database searching, peptides were identified as LDSPSEGRAPG and LDSPSEGRAPGAD, respectively. MS/MS spectra were acquired with linear trap quadrupole-Orbitrap mass spectrometry. The sequences of these peptides are displayed with the fragment ions observed in the spectra.

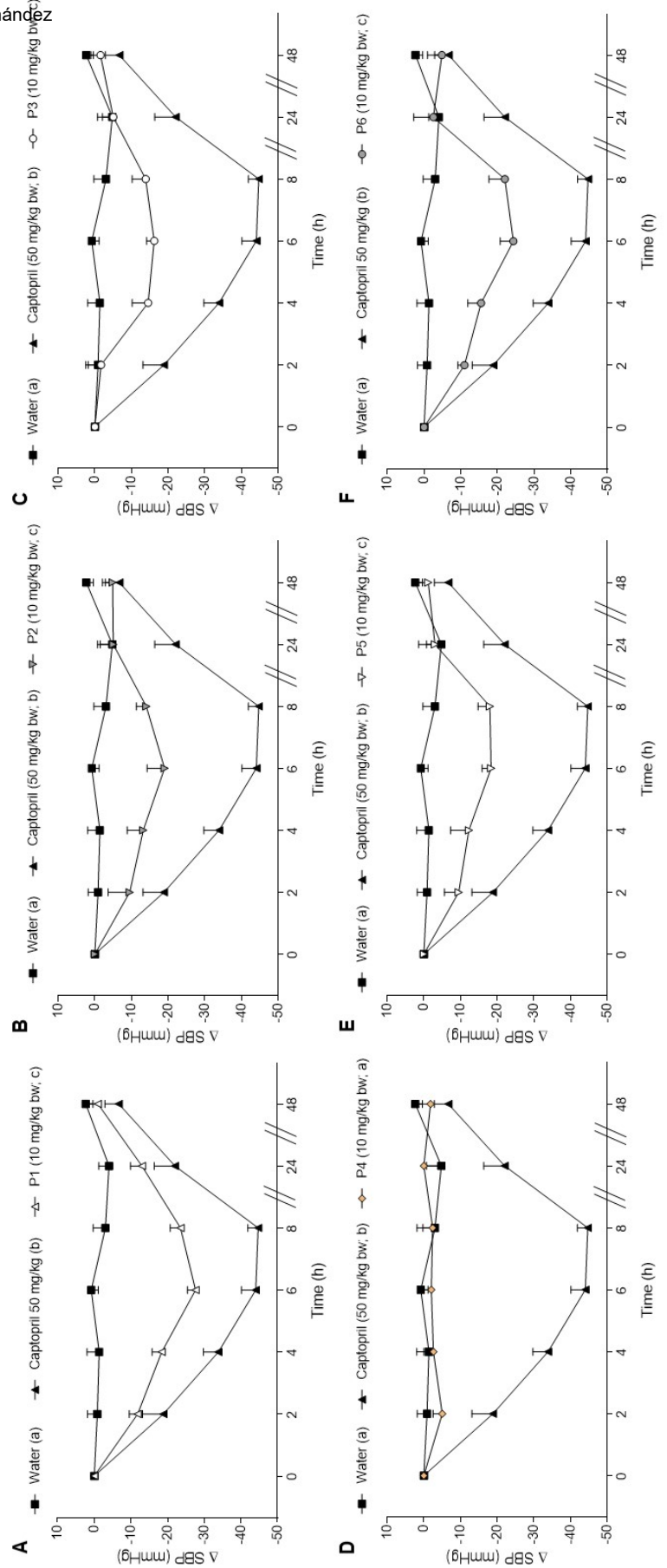

Figure 3. Changes in systolic blood pressure (SBP) in spontaneously hypertensive rats after administration of water, Captopril (50 mg/kg bw) or synthetic peptides (10 mg/kg bw): FKTTDQQTRTTVA, NPKLVTIV, TVTNPARIA, PAGELHP, LDSPSEGRAPG, LDSPSEGRAPGAD. Data are expressed as the mean ± SEM ($n \geq 6$). Different letters represent significant differences ($p < 0.05$). p was estimated by two-way ANOVA.

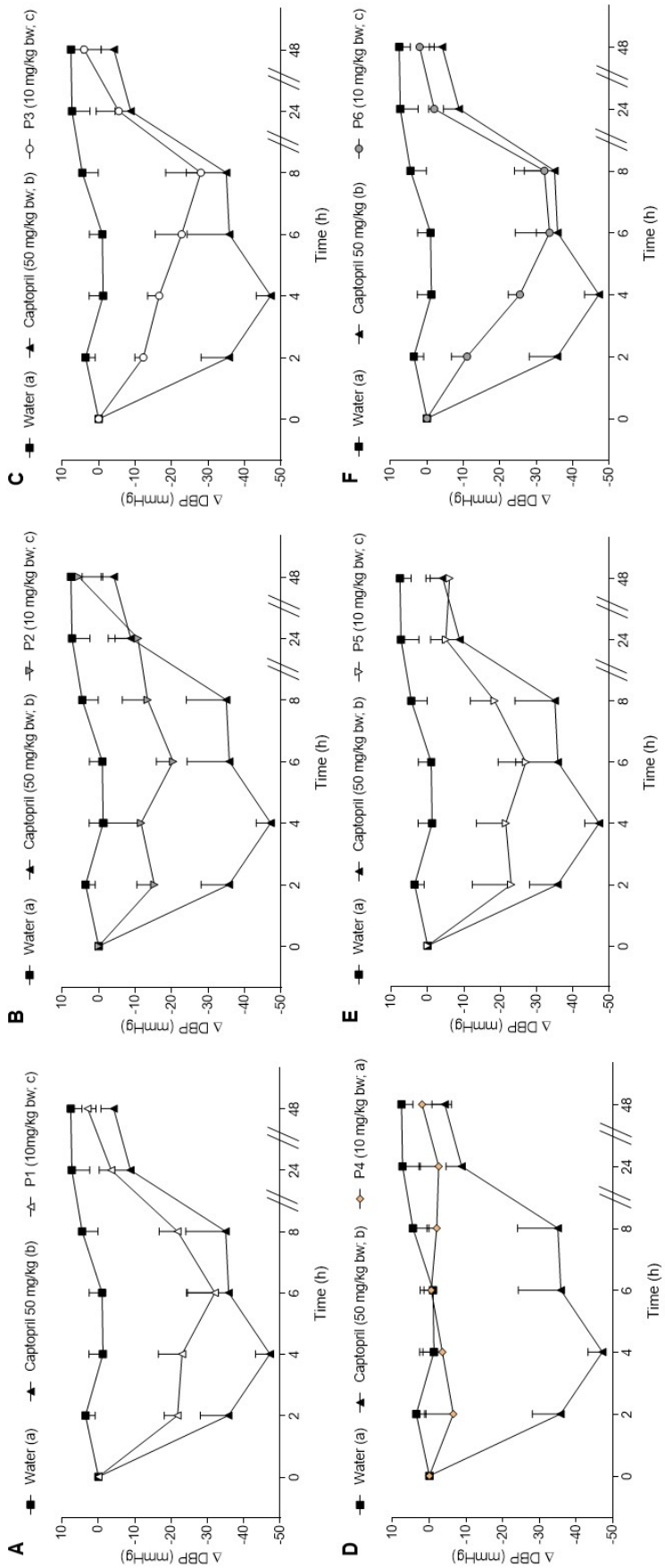

Figure 4. Changes in diastolic blood pressure (DBP) in spontaneously hypertensive rats after administration of water, Captopril (50 mg/kg bw) or synthetic peptides (10 mg/kg bw): FKTTDQQTRTTVA, NPKLVTIV, TVTNPARIA, PAGELHP, LDSPSEGRAPG, LDSPSEGRAPGAD. Data are expressed as the mean ± SEM (n ≥ 6). Different letters represent significant differences ($p < 0.05$). p was estimated by two-way ANOVA.

CHAPTER 2:

To evaluate the antihypertensive effect of WL dealcoholization and stablish the mechanisms involved in their BP-lowering effect

Manuscript 4:

Objective:

To determinate the most effective antihypertensive dose of the WL after an acute administration to SHR, evaluate the role of alcohol in their antihypertensive effect administrating dried WL to the animals and discard a possible hypotensive effect of the free-alcohol WL in normotensive WKY rats. Finally, to evaluate its antioxidant effects.

Blood pressure-lowering effect of wine lees: Dose-response study, effect of dealcoholization and possible mechanisms of action

Raúl López-Fernández-Sobrino, Jorge R. Soliz-Rueda, Manuel Suárez, Miquel Mulero, Lluís Arola, Francisca I. Bravo * and Begoña Muguerza

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Abstract: The antihypertensive effect of wine lees (WL) has been previously evidenced. In this study, the antihypertensive properties of different doses of WL were evaluated in spontaneously hypertensive rats (SHR). In addition, the blood pressure (BP)-lowering effect of dried (dealcoholized) WL powder (WLPW) and the mechanisms involved in its functionality were investigated. Furthermore, a possible hypotensive effect of WLPW was discarded in Wistar–Kyoto (WKY) rats. The administration of WL at different doses caused a dose-dependent decrease in BP of SHR up to 5.0 mL/kg bw, exhibiting the maximum decrease at 6 h post-administration. WLPW caused a greater drop in BP than WL, showing an antihypertensive effect higher and more prolonged than the drug Captopril. Moreover, the BP-lowering effect of WLPW was specific to the hypertensive state since an undesirable hypotensive effect in normotensive WKY rats was ruled out. Finally, WLPW improved oxidative stress and increased the activity of the antioxidant endogen system of SHR. These results suggest that WLPW could be used as functional ingredient for foods or nutraceuticals to ameliorate hypertension. Nevertheless, further clinical studies are needed to evaluate its long-term antihypertensive efficiency.

Keywords: angiotensin-converting enzyme activity; antihypertensive activity; antioxidant activity; spontaneously hypertensive rats; winery byproducts

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1. Introduction

Hypertension (HTN) is one of the main causes of premature death in the world. According to the WHO, 1.13 billion people suffer from HTN and 4 in 5 hypertensive people do not have it under control. Uncontrolled HTN can increase the risk of suffering other diseases such as stroke, coronary heart disease and renal failure [1]. HTN can be caused by different factors including alterations in the renin-angiotensin-aldosterone system (RAAS) components [2]. This system is essential in the regulation of blood pressure (BP), sodium–potassium balance and fluid volume [3]. Thus, increases in the angiotensin-converting enzyme (ACE) levels, a key component of RAAS, induces vasoconstriction and high BP [4,5]. Specifically, ACE produces the release of the vasoconstrictor angiotensin (Ang) II and the degradation of the vasodilator bradykinin [5]. In fact, the inhibition of its enzymatic activity is usually used as therapeutic treatment of HTN [6].

In addition, many studies suggest a close relationship between high levels of oxidative stress and HTN [7]. In fact, restoration of the oxidative balance has been demonstrated to be effective in reducing BP [8,9]. Oxidative stress is an imbalance

between oxidants and antioxidants in favor of the oxidants or reactive oxygen species (ROS). Uncontrolled overproduction of ROS has been related to reduction of vasodilator effects [10]. It produces a decrease in the endothelium-derived nitric oxide (NO) levels since ROS react with the vasodilator NO, producing peroxynitrite [11].

High intake of vegetables, fruits, plant-based beverages or juices has been associated with beneficial effects on BP [12]. More specifically, grape or grape-derived products have shown cardiovascular protective effects, including a reported reduction on BP in humans after the consumption of grape juice [13]. In addition, epidemiological studies have shown that a moderate consumption of wine can also reduce different cardiovascular events [14]. These effects have been associated with some of the phenolic compounds present in grapes such as the flavonoid quercetin or other phenolic compounds as resveratrol [15]. Nevertheless, these phenolic compounds are also present in grape processing byproducts. In fact, grape seeds have been effectively used to obtain phenolic extracts rich in proanthocyanidins with demonstrable antihypertensive effects in hypertensive rats and humans [16–20], although not always dose-dependently [16]. Their BP-lowering properties are mainly attributed to changes in endothelium-derived NO availability [18,21].

Wine lees (WL) are another byproduct of the winery industry. They are the sediments that remain at the bottom of wine fermenters once wine has been elaborated and racked [22]. Recently, our group has evidenced the antihypertensive effect of WL from grapes of the Cabernet variety in spontaneously hypertensive rats (SHR) after an acute administration [23]. Animals were administered the soluble fraction of WL obtained by centrifugation. This fraction is not used by the winery since the removal of solid waste from WL, for example by a filtration process, is not a profitable process for the wine industry. The BP-lowering effect of these WL was mainly associated with two phenolic families, namely flavanols and anthocyanins. In addition, a potential hypotensive effect of this WL was ruled out in normotensive rats, linking their BP-lowering effect to a hypertensive state [23]. In addition, the efficacy of enzyme-assisted extraction using Flavourzyme® to release phenolic compounds from a non-soluble fraction of WL was also recently shown in other study from our group [24]. The results demonstrated that enzymatic protein hydrolysis was a useful methodology to maximize the extraction of phenolic compounds from WL and to obtain extracts with enhanced functionalities. Both studies highlight the potential of WL and their derived products to manage HTN, opening the door to their commercial use not only within the wine industry, but also for other industrial sectors such as nutraceutical and functional food companies. In this regard, the cheap and easy process for the obtainment of the soluble fraction of WL at an industrial level and the low dose necessary to achieve its antihypertensive effect [23], opens the door to its commercial use as a great value-added product. Nevertheless, the drying of the soluble fraction of WL is essential for its use as nutraceutical or food ingredient. In this process, WL will lose water, but also ethanol, which reaches values similar to the wine of at least 8.5% [25]. It has been evidenced that alcohol intake can modify BP depending on dose and time administration [26]. Thus, the WL drying process could potentially modify their antihypertensive properties due to the removal of alcohol.

Considering all this evidence, the aims of the current study were to investigate in SHR the antihypertensive effect of the dried (dealcoholized) WL powder (WLPW) at the most effective dose and the underlying mechanisms taking part of its antihypertensive effect. In addition, a potential hypotensive effect of this free-alcohol WL was evaluated in normotensive Wistar–Kyoto (WKY) rats to be discarded.

2. Materials and Methods

2.1. Chemicals and Reagents

N-hippuryl-His-Leu (Hip-His-Leu) (PubChem CID: 94418), ACE (peptidyl-dipeptidase A, E.C. 3.4.15.1) (PubChem CID: 329770629), glutathione-S transferase from

horse liver (PubChem CID: 114886), monochlorobimane (PubChem CID: 114886), 2′,7′-dichlorofluorescein diacetate (PubChem CID: 24894058), 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH, PubChem CID: 57654141), N-1-naftiletilendiamine (PubChem CID: 329754555), HPLC grade acetonitrile and trifluoroacetic acid were provided by Sigma Aldrich (Madrid, Spain). Heparin (PubChem CID: 772) was provided by DeltaLab (Barcelona, Spain). Quercetin, gallic acid, (+)-catechin, *p*-coumaric acid and (−)-epicatechin were purchased from Fluka/Sigma-Aldrich; caffeic acid, malvidin-3-*O*-glucoside, vanillic acid, procyanidin dimer B2 and 4-hydroxybenzoic acid were purchased from Extrasynthése (Lyon, France); resveratrol was purchased from Carl Roth (Karlsruhe, Germany); cyanidin-3-*O*-rutinoside, ferulic acid and peonidin-3-*O*-rutinoside were purchased from PhytoLab (Vestenbergsgreuth, Germany). The rest of chemical solvents used in this study were of analytical grade.

2.2. Obtaining and Characterisation of the Wine Lees Samples

WL were supplied by the cellar Grandes Vinos y Viñedos from Cariñena (Spain), an area recognized by a Protected Designation of Origen (PDO). They were collected immediately after the wine transfer, which was elaborated with grapes of Cabernet variety. These lees were further centrifuged at 3000× *g* for 15 min at 4 °C to obtain the supernatant. It was kept at −20 °C until their analysis. The content of ethanol of this supernatant, determined using a micro ebulliometer (μEbu from GAP systems, Barcelona, Spain), was 9.8%. One aliquot of this supernatant was freeze-dried to remove ethanol and water, obtaining the WLPW. This powder was kept at room temperature until its analysis.

The content of ethanol of WLPW was 0.0%. Determination of moisture was carried out according to AOAC official methods [27]. Total protein content was determined by Kjeldahl method using the factor 6.25 [27]. Gallic acid was used as standard for total phenolic content quantification following the Folin–Ciocalteu method [24]. The results were expressed as gallic acid equivalents (mg) per gram of dry weight (mg GAE/g). All the analyses were carried out at least in duplicate. Table 1 shows WLPW characterization as well as its ACEi and antioxidant activities.

Table 1. Characterization of wine lees powder (WLPW).

Parameters	WLPW
Moisture	7.85 ± 1.49%
Total protein content [a]	24.08 ± 6.20%
Total phenolic content [a]	82.40 ± 0.80 mg GAE/g
ACEi activity (IC_{50}) [a]	13.38 ± 0.91 μg/mL
ACEi activity (IC_{50})	3.23 ± 0.25 μg prot/mL
Antioxidant activity (EC_{50}) [a]	5.50 ± 0.58 μg/mL

[a] Results are shown per dry weight. Abbreviations: ACEi: angiotensin-converting enzyme inhibitory, EC_{50}: the half maximal effective concentration, GAE: gallic acid equivalents, IC_{50}: the half maximal inhibitory concentration.

ACEi activity was measured as previously reported by our group [23]. Fluorescence measurement at 60 min (37 °C) as well as λex 360 nm and λem 400 nm were used to determine the activity. ACEi activity was expressed as IC_{50} (μg prot/mL and μg of dry weight/mL). Data are represented as a mean value ± SD of three determinations.

Finally, the antioxidant activity was determined by DPPH method according to López-Fernández-Sobrino et al. [24]. An aliquot of 500 μL of the sample at different concentrations (0–400 μg/mL) was mixed with 200 mL of DPPH 0.5 mM diluted in ethanol. The mixture was immediately shaken and incubated at room temperature for 30 min under darkness conditions. Absorbance was measured at 517 nm. Results were expressed as EC_{50} of radical scavenging activity (μg of dry weight/mL). Analyses were carried out in triplicate.

WLPW was analyzed by a UHPLC-ESI-Q-TOF-MS system using 1290 UHPLC Infinity II series coupled to a Q-TOF/MS 6550 (Agilent Technologies, Palo Alto, CA, USA)

according to López-Fernández-Sobrino et al. [23]. Both negative and positive ionization ([M-H]− or [M-H]+) were used to identify parental ions and fragmentation patterns. Quantification was carried out using calibration curves with commercial phenolic compounds. A semi-quantitative analysis was done when the phenolic compound was not available, using the calibration curve of the commercial compound with the most similar structure to the analyzed compound. Individual non-anthocyanin and anthocyanin compounds identified in the WLPW are shown in Table 2, Table3, respectively.

Table 2. Non-anthocyanin compounds found in wine lees powder by UHPLC-(ESI-)-Q-TOF-MS.

Compound	Quantity (μg/g)
Flavanols	
Catechin	3905.20 ± 19.20
Catechin gallate [a]	32.00 ± 0.40
Epicatechin	1739.20 ± 6.12
(Epi)catechin *O*-glucoside iso1 [b]	20.00 ± 0.01
(Epi)catechin *O*-glucoside iso2 [b]	13.20 ± 0.00
(Epi)catechin *O*-glucoside iso3 [b]	58.80 ± 1.17
Procyanidin dimer B2	1384.00 ± 0.40
Procyanidin dimer iso1 [c]	2568.40 ± 6.12
Procyanidin dimer iso2 [c]	570.40 ± 2.50
Procyanidin dimer iso3 [c]	118.40 ± 0.83
Procyanidin dimer iso4 [c]	512.00 ± 4.25
Procyanidin dimer iso5 [c]	172.80 ± 0.89
Procyanidin trimer iso1 [c]	651.20 ± 4.12
Procyanidin trimer iso2 [c]	574.00 ± 10.60
Procyanidin trimer iso3 [c]	244.40 ± 2.32
Procyanidin trimer iso4 [c]	128.80 ± 4.23
Procyanidin trimer iso5 [c]	551.60 ± 3.81
Flavonols	
Quercetin	1471.20 ± 4.85
Quercetin-3-*O*-glucoside [d]	65.20 ± 0.42
Quercetin-3-*O*-glucuronide [d]	96.80 ± 0.82
Kaempferol [d]	206.00 ± 1.63
Kaempferol-3-*O*-glucuronide [d]	19.20 ± 0.38
Isorhamnetin [d]	446.40 ± 2.51
Phenolic Acids	
Gallic acid	4834.80 ± 96.60
Caffeic acid	130.80 ± 0.84
Caffeic acid *O*-glucoside iso1 [e]	22.00 ± 0.80
Caffeic acid *O*-glucoside iso2 [e]	26.40 ± 1.20
p-Coumaric acid	137.60 ± 0.74
4-Hydroxybenzoic acid	66.80 ± 2.32
Ferulic acid	30.00 ± 0.47
Vanillic acid	93.20 ± 2.61
Stilbenes	
trans-Resveratrol [f]	184.00 ± 0.80
Resveratrol iso1 [f]	118.00 ± 0.40
Resveratrol *O*-glucoside iso1 [f]	10.80 ± 0.40
Resveratrol *O*-glucoside iso2 [f]	54.00 ± 1.60
Piceatannol [f]	168.00 ± 2.17
Piceatannol 3-*O*-glucoside iso1 [f]	8.80 ± 0.00
Piceatannol 3-*O*-glucoside iso2 [f]	2.40 ± 0.00
Viniferin-iso1 [f]	10.80 ± 0.00
Viniferin-iso2 [f]	32.40 ± 0.45

[a,b,c, d, e, f] indicate a semi-quantitative analysis using a calibration curve of standard ([a]) catechin, ([b]) epicatechin, ([c]) procyanidin dimer B2, ([d]) quercetin, ([e]) caffeic acid and ([f]) resveratrol.

Table 3. Anthocyanins found in wine lees powder by UHPLC-(ESI+)-Q-TOF-MS.

Anthocyanins	Quantity (μg/g)
Gallocatechin-Malvidin-3-glucoside dimer [a]	9.86 ± 0.04
Malvidin-3-glucoside-(epi)catechin [a]	44.43 ± 0.13
Delphinidin-3-glucoside [b]	147.58 ± 1.82
Cyanidin-3-glucoside [b]	9.05 ± 0.81
Petunidin-3-glucoside [c]	201.21 ± 2.26
Petunidin-3-glucoside-pyruvic acid [c]	3.56 ± 0.04
Peonidin-3-glucoside [c]	108.84 ± 2.50
Malvidin-3-glucoside [a]	2426.95 ± 20.01
Peonidin-3-glucoside-pyruvic acid [c]	1.63 ± 0.03
Delphinidin-(6-acetyl)-3-glucoside [b]	36.33 ± 0.90
Visitin A (malvidin-3-glucoside-pyruvic acid) [a]	49.07 ± 0.13
Visitin B (malvidin-3-glucoside-acetaldehyde) [a]	122.52 ± 0.59
Malvidin-3-glucoside-ethyl-(epi)catechin [a]	14.61 ± 0.03
Cyanidin-(6-acetyl)-3-glucoside [b]	8.14 ± 0.23
Acetylvisitin A [a]	31.41 ± 0.44
Malvidin-3-glucoside-ethyl-(epi)catechin [a]	55.06 ± 0.24
Petunidin-(6-acetyl)-3-glucoside [c]	51.55 ± 1.87
Malvidin-3-glucoside-ethyl-(epi)catechin [a]	81.77 ± 0.73
Acetylvisitin B [a]	66.45 ± 0.44
Peonidin-(6-acetyl)-3-glucoside [c]	52.79 ± 1.24
Delphinidin-(6-coumaroyl)-3-glucoside [b]	17.47 ± 0.27
Malvidin-(6-acetyl)-3-glucoside [a]	1135.64 ± 0.84
Coumaroylvisitin A [a]	8.01 ± 0.07
Malvidin-(6-caffeoyl)-3-glucoside [a]	14.56 ± 0.27
Cyanidin-(6-coumaroyl)-3-glucoside [b]	3.96 ± 0.16
Catechin-ethyl-Malvidin-3-acetylglucoside dimer [a]	35.09 ± 0.31
Petunidin-(6-coumaroyl)-3-glucoside [c]	29.79 ± 0.36
Pinotin A (malvidin-3-glucoside-vinylcatechol) [a]	33.59 ± 0.51
Malvidin-glucoside-vinyl-catechin [a]	6.12 ± 0.03
Coumaroylvisitin B [a]	36.48 ± 0.28
Malvidin-3-glucoside-vinylguaiacol [a]	23.78 ± 0.20
Catechin-ethyl-malvidin-3-coumaroylglucoside dimer [a]	27.39 ± 0.11
Catechin-ethyl-malvidin-3-acetylglucoside dimer [a]	5.78 ± 0.06
Peonidin-(6-coumaroyl)-3-glucoside [c]	37.78 ± 1.08
Malvidin-(6-coumaroyl)-3-glucoside [a]	430.71 ± 0.60
Malvidin-glucoside-vinyl-catechin [a]	6.5 ± 0.02
Acetyl-pinotin A [a]	0.28 ± 0.00
Malvidin 3-*O*-glucoside 4-vinylphenol (Pigment A) [a]	25.58 ± 0.08
Catechin-ethyl-malvidin-3-coumaroylglucoside dimer [a]	4.74 ± 0.01
Malvidin acetyl 3-*O*-glucoside 4-vinylphenol (Acetyl-pigment A) [a]	15.32 ± 0.17

[a,b,c] indicate a semi-quantitative analysis using a calibration curve of standard ([a]) malvidin-3-*O*-glucoside, ([b]) cyanidin-3-*O*-rutinoside and ([c]) peonidin-3-*O*-rutinoside.

2.3. Experimental Procedure in Rats

Male SHR and WKY rats (17–20 weeks old, weighing 300–350 g) were obtained from Charles River Laboratories España S.A. (Barcelona, Spain) and individually housed under standard conditions (23 °C, 50% of humidity and 12 h light/dark cycles) with ad libitum access to tap water and standard chow (A04 Panlab, Barcelona, Spain). After 10 days of adaptation, four different experiments were carried out (Figure 1). Treatments were administered between 8:00–9:00 am in all the experiments.

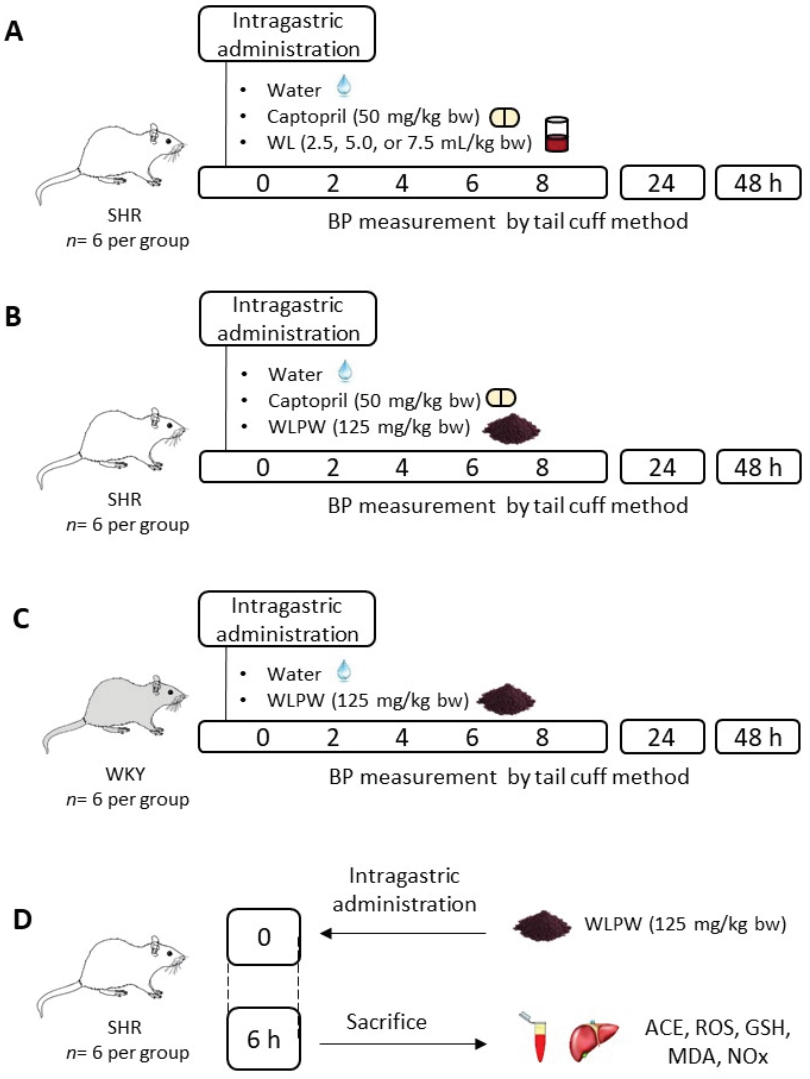

Figure 1. Experimental design of the different in vivo studies. (**A**) Effect of 3 different doses (2.5. 5.0 and 7.5 mL/kg bw) of wine lees (WL) on blood pressure (BP) in spontaneously hypertensive rats (SHR). (**B**) Effect of dried WL powder (WLPW) on BP in SHR. (**C**) Effect of WLPW on BP in normotensive Wistar Kyoto rats (WKY) and (**D**) mechanisms involved in the antihypertensive effect of WLPW in SHR at 6 h post-administration. ACE: angiotensin-converting enzyme; ROS: reactive oxygen species; GSH: reduced glutathione; MDA: malondialdehyde; NOx: nitric oxide metabolites.

The first study was performed in order to evaluate the antihypertensive effect of different doses of the WL in SHR (Figure 1A). The three tested doses (2.5, 5.0 and 7.5 mL/kg bw (equivalent to 62.5, 125 and 187.5 mg/kg bw)) were administered by oral gavage to SHR (n = 6). Water and Captopril (50 mg/kg bw) were used as negative (n = 6) and positive controls (n = 6), respectively.

The second study (Figure 1B) was carried out to determine the antihypertensive effect of the dried WL. SHR were administered WLPW (125 mg/kg bw), Captopril (50

mg/kg bw) or water (n = 6 per group) in a single dose by oral gavage. Captopril and water were given as positive and negative controls, respectively. Tap water was used to dissolve the treatments. In addition, in the third study the effect on BP of WLPW at the same dose was also evaluate in normotensive WKY rats (Figure 1C). Water or 125 mg/kg bw of WLPW (n = 6 per group) were administered at a single dose by oral gavage to WKY rats.

In the three studies systolic and diastolic blood pressure (SBP and DBP, respectively) were recorded by the tail-cuff method before and after 2, 4, 6, 8, 24 and 48 h from administration [28]. Decreases of SBP and DBP were calculated as the difference between SBP or DBP mean values after and before treatment administration for each rat. Data were expressed as the mean values ± SEM for a minimum of six experiments.

Finally, the mechanisms involved in WLPW antihypertensive effect were investigated in the fourth study (Figure 1D). SHR were administered water or a single dose of the WLPW (125 mg/kg bw) and sacrificed after 6 h post-administration. Blood was collected in heparin tubes and then, plasma was obtained by blood centrifugation at 1500× *g*, 15 min, 4 °C. Livers were extracted and immediately frozen at −80 °C.

All animal procedures carried out in this study were in accordance with the European Communities Council Directive (86/609/EEC) and approved by both the Animal Ethics Review Committee for Animal Experimentation of the Universitat Rovira i Virgili and the Generalitat de Catalunya (permission number 10780).

2.4. Determination of Plasma ACE Activity

Plasma ACE activity was carried out according to Mas-Capdevila et al. [29]. Commercial ACE was used as standard. Plasma ACE activity (mU ACE/mL) was expressed as the mean ± SEM from at least three replicates.

2.5. Reduced Glutathione Assay

Hepatic reduced glutathione (GSH) was analyzed following the monochlorobimane fluorometric method [30]. Briefly, 90 µL of liver homogenized supernatant was mixed with monochlorobimane (100 mM) and 10 µL of the glutathione S-transferase catalytic solution (1 U/mL). The levels of GSH were expressed as the mean ± SEM in µmol GSH/g tissue protein from at least three replicates. Liver protein content was determined by the bicinchoninic acid using the standard Pierce BCA protein assay (ThermoFisher Scientific, Madrid, Spain) following the manufacturer's instructions in a microplate format. A calibration standard curve was prepared with seroalbumin bovine.

2.6. Malondialdehyde Production

Plasma malondialdehyde (MDA) was measured by thiobarbituric acid assay according to Mas-Capdevila et al. [31] with some modifications. A total of 150 µL of plasma was mixed with 150 µL of TBA-HCl (trichloroacetic acid 1.21 M, HCL 0.6 M), incubated for 20 min at 4 °C and centrifuged at 1500× *g*, 4 °C for 25 min. Finally, 125 µL of supernatant was mixed with 25 µL of tribarbituric acid (120 mM in Tris 260 mM, pH 7). Spectrophotometric measurements at 540 nm were made at room temperature. Plasma thiobarbituric acid reactive substances (TBARS) were expressed as nM of MDA.

2.7. Reactive Oxygen Species

Hepatic ROS were measured according to Gabbia et al. [32]. Briefly, a piece of liver tissue (200 mg) was homogenized with 1.5 mL of ice-cold Tris-HCl buffer (40 Mm, pH = 7.4). Then, 100 µL of homogenate was mixed with 1 mL of Tris-HCl buffer and 5 µL of 2′,7′-dichlorofluorescein diacetate (10 µM final concentration). A 100 µL sample of liver homogenate was mixed with 1 mL of Tris-HCl buffer and used as control of tissue autofluorescence. Samples were incubated for 40 min at 37 °C. Finally, 200 µL of each sample was transferred to a 96-well multiplate and fluorescence intensity was measured (λex = 485 nm and λem = 525 nm).

2.8. Nitric Oxide Metabolites in Plasma

Nitric oxide metabolites (NOx) were determined in plasma following the method described by Grisham et al. [33] with some modifications. First, plasma samples were mixed with ethanol (1:3) and centrifuged at 4 °C and 10,000× *g* for 15 min to remove proteins. Then, 75 µL of the obtained supernatant was plated and 100 µL of reactive A (composed by 1.5 g of sulphanilamide, 50 mL of HCL 6.5 M and 50 mL of Milli-Q water) was added. Samples were incubated at 4 °C for 10 min. Then, 50 µL of N-1-naftiletilendiamine at a concentration of 3.64 g/L was added and incubated at 37 °C in dark conditions for 30 min. Spectrophotometric measurements at 540 nm were conducted at 37 °C. Sodium nitrite was used to perform a standard calibration curve. The results were expressed in µM of NOx and were measured per triplicate.

2.9. Statistical Analysis

BP differences were analyzed by a two-way analysis of variance (ANOVA) followed by a post hoc Tukey test in the studies with SHR. One-way ANOVA was used to identify BP differences between water and WLPW groups in WKY. Statistical differences between treatments in GSH, MDA and ROS levels were analyzed by Student's t-test. All the analyses were performed using GraphPad Prism 7.04 for Windows (GraphPad Software, San Diego, CA, USA). Outliers were determined using Grubbs' test. Differences between groups were considered significant when $p < 0.05$.

3. Results

3.1. Effect of Different Doses of Wine Lees on Blood Pressure in Hypertensive Rats

Initial values of the SBP and DBP in the animals were 190.4 ± 6.2 mmHg and 154.8 ± 14.1 mmHg (mean ± SD), respectively, showing a hypertensive condition. Figure 2A,B shows the effect of three different doses (2.5, 5.0 and 7.5 mL/kg bw) of WL on SBP and DBP, respectively, in SHR. As expected, water administration did not affect BP levels. In contrast, Captopril administration led to a clear decrease in both SBP and DBP, reaching the maximum decrease at 6 h post-administration. WL administration at difference doses decreased BP, reaching the maximum effect at 6 h post-administration. The most potent WL doses were 5.0 mL/kg bw and 7.5 mL/kg bw, with their BP-lowering properties similar to the effect caused by Captopril administration. Regarding DBP, all the tested doses produced a significant reduction in this parameter in comparison with the observed water group. No significant differences on DBP were found between WL administration at different doses and Captopril.

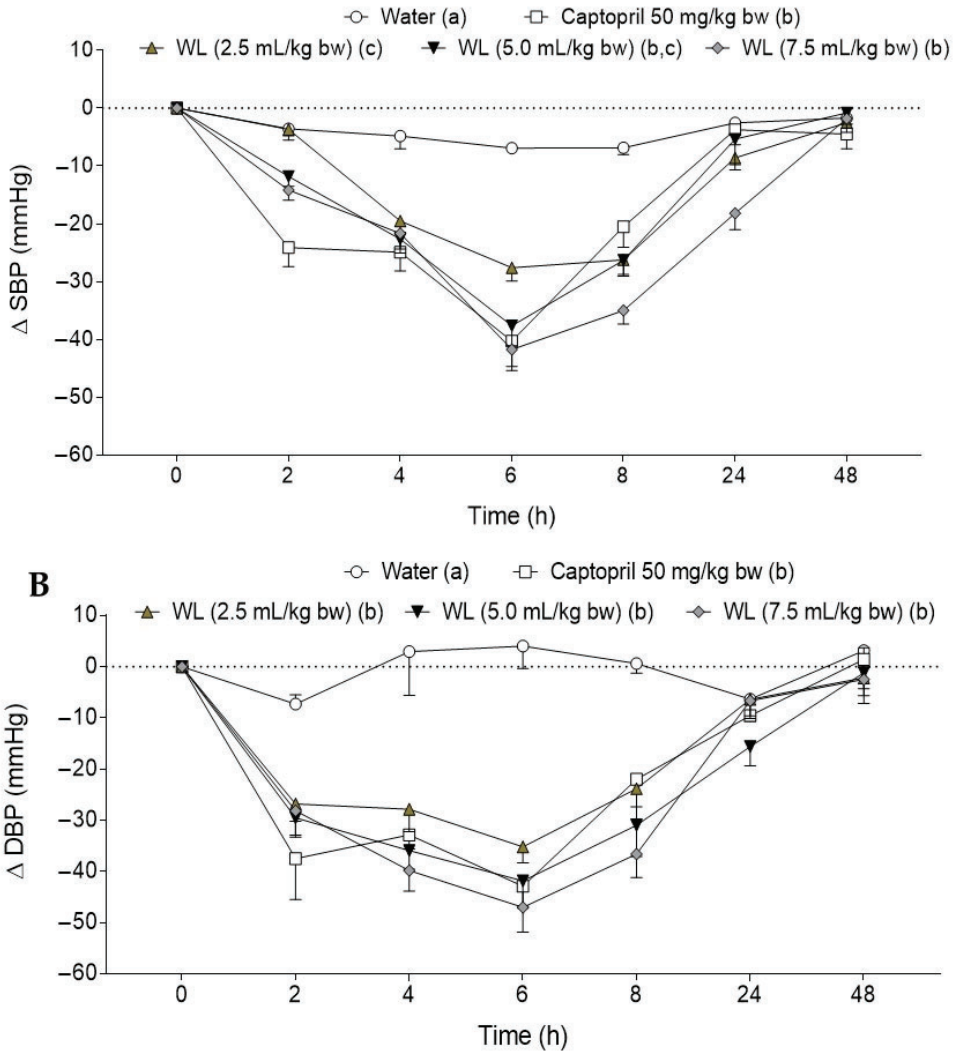

Figure 2. (**A**) Changes in systolic blood pressure (SBP) and (**B**) diastolic blood pressure (DBP) caused in spontaneous hypertensive rats by the administration of water, Captopril (50 mg/kg bw) or different doses of wine lees: 2.5 mL/kg bw. 5.0 mL/kg bw and 7.5 mL/kg bw. Data are expressed as mean ($n = 6$) ± SEM. Significant differences ($p < 0.05$) are represented by different letters in the legend and p was estimated by two-way ANOVA. Tukey test was used as post hoc.

3.2. *Effect of Dried Wine Lees on Blood Pressure in Hypertensive and Normotensive Rats*

The antihypertensive effect of WLPW was tested in SHR after an oral acute dose of 125 mg/kg bw. This dose was equivalent to the dose of 5.0 mL/kg bw of WL in dry weight. As shown in Figure 3, WLPW administration caused a potent reduction on SBP and DBP, showing a reduction of SBP higher than that caused by Captopril and a decrease of DBP similar to that exhibited by the drug. The maximum decrease in BP occurred 6 h after administration. Initial values of SBP and DBP did not recover until 48 h post-administration.

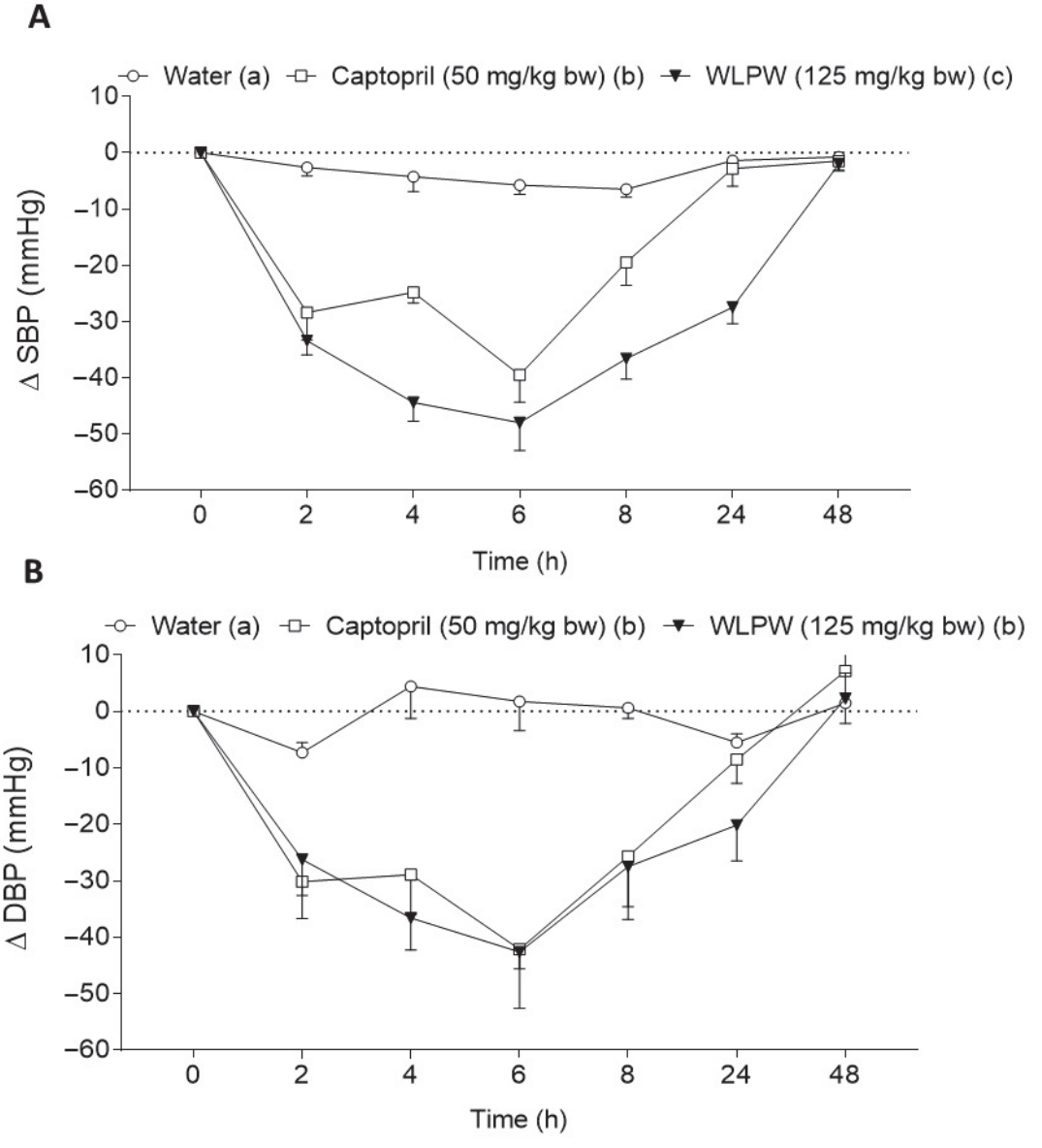

Figure 3. (**A**) Changes in systolic blood pressure (SBP) and (**B**) diastolic blood pressure (DBP) caused in spontaneously hypertensive rats by the acute administration of water, Captopril (50 mg/kg bw) or dried wine lees powder (WLPW) (125 mg/kg bw). Data are expressed as mean ($n = 6$) ± SEM. Significant differences ($p < 0.05$) are represented by different letters and p was estimated by two-way ANOVA. Tukey test was used as post hoc.

In addition, the effect of WLPW was also tested in normotensive rats to rule out a possible hypotensive effect. Initial values of SBP and DBP of these animals were 118.8 ± 3.6 mmHg and 89.2 ± 3.2 mmHg, respectively. The BP of the rats that ingested WLPW (125 mg/kg bw) was not significantly different to BP of the water group (Figure 4).

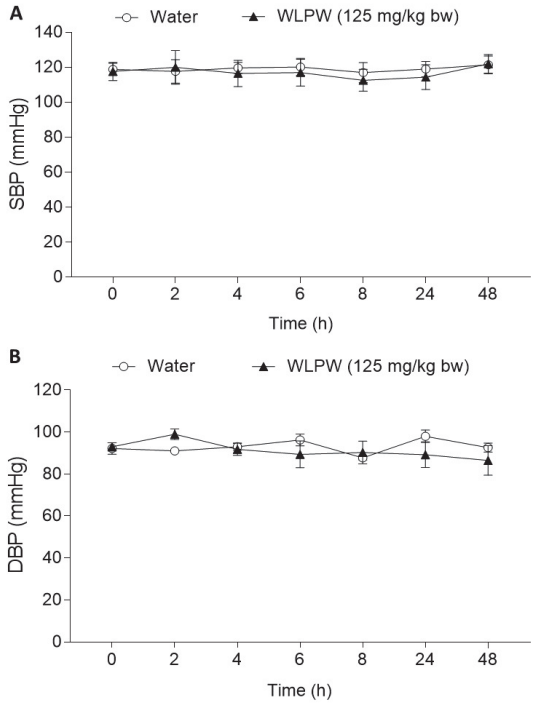

Figure 4. (**A**) Systolic blood pressure (SBP) and (**B**) diastolic blood pressure (DBP) of the Wistar–Kyoto rats before and after a single administration of water or dried wine lees powder (WLPW) (125 mg/kg bw). Data are expressed as mean (n = 6) ± SEM. No significant differences ($p < 0.05$) were found between both groups (two-way ANOVA).

3.3. Mechanisms Involved in the Antihypertensive Effect of the Wine Lees Extract

Figure 5 shows the plasma ACE activity of SHR 6 h after water or WLPW (125 mg/kg bw) administration. ACE activity in plasma did not change significantly between the WLPW- and water-administered groups.

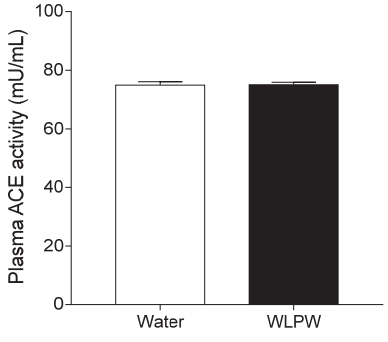

Figure 5. Plasma angiotensin-converting enzyme (ACE) activity in spontaneously hypertensive rats 6 h after administration of 125 mg/kg bw dried wine lees powder (WLPW) or water. Data are expressed as mean (n = 6) ± SEM. No significant differences ($p < 0.05$) were found between both groups (Student's t-test).

In addition, the antioxidant effect of WLPW was also studied as a potential mechanism involved in the antihypertensive effect of this sample. Plasma MDA and NO values are shown in Figure 6A,B, respectively. WLPW produced a decrease in plasma MDA, while NO levels significantly increased after WLPW administration. Liver GSH and ROS levels are shown in Figure 6C,D, respectively. The administration of WLPW caused a decrease in hepatic ROS levels compared to animals administered water. In addition, WLPW produced a significant increase of hepatic GSH levels in respect to those observed by the water group.

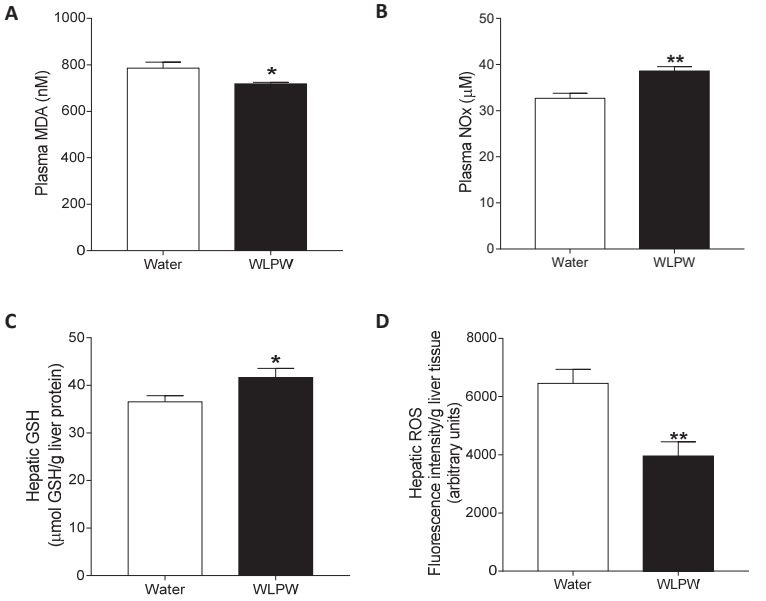

Figure 6. Levels of (**A**) plasma malondialdehyde (MDA) (**B**) plasma nitric oxide metabolites (NOx), (**C**) hepatic reduced glutathione (GSH) and (**D**) hepatic reactive oxygen species (ROS) in spontaneously hypertensive rats 6 h after administration of 125 mg/kg bw dried wine lees powder (WLPW) or water. Data are expressed as mean ($n = 6$) ± SEM. Significant differences are represented with (*) or (**) for $p < 0.05$ or $p < 0.01$, respectively. p was estimated by Student's t-test.

4. Discussion

Phenolic compounds are among the most explored natural compounds due to their beneficial health effects, including antihypertensive properties [34]. Evidence indicates that they can be effective in the prevention of oxidative stress-related diseases. These functionalities are mainly attributed to their potent antioxidant effects and depend on different factors such as type of phenolic compounds, treatment duration or dosage [35]. Nevertheless, they do not always act in a dose-dependent manner. Hormesis is a biological phenomenon that explains why a bioactive compound, when it is given at a low concentration, elicits a positive response, while when the compound is given at a higher concentration this response is diminished and may even be toxic, [36]. Phenolic compounds exhibit this dose–response behavior and they are considered as hormetic dietary phytochemicals [37]. They exhibit both antioxidant and pro-oxidant activities, depending on their concentration and the nature of the cellular microenvironment [38,39]. In this regard, this biphasic dose–response phenomenon has been reported in the antihypertensive activity of flavanol monomers [40], flavanol-rich grape seed [16,18] or cocoa [28] extracts and other flavanol-rich food such as cocoa [41]. In all these studies, the

highest dose of flavanol compounds produced a smaller drop in BP than lower doses. We have previously demonstrated in SHR the BP-lowering effect of the soluble fraction of WL [23]. In the current research, the dose–response study showed that all the tested doses of WL exhibited an antihypertensive effect, reducing both SBP and DBP values, with the maximum BP decreases at 6 h post-administration. The BP-lowering effect was dose-dependently up to 5.0 mL/kg bw. WL doses of 5.0 and 7.5 mL/kg bw caused comparable BP-lowering effects in SHR, with their antihypertensive effect similar to that shown by the animals administered Captopril. However, this dose–response behavior was different to the found in the aforementioned studies using pure flavanol compounds or flavanol-rich extracts or foods. This fact may be due to the use of lower doses of WL in respect to the ones used in those reported studies. For example, the results of our current study showed that the most effective dose for WL was 125 mg/kg bw (dose equivalent to 5.0 mL/kg bw), while a higher dose (375 mg/kg bw) was reported as the most effective antihypertensive dose for a flavanol-rich grape seed extract in SHR [17]. Nevertheless, it is remarkable that the BP decrease produced by WL was similar to one reported for that flavanol-rich grape seed extract, considering that the used WL dose was much lower [17]. Furthermore, the participation in the WL antihypertensive effect of other phenolic compounds, in addition to flavanols, such as anthocyanins, could also be involved in the different dose–response antihypertensive effect observed for this winery product.

WL were further dried to facilitate the potential use of WL as a nutraceutical or food ingredient, obtaining a WLPW. In addition to phenolic compounds, WL contain other components that could produce BP changes after their consumption, such as alcohol. In this regard, different studies carried out testing the effects of alcoholic drinks showed that alcohol can modify BP depending on the dose and intake duration [26]. Thus, as a consequence of the drying process, alcohol is removed from WL and can alter the antihypertensive effect of WL. To evaluate the effectiveness of the dried WL, they were administered to SHR in an acute dose of 125 mg/kg bw. WLPW demonstrated a greater antihypertensive effect than WL and even than the antihypertensive drug Captopril. According to these findings, Chiva-Blanch et al. reported greater BP reduction of a dealcoholized red wine compared to the corresponding red wine in a study carried out for 4 weeks in subjects with high cardiovascular risk, who also presented diabetes mellitus or three or more CVD risk factors [42]. In addition to the potent BP drop caused by WLPW, it is noteworthy that its antihypertensive effect remained 24 h post-administration. Furthermore, since the BP-lowering effect of WLPW was even more potent than the drug Captopril, it was considered essential to rule out a potential hypotensive effect on normotensive animals. The obtained results showed that the WLPW antihypertensive effect was specific to a hypertensive state, since no BP-lowering effects were observed in normotensive rats as has been previously evidenced for WL [23] or other products rich in phenolic compounds [16,43,44]. These results showed the great potential of a low dose of dried WL to HTN management. This dose corresponds to an intake of 1.8 g/day in humans, using a translation of animal to human doses [45]. Although experimental results in animals cannot be directly translated to humans, the fact that WLPW exhibits antihypertensive effects at this dose could allow for its use in nutraceutical and functional food sectors, promoting its revalorization. As was previously demonstrated for the soluble fraction of WL, the BP-lowering effect of WLPW may also be related to its high content of anthocyanins and flavanols [23]. Specifically, its effect was attributed to the flavanols catechin, epicatechin and procyanidins as well as the anthocyanin malvidin-3-glucoside [23], which have been reported to cause a BP reduction in hypertensive animals and humans [46–48] or vasodilation [49]. Nevertheless, as the bioactivity of phenolic compounds is linked with their metabolic-derived compounds, an additional study to identify the phase-II and gut microbiota-derived metabolites responsible for the WLPW antihypertensive effect would be of interest.

It is known that phenolic compounds can exert their antihypertensive effects by different mechanisms such as acting on the RAAS. This system is one of the main BP regulatory mechanisms, where ACE plays an important role. Inhibition of ACE activity avoids the overproduction of the vasoconstrictor Ang II, which is associated with a hypertensive condition [4]. Thus, in order to understand the underlying mechanisms involved in the antihypertensive effect of WL, plasma ACE activity was evaluated in SHR at the time of its maximum antihypertensive effect (6 h post-administration). No ACE activity changes were found in the plasma of animals administered 125 mg/kg bw of WLPW in respect to those administered water. These results could seem contradictory since the *in vitro* ACEi properties of WL have been reported [23]. However, the lack of correspondence between *in vitro* ACEi activity and its plasma ACE activity has been shown for other phenolic-rich extracts. In this regard, Quiñones et al. did not observe changes in plasma ACE activity of the antihypertensive GSPE (grape seed proanthocyanidins extract) in SHR, although this extract exhibited a potent *in vitro* ACEi activity [16]. Similar findings were reported for a quercetin-rich onionskin extract. This extract showed a great *in vitro* ACEi activity and antihypertensive effects in a randomized double-blinded placebo-controlled cross-over trial with pre-hypertensive patients, but plasma ACE activity in this patients was similar to non-treated subjects [50]. Nevertheless, the results of this study did not rule out the involvement of ACE, acting before 6 h post-administration in the antihypertensive effect of the WLPW.

Because of the potent *in vitro* antioxidant effect showed by WLPW, an improvement in oxidative stress could be involved in the antihypertensive effect of the dealcoholized WL. Increased oxidative stress has been linked to HTN development [51]. Elevated ROS levels are associated with endothelial dysfunction [52,53], since ROS can directly scavenge NO and avoid NO-dependent vasodilatation [54]. In this regard, it has been evidenced that one of the mechanisms involved in the antihypertensive effect of phenolic compounds is acting like radical scavengers and stimulating levels of endogenous antioxidants [8,36,55,56]. GSH is the most important antioxidant synthetized in cells, and plays an important role in the protection of cells from oxidative damage [57]. In this sense, the acute and long-term administration of a flavanol-rich grape seed extract (a grape byproduct extract) at a dose of 500 and 375 mg/kg bw, respectively, produced an increase of hepatic GSH in SHR [16,20]. Moreover, the administration of 1 g/kg bw of WL has also been demonstrated to increase hepatic GSH levels in healthy mice and in hypercholesterolemic mice. Furthermore, WL polyphenols also increase catalase activity in the liver, demonstrating their antioxidant properties [58]. According to these previous studies, our findings showed an increase of hepatic GSH and an increase of plasma NO. Furthermore, hepatic ROS levels were also found reduced in SHR administered WLPW, which would be associated with the increase of NO levels since it is known that ROS reduce endothelial NO availability [43].

Additionally, excessive ROS levels also produce lipid peroxidation, which generates lipid peroxyl radicals. In the last stages of lipid peroxidation of a biological membrane, MDA is produced by oxidation of polyunsaturated fatty acids within low-density lipoprotein (LDL) [33,34]. Hepatic MDA levels are considered a marker of tissue damage and failure of the antioxidant defense mechanisms [51]. Furthermore, MDA plays an important role in endothelial dysfunction, since it causes the inhibition of eNOS activity and expression, reducing NO availability [59]. Supplementation with red wine pomace has been shown to reduce the plasma MDA levels and increase plasma NO levels and eNOS activity in SHR [43]. In addition, studies carried out by Jurcevic et al. demonstrated the antioxidant capacity of WL phenolic compounds with a reduction of hepatic MDA levels in hypercholesterolemic mice [58]. All these results are in concordance with our findings, since plasma MDA levels were also reduced in SHR after administration of WLPW. It should be mentioned that MDA was measured by TBARS method and this methodology may limit the likelihood of detecting true differences in the level of plasma lipid peroxidation due to the specificity of the plasma TBARS assay being relatively low [60].

Therefore, all these results show that the improvement in oxidative stress and redox state would be one of the mechanisms involved in the antihypertensive effect of WLPW.

5. Conclusions

Results showed that the most effective antihypertensive dose of WL was 5.0 mL/kg bw, exhibiting similar effects than the ones showed by the antihypertensive drug Captopril. In addition, a significant enhancement of the BP-lowering effect was observed after the drying of WL due to the alcohol elimination during this drying process. A dose of 125 mg/kg bw, equivalent to 1.8 g/day in humans, caused a BP-lowering effect more potent than that obtained with Captopril and its effect was specific to the hypertensive state. Furthermore, it was evidenced that the WLPW effect on BP was mediated by a reduction in oxidative stress and an improvement of redox state and endothelial function. Nevertheless, further studies are needed to understand more deeply the effect of the extract in endothelial function and to evaluate its antihypertensive effect after a long-term administration. The evidence shows the potential use of WLPW as a nutraceutical or functional food ingredient in HTN prevention.

6. Patents

Patent application "Wine lees, derivatives thereof and their uses": application number EP20382358.8 and PCT/EP2021/053051.

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Institutional Review Board Statement: All animal procedures carried out in this study were in accordance with the European Communities Council Directive (86/609/EEC) and approved by both the Animal Ethics Review Committee for Animal Experimentation of the Universitat Rovira i Virgili and the Generalitat de Catalunya (permission number 10780).

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

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References

1. WHO. Hypertension. Available online: https://www.who.int/news-room/fact-sheets/detail/hypertension (accessed on 29 January 2021).
2. Nishiyama, A.; Kobori, H. Independent Regulation of Renin–Angiotensin–Aldosterone System in the Kidney. *Clin. Exp. Nephrol.* **2018**, *22*, 1231–1239, doi:10.1007/s10157-018-1567-1.
3. Te Riet, L.; van Esch, J.H.M.; Roks, A.J.M.; Van Den Meiracker, A.H.; Danser, A.H.J. Hypertension: Renin-Angiotensin-Aldosterone System Alterations. *Circ. Res.* **2015**, *116*, 960–975, doi:10.1161/CIRCRESAHA.116.303587.
4. Brown, N.J.; Vaughan, D.E. Angiotensin-Converting Enzyme Inhibitors. *Circulation* **1998**, *97*, 1411–1420, doi:10.1161/01.CIR.97.14.1411.
5. Shi, L.; Mao, C.; Xu, Z.; Zhang, L. Angiotensin-Converting Enzymes and Drug Discovery in Cardiovascular Diseases. *Drug Discov. Today* **2010**, *15*, 332–341, doi:10.1016/j.drudis.2010.02.003.

6. Girgih, A.T.; Nwachukwu, I.D.; Hasan, F.; Fagbemi, T.N.; Gill, T.; Aluko, R.E. Kinetics of the Inhibition of Renin and Angiotensin I-Converting Enzyme by Cod (Gadus Morhua) Protein Hydrolysates and Their Antihypertensive Effects in Spontaneously Hypertensive Rats. *Food Nutr. Res.* **2015**, *59*, doi:10.3402/fnr.v59.29788.
7. Chaudhary, P.; Pandey, A.; Azad, C.S.; Tia, N.; Singh, M.; Gambhir, I.S. Association of Oxidative Stress and Endothelial Dysfunction in Hypertension. *Anal. Biochem.* **2020**, *590*, 113535, doi:10.1016/j.ab.2019.113535.
8. Sies, H. Oxidative Stress: Oxidants and Antioxidants. *Exp. Physiol.* **1997**, *82*, 291–295, doi:10.1113/expphysiol.1997.sp004024.
9. Hsu, C.N.; Tain, Y.L. Early Origins of Hypertension: Should Prevention Start before Birth Using Natural Antioxidants? *Antioxidants* **2020**, *9*, 1–22, doi:10.3390/antiox9111034.
10. Chen, Q.; Wang, Q.; Zhu, J.; Xiao, Q.; Zhang, L. Reactive Oxygen Species: Key Regulators in Vascular Health and Diseases. *Br. J. Pharmacol.* **2018**, *175*, 1279–1292, doi:10.1111/bph.13828.
11. Kizhakekuttu, T.J.; Widlansky, M.E. Natural Antioxidants and Hypertension: Promise and Challenges. *Cardiovasc. Ther.* **2010**, *28*, e20–e32, doi:10.1111/j.1755-5922.2010.00137.x.
12. Trigo, J.P.; Alexandre, E.M.C.; Saraiva, J.A.; Pintado, M.E. High Value-Added Compounds from Fruit and Vegetable by-Products-Characterization, Bioactivities, and Application in the Development of Novel Food Products. *Crit. Rev. Food Sci. Nutr.* **2020**, *60*, 1388–1416, doi:10.1080/10408398.2019.1572588.
13. Wightman, J.D.; Heuberger, R.A. Effect of Grape and Other Berries on Cardiovascular Health. *J. Sci. Food Agric.* **2015**, *95*, 1584–1597, doi:10.1002/jsfa.6890.
14. Levantesi, G.; Marfisi, R.; Mozaffarian, D.; Franzosi, M.G.; Maggioni, A.; Nicolosi, G.L.; Schweiger, C.; Silletta, M.; Tavazzi, L.; Tognoni, G.; et al. Wine Consumption and Risk of Cardiovascular Events after Myocardial Infarction: Results from the GISSI-Prevenzione Trial. *Int. J. Cardiol.* **2013**, *163*, 282–287, doi:10.1016/j.ijcard.2011.06.053.
15. Haseeb, S.; Alexander, B.; Baranchuk, A. Wine and Cardiovascular Health. *Circulation* **2017**, *136*, 1434–1448, doi:10.1161/CIRCULATIONAHA.117.030387.
16. Quiñones, M.; Guerrero, L.; Suarez, M.; Pons, Z.; Aleixandre, A.; Arola, L.; Muguerza, B. Low-Molecular Procyanidin Rich Grape Seed Extract Exerts Antihypertensive Effect in Males Spontaneously Hypertensive Rats. *Food Res. Int.* **2013**, *51*, 587–595, doi:10.1016/j.foodres.2013.01.023.
17. Pons, Z.; Guerrero, L.; Margalef, M.; Arola, L.; Arola-Arnal, A.; Muguerza, B. Effect of Low Molecular Grape Seed Proanthocyanidins on Blood Pressure and Lipid Homeostasis in Cafeteria Diet-Fed Rats. *J. Physiol. Biochem.* **2014**, *70*, 629–637, doi:10.1007/s13105-014-0329-0.
18. Pons, Z.; Margalef, M.; Bravo, F.I.; Arola-Arnal, A.; Muguerza, B. Acute Administration of Single Oral Dose of Grape Seed Polyphenols Restores Blood Pressure in a Rat Model of Metabolic Syndrome: Role of Nitric Oxide and Prostacyclin. *Eur. J. Nutr.* **2015**, *55*, 749–758, doi:10.1007/s00394-015-0895-0.
19. Odai, T.; Terauchi, M.; Kato, K.; Hirose, A.; Miyasaka, N. Effects of Grape Seed Proanthocyanidin Extract on Vascular Endothelial Function in Participants with Prehypertension: A Randomized, Double-Blind, Placebo-Controlled Study. *Nutrients* **2019**, *11*, 2844, doi:10.3390/nu11122844.
20. Mas-Capdevila, A.; Iglesias-Carres, L.; Arola-Arnal, A.; Suárez, M.; Bravo, F.I.; Muguerza, B. Changes in Arterial Blood Pressure Caused by Long-Term Administration of Grape Seed Proanthocyanidins in Rats with Established Hypertension. *Food Funct.* **2020**, *11*, 8735–8742, doi:10.1039/D0FO00981D.
21. Quiñones, M.; Guerrero, L.; Fernández-Vallinas, S.; Pons, Z.; Arola, L.; Aleixandre, A.; Muguerza, B. Involvement of Nitric Oxide and Prostacyclin in the Antihypertensive Effect of Low-Molecular-Weight Procyanidin Rich Grape Seed Extract in Male Spontaneously Hypertensive Rats. *J. Funct. Foods* **2014**, *6*, 419–427, doi:10.1016/j.jff.2013.11.008.
22. Pérez-Serradilla, J.A.; de Castro, M.D.L. Role of Lees in Wine Production: A Review. *Food Chem.* **2008**, *111*, 447–456, doi:10.1016/j.foodchem.2008.04.019.
23. López-Fernández-Sobrino, R.; Soliz-Rueda, J.R.; Margalef, M.; Arola-Arnal, A.; Suárez, M.; Bravo, F.I.; Muguerza, B. ACE Inhibitory and Antihypertensive Activities of Wine Lees and Relationship among Bioactivity and Phenolic Profile. *Nutrients* **2021**, *13*, 679, doi:10.3390/nu13020679.
24. López-Fernández-Sobrino, R.; Margalef, M.; Torres-Fuentes, C.; Ávila-Román, J.; Aragonès, G.; Muguerza, B.; Bravo, F.I. Enzyme-Assisted Extraction to Obtain Phenolic-Enriched Wine Lees with Enhanced Bioactivity in Hypertensive Rats. *Antioxidants* **2021**, *10*, 517, https://doi.org/10.3390/antiox10040517.
25. OIV. *Código Prácticas Enológicas*; OIV: Paris, France, 2016; Volume 33.
26. Arranz, S.; Chiva-Blanch, G.; Valderas-Martínez, P.; Medina-Remón, A.; Lamuela-Raventós, R.M.; Estruch, R. Wine, Beer, Alcohol and Polyphenols on Cardiovascular Disease and Cancer. *Nutrients* **2012**, *4*, 759–781, doi:10.3390/nu4070759.
27. Cunniff, P. Official Method 969.33 of AOAC International. In *Official Methods of Analysis of AOAC Internationa*; Association of Official Analytical Chemists: Arlington, VA, USA, 1995.
28. Quiñones, M.; Miguel, M.; Muguerza, B.; Aleixandre, A. Effect of a Cocoa Polyphenol Extract in Spontaneously Hypertensive Rats. *Food Funct.* **2011**, *2*, 649–653, doi:10.1039/C1FO10119F.
29. Mas-Capdevila, A.; Pons, Z.; Aleixandre, A.; Bravo, F.I.; Muguerza, B. Dose-Related Antihypertensive Properties and the Corresponding Mechanisms of a Chicken Foot Hydrolysate in Hypertensive Rats. *Nutrients* **2018**, *10*, 1295, doi:10.3390/nu10091295.
30. Kamencic, H.; Lyon, A.; Paterson, P.G.; Juurlink, B.H.J. Monochlorobimane Fluorometric Method to Measure Tissue Glutathione. *Anal. Biochem.* **2000**, *286*, 35–37, doi:10.1006/abio.2000.4765.

31. Mas-Capdevila, A.; Iglesias-Carres, L.; Arola-Arnal, A.; Suarez, M.; Muguerza, B.; Bravo, F. I. Long-Term Administration of Protein Hydrolysate from Chicken Feet Induces Antihypertensive Effect and Confers Vasoprotective Pattern in Diet-Induced Hypertensive Rats. *J. Funct. Foods* **2019**, *55*, doi:10.1016/j.jff.2019.02.006.
32. Gabbia, D.; Pozzo, L.; Zigiotto, G.; Roverso, M.; Sacchi, D.; Pozza, A.D.; Carrara, M.; Bogialli, S.; Floreani, A.; Guido, M.; et al. Dexamethasone Counteracts Hepatic Inflammation and Oxidative Stress in Cholestatic Rats via CAR Activation. *PLoS ONE* **2018**, *13*, 1–21, doi:10.1371/journal.pone.0204336.
33. Grisham, M.B.; Johnson, G.G.; Gautreaux, M.D.; Berg, R.D. Measurement of Nitrate and Nitrite in Extracellular Fluids: A Window to Systemic Nitric Oxide Metabolism. *Methods* **1995**, *7*, 84–90, doi:10.1006/meth.1995.1012.
34. Fraga, C.G.; Croft, K.D.; Kennedy, D.O.; Tomás-Barberán, F.A. The Effects of Polyphenols and Other Bioactives on Human Health. *Food Funct.* **2019**, *10*, 514–528, doi:10.1039/c8fo01997e.
35. Ahmad, K.A.; Yuan Yuan, D.; Nawaz, W.; Ze, H.; Zhuo, C.X.; Talal, B.; Taleb, A.; Mais, E.; Qilong, D. Antioxidant Therapy for Management of Oxidative Stress Induced Hypertension. *Free Radic. Res.* **2017**, *51*, 428–438, doi:10.1080/10715762.2017.1322205.
36. Calabrese, V.; Cornelius, C.; Trovato-Salinaro, A.; Cambria, M.; Locascio, M.; Rienzo, L.; Condorelli, D.; Mancuso, C.; De Lorenzo, A.; Calabrese, E. The Hormetic Role of Dietary Antioxidants in Free Radical-Related Diseases. *Curr. Pharm. Des.* **2010**, *16*, 877–883, doi:10.2174/138161210790883615.
37. Son, T.G.; Camandola, S.; Mattson, M.P. Hormetic Dietary Phytochemicals. *NeuroMol. Med.* **2008**, *10*, 236–246, doi:10.1007/s12017-008-8037-y.
38. Halliwell, B. Are Polyphenols Antioxidants or Pro-Oxidants? What Do We Learn from Cell Culture and in Vivo Studies? *Arch. Biochem. Biophys.* **2008**, *476*, 107–112, doi:10.1016/j.abb.2008.01.028.
39. Eghbaliferiz, S.; Iranshahi, M. Prooxidant Activity of Polyphenols, Flavonoids, Anthocyanins and Carotenoids: Updated Review of Mechanisms and Catalyzing Metals. *Phyther. Res.* **2016**, *30*, 1379–1391, doi:10.1002/ptr.5643.
40. Quiñones, M.; Margalef, M.; Arola-Arnal, A.; Muguerza, B.; Miguel, M.; Aleixandre, A. The Blood Pressure Effect and Related Plasma Levels of Flavan-3-Ols in Spontaneously Hypertensive Rats. *Food Funct.* **2015**, *6*, 3479–3489, doi:10.1039/c5fo00547g.
41. Cienfuegos-Jovellanos, E.; del Mar Quiñones, M.; Muguerza, B.; Moulay, L.; Miguel, M.; Aleixandre, A. Antihypertensive Effect of a Polyphenol-Rich Cocoa Powder Industrially Processed to Preserve the Original Flavonoids of the Cocoa Beans. *J. Agric. Food Chem.* **2009**, *57*, 6156–6162, doi:10.1021/jf804045b.
42. Chiva-Blanch, G.; Urpi-Sarda, M.; Ros, E.; Arranz, S.; Valderas-Martínez, P.; Casas, R.; Sacanella, E.; Llorach, R.; Lamuela-Raventos, R.M.; Andres-Lacueva, C.; et al. Dealcoholized Red Wine Decreases Systolic and Diastolic Blood Pressure and Increases Plasma Nitric Oxide: Short Communication. *Circ. Res.* **2012**, *111*, 1065–1068, doi:10.1161/CIRCRESAHA.112.275636.
43. Del Pino-García, R.; Rivero-Pérez, M.D.; González-Sanjosé, M.L.; Croft, K.D.; Muñiz, P. Antihypertensive and Antioxidant Effects of Supplementation with Red Wine Pomace in Spontaneously Hypertensive Rats. *Food Funct.* **2017**, *8*, 2444–2454, doi:10.1039/c7fo00390k.
44. Lin, X.; Han, T.; Fan, Y.; Wu, S.; Wang, F.; Wang, C. Quercetin Improves Vascular Endothelial Function through Promotion of Autophagy in Hypertensive Rats. *Life Sci.* **2020**, *258*, 118106, doi:10.1016/j.lfs.2020.118106.
45. Reagan-Shaw, S.; Nihal, M.; Ahmad, N. Dose Translation from Animal to Human Studies Revisited. *FASEB J.* **2008**, *22*, 659–661, doi:10.1096/fj.07-9574lsf.
46. Alañón, M.E.; Castle, S.M.; Serra, G.; Lévèques, A.; Poquet, L.; Actis-Goretta, L.; Spencer, J.P.E. Acute Study of Dose-Dependent Effects of (−)-Epicatechin on Vascular Function in Healthy Male Volunteers: A Randomized Controlled Trial. *Clin. Nutr.* **2020**, *39*, 746–754, doi:10.1016/j.clnu.2019.03.041.
47. Rodríguez-Mateos, A.; Weber, T.; Skene, S.S.; Ottaviani, J.I.; Crozier, A.; Kelm, M.; Schroeter, H.; Heiss, C. Assessing the Respective Contributions of Dietary Flavanol Monomers and Procyanidins in Mediating Cardiovascular Effects in Humans: Randomized, Controlled, Double-Masked Intervention Trial. *Am. J. Clin. Nutr.* **2018**, *108*, 1229–1237, doi:10.1093/ajcn/nqy229.
48. Pons, Z.; Margalef, M.; Bravo, F.I.; Arola-Arnal, A.; Muguerza, B. Grape Seed Flavanols Decrease Blood Pressure via Sirt-1 and Confer a Vasoprotective Pattern in Rats. *J. Funct. Foods* **2016**, *24*, 164–172, doi:10.1016/j.jff.2016.03.030.
49. Calfío, C.; Huidobro-Toro, J.P. Potent Vasodilator and Cellular Antioxidant Activity of Endemic Patagonian Calafate Berries (Berberis Microphylla) with Nutraceutical Potential. *Molecules* **2019**, *24*, 2700, doi:10.3390/molecules24152700.
50. Brüll, V.; Burak, C.; Stoffel-Wagner, B.; Wolffram, S.; Nickenig, G.; Müller, C.; Langguth, P.; Alteheld, B.; Fimmers, R.; Naaf, S.; et al. Effects of a Quercetin-Rich Onion Skin Extract on 24 h Ambulatory Blood Pressure and Endothelial Function in Overweight-to-Obese Patients with (Pre-)Hypertension: A Randomised Double-Blinded Placebo-Controlled Cross-over Trial. *Br. J. Nutr.* **2015**, *114*, 1263–1277, doi:10.1017/S0007114515002950.
51. Uzun, H.; Karter, Y.; Aydin, S.; Çurgunlu, A.; Şimşek, G.; Yücel, R.; Vehiyd, S.; Ertürk, N.; Kutlu, A.; Benian, A.; et al. Oxidative Stress in White Coat Hypertension; Role of Paraoxonase. *J. Hum. Hypertens.* **2004**, *18*, 523–528, doi:10.1038/sj.jhh.1001697.
52. Rodrigo, R.; Prat, H.; Passalacqua, W.; Araya, J.; Guichard, C.; Bächler, J.P. Relationship between Oxidative Stress and Essential Hypertension. *Hypertens. Res.* **2007**, *30*, 1159–1167.
53. Tanito, M.; Nakamura, H.; Kwon, Y.W.; Teratani, A.; Masutani, H.; Shioji, K.; Kishimoto, C.; Ohira, A.; Horie, R.; Yodoi, J. Enhanced Oxidative Stress and Impaired Thioredoxin Expression in Spontaneously Hypertensive Rats. *Antioxid. Redox Signal.* **2004**, *6*, 89–97, doi:10.1089/152308604771978381.
54. Rubanyi, G.M.; Vanhoutte, P.M. Superoxide Anions and Hyperoxia Inactivate Endothelium-Derived Relaxing Factor. *Am. J. Physiol. Circ. Physiol.* **1986**, *250*, H822–H827, doi:10.1152/ajpheart.1986.250.5.h822.

55. Crowley, S.D. The Cooperative Roles of Inflammation and Oxidative Stress in the Pathogenesis of Hypertension. *Antioxid. Redox Signal.* **2014**, *20*, 102–120, doi:10.1089/ars.2013.5258.
56. Godos, J.; Vitale, M.; Micek, A.; Ray, S.; Martini, D.; del Rio, D.; Riccardi, G.; Galvano, F.; Grosso, G. Dietary Polyphenol Intake, Blood Pressure, and Hypertension: A Systematic Review and Meta-Analysis of Observational Studies. *Antioxidants* **2019**, *8*, 152, doi:10.3390/antiox8060152.
57. Forman, H.J.; Zhang, H.; Rinna, A. Glutathione: Overview of Its Protective Roles, Measurement, and Biosynthesis. *Mol. Asp. Med.* **2009**, *30*, 1–12, doi:10.1016/j.mam.2008.08.006.
58. Jurcevic, I.L.; Dora, M.; Guberovic, I.; Petras, M.; Brncic, S.R.; Dikic, D. Polyphenols from Wine Lees as a Novel Functional Bioactive Compound in the Protection against Oxidative Stress and Hyperlipidaemia. *Food Technol. Biotechnol.* **2017**, *55*, 109–116, doi:10.17113/ft b.55.01.17.4894.
59. Li, D.; Mehta, J.L. 3-Hydroxy-3-Methylglutaryl Coenzyme a Reductase Inhibitors Protect against Oxidized Low-Density Lipoprotein-Induced Endothelial Dysfunction. *Endothel. J. Endothel. Cell Res.* **2003**, *10*, 17–21, doi:10.1080/10623320303355.
60. Moselhy, H.F.; Reid, R.G.; Yousef, S.; Boyle, S.P. A Specific, Accurate, and Sensitive Measure of Total Plasma Malondialdehyde by HPLC. *J. Lipid Res.* **2013**, *54*, 852–858, doi:10.1194/jlr.D032698.

Manuscript 5:

Objective:

To establish the mechanisms underlying in the BP-lowering effect of the alcohol-fee WL in SHR. Since it has been evidenced that phenolic compounds can exhibit a BP-lowering effect by acting on components of the ACE system, improving endothelial function and reducing ROS, these antihypertensive mechanisms will be investigated

Blood pressure-lowering effect of wine lees phenolic compounds is mediated by endothelial-derived factors: role of sirtuin-1

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Keywords: Hypertension, nitric oxide, sirtuin, reactive oxygen species, endothelium, prostacyclin

Abbreviations: CVD, cardiovascular disease; BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; SHR, spontaneously hypertensive rats; WLP, wine lees powder; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; PGI_2, prostaglandin I_2

ABSTRACT

The antihypertensive effect of a wine lees powder (WLPW) from a Cabernet grape variety was recently demonstrated. This activity was related to its high content in phenolic compounds. This study investigates the involvement of endothelial-derived factors and SIRT1 in its bioactivity. Spontaneously hypertensive rats (SHR) were orally administered water or WLPW (125 mg/kg bw). Posteriorly, both groups were intraperitoneally administered saline, Nω-nitro-L-arginine methyl ester (L-NAME), a nitric oxide (NO) synthesis inhibitor, indomethacin, a prostacyclin synthesis inhibitor, or sirtinol, an inhibitor of sirtuins. Blood pressure (BP) was recorded before and 6h after WLPW administration. In an additional experiment, SHR were administered water or WLPW and endothelial expressions of *eNos*, *Sirt1*, *Nox4* and *Et1* were determined. The BP-lowering properties of WLPW were abolished by L-NAME and partially reduced by indomethacin, demonstrating that WLPW antihypertensive effect was mediated by changes in NO availability, although prostacyclin also contributed to this activity. Moreover, BP-lowering effect was reduced by sirtinol, indicating that WLPW decreased BP in a SIRT1-dependent manner. Furthermore, WLPW upregulated *eNos* and *Sirt1* and downregulated *Nox4* and *Et1* endothelial gene expression. These results evidence the vasoprotective effect of WLPW and show that its antihypertensive effect in SHR is endothelium dependent and mediated by SIRT1.

1. Introduction

Endothelium plays an important role in the regulation of vascular tone and blood fluidity by balancing the production of endothelium-derived vasodilator and vasoconstrictor factors [1]. Alterations in its functionality, called endothelial dysfunction, are associated to different diseases including hypertension (HTN). This dysfunction produces an imbalance between vasodilator and vasoconstrictor factors by decreasing the availability of vasodilators, mainly nitric oxide (NO) and/or increasing the production of vasoconstrictor factors [2].

NO is the main endothelial-derived vasodilator factor involved in vascular tone regulation [3]. Its endothelium production is mediated by the endothelial NO synthase (eNOS), that converts L-arginine in NO [4]. Activation of this enzyme depends on intracellular Ca^{2+} levels, which mediate eNOS detachment from caveolin [5]. In response to oxidative stress, endothelium-derived NO production is enhanced by Sirtuin 1 (SIRT1), a NAD^{+}-dependent deacetylase, which increases *eNos* transcription and enzymatic activity of eNOS via its deacetylation [6][7]. Furthermore, SIRT1 also decrease the NADPH oxidase (NOX)-dependent production of reactive oxygen species (ROS), acting on gene expression and activity of the vascular NOX subunits p22phox and 4 (NOX4) [8]. An excess in ROS levels leads to endothelial dysfunction and vasoconstriction since ROS react to NO, producing peroxynitrite and decreasing NO bioavailability [2]. In addition to NO, prostaglandin I_2 (PGI_2), also namely as prostacyclin, is also an important vasodilator implicated in endothelial regulation [9]. However, it seems that PGI_2 exerts its effect when NO levels are reduced [10]. Endothelial PGI_2 is produced by cyclooxygenase (COX) isomer 2, which catalyses the conversion of arachidonic acid to prostaglandin H_2 (PGH_2). PGH_2 is further transformed into PGI_2 by prostacyclin synthase [11]. Opposite to endothelial-derived relaxing factors, endothelin 1 (ET-1) is an endothelial-derived vasoconstrictor factor, which is overexpressed in the vasculature of

different hypertensive models [12]. ET-1 is produced by the proteolytic cleavage of its precursor, big ET-1, by endothelin converting enzyme [13].

We have previously demonstrated the antihypertensive effect of wine lees (WL) from Cabernet grape variety in spontaneously hypertensive rats (SHR). The BP-lowering effect was related to the higher content of flavanols and anthocyanins present in these WL respect to the other varieties studied [14]. A dose of 125 mg/kg bw of dealcoholized WL powder (WLPW), which would be equivalent to 1.8 g/day in humans, caused an antihypertensive effect more potent than the obtained with the antihypertensive drug captopril [15]. This potent BP-lowering property was associated to an improvement of oxidative stress state of WLPW-treated animals and attributed to its high content in flavanols and anthocyanins [15]. Nevertheless, different studies have shown that grape phenolic compounds can also exert their antihypertensive effect through an improvement of endothelial dysfunction. In this regard, Kondrashov *et al*. observed an increase in eNOS activity after an acute administration of a red wine extract to SHR [16]. In addition, long-term administration of a grape seed proanthocyanidin extract (GSPE) to diet-induced hypertensive rats produced an antihypertensive effect and conferred a vasoprotective pattern, including an overexpression of endothelial *Sirt1* [17]. According to this, the acute antihypertensive effect of GSPE in SHR was completely abolished by sirtinol, an inhibitor of sirtuins, indicating that grape seed flavanols decrease BP in a SIRT1-dependent manner. Thus, the aim of this study was to evaluate the involvement of endothelial-derived factors on the BP-lowering effect of WLPW and to study a potential role of SIRT1.

2. Materials and methods

2.1. Obtaining and Characterization of wine lees

WL from grapes of Cabernet variety were provided by Grandes Vinos y Viñedos, S.A, located in the Cariñena P.O.D area (Zaragoza, Spain). WL were centrifuged at 3,000 × *g* for 15 min at 4 °C. The supernatant was collected, freeze-dried and

grounded to obtain the WLPW. Finally, WLPW was kept at room temperature and protected from light exposure and humidity until its administration to animals.

Humidity, total protein content, measured by Kjeldahl method, and total phenolic content, measured by Folin-Ciocalteu method, of WLPW were 7.85 ± 1.49 %, 222.61 ± 5.73 mg/g of wet weight and 76.40 ± 0.74 mg gallic acid equivalents (GAE)/g of wet weight, respectively [15]. Tables S1 and S2 show the WLPW phenolic profile. Individual phenolic compounds were characterised by using a UHPLC-ESI-Q-TOF-MS system using 1290 UHPLC Infinity II series coupled to a Q-TOF/MS 6550 (Agilent Technologies, Palo Alto, CA, USA). Both negative and positive ionization ([M-H]- or [M-H]+) were used to identify parental ions and fragmentation patterns.

2.2. Dosage regimen and experimental procedure in animal

Male SHR (19–22 weeks old) weighing between 340-390 g and purchased from Charles River Laboratories España S.A. (Barcelona, Spain) were used in this study. They were singly housed in animal quarters at 22 °C and 50% of humidity with a light/dark period of 12 h. Tap water and standard diet (A04 Panlab, Barcelona, Spain) were provided *ad libitum* during the experiments. Figure 1 shows a graphical representation of the two experimental designs used in this study.

Rats were administered tap water or 125 mg/kg bw WLPW (human equivalent dose 1.8 g/day) dissolved in tap water by oral gavage between 8:00 and 9:00 a.m. Total volume administered to rats was 1.5 mL. Four hours after oral administration of these treatments, animals were intraperitoneal administered with 1 mL of saline solution, 30 mg/kg bw L-NAME (PubChem CID: 135193; Sigma-Aldrich), 5 mg/kg bw indomethacin (PubChem CID: 3715; Sigma-Aldrich) or 1 mg/kg bw sirtinol (PubChem CID: 2827646; Sigma-Aldrich), obtaining the following working groups: Water + Saline, Water + L-NAME, Water + Indomethacin, Water + Sirtinol, WLPW + Saline, WLPW + L-NAME, WLPW +

Indomethacin, WLPW + Sirtinol (n=6 per group). All treatments were prepared in saline solution.

Systolic and diastolic BP (SBP and DBP respectively) were measured by *tail cuff* method following the method described by Quiñones *et al*. [18] before and 6 h after oral administration of water or WLPW to SHR. To minimize stress-induced variations in BP and guarantee a correct BP values, animals were trained for 2 weeks after a 10-days adaptation period and all measurements were taken in a peaceful environment and by the same person.

In the second study, male SHR (19–22 weeks old) weighing between 336-392 g and purchased from Charles River Laboratories España S.A. (Barcelona, Spain) were also used. Diet and housing conditions were the same as the first mentioned study. Animals were divided in two groups and were administered tap water (1.5 mL) or WLPW (125 mg/kg bw, dissolved in 1.5 mL of tap water) by oral gavage (n = 6 per group). Six-hour post-administration, animals were sacrificed by live decapitation. Aorta was excised and immediately frozen in liquid nitrogen for further study.

The animal protocols followed in this study were conducted in accordance with the European Communities Council Directive (86/609/EEC) and approved by the Animal Ethics Review Committee for Animal Experimentation of the Universitat Rovira i Virgili and further approved by Generalitat de Catalunya (permission number 10780).

2.3. RNA Extraction and mRNA Quantification by Real-Time qPCR

The frozen aorta was homogenized in a TissueLyser (Qiagen, Barcelona, Spain) without buffer and with stainless steel balls. Then, lysis buffer was immediately added for RNA extraction using the RNeasy Mini Kit (RNeasy Mini Kit, Qiagen). Total extracted RNA was quantified using a Nanodrop 100 Spectrophotometer (ThermoFisher Scientific, Madrid, Spain).

mRNA reverse transcription was carried out by using the High Capacity cDNA Reverse Transcription Kit (AppliedBiosystems, Madrid, Spain). Quantitative PCR amplification and detection were performed in a 96-well plate using SYBR PCR Premix Reagent Ex Taq™ (Takara, Barcelona, Spain) and the CFX96 Touch Real Time PCR System (Bio-Rad, Barcelona, Spain) following the manufacturer procedures.

Relative mRNA levels of e*Nos*, *Sirt1*, *Nox4*, and *Et1* were analysed by real-time PCR using peptidyl prolyl isomerase A (*Ppia*) as the housekeeping gene. Table 1 shows the primers used, which were obtained from Biomers (Söflinger, Germany). Melting curve analysis and gel electrophoresis separation (3% agarose) were used to verified primer specificity and amplicon size, respectively. qPCR efficiency was calculated by evaluating a 2-fold dilution series of aortic cDNA and calculated by E = 10(1/slope). The results were expressed as the logarithm of the cDNA concentration vs. the obtained Ct. The relative expression or relative quantification was calculated by $RQ = (E_{target})^{\Delta Ct\ (target)} / (E_{reference})^{\Delta Ct\ (reference)}$ where ΔCt (target) = Ct (target gene calibrator) – Ct (target gene in test) and ΔCt (reference) = Ct (reference gene calibrator) – Ct (reference gene in test). Each sample was performed at least in duplicate.

2.4. Statistical analysis

BP differences produced by the treatments were analysed by a one-way analysis of variance (ANOVA). Student's T-test was used to evaluate differences between groups in gene expression. All the analyses were performed using GraphPad Prism 7.04 for Windows (GraphPad Software, San Diego, California). Outliers were determined by using Grubbs' test. Differences between groups were considered significant when $p < 0.05$.

3. Results

In the first study, the effects of WL phenolic compounds on BP in rats treated with L-NAME, indomethacin or sirtinol were investigated. Initial values of SBP and DBP in these animals were 193 ± 6.8 and 147 ± 8.2 mmHg, respectively.

Figure 2, 3 and 4 show the BP changes in SHR 6 h after oral administration of water or WLPW (125 mg/kg bw), and additionally treated intraperitoneally with saline solution, L-NAME, indomethacin or sirtinol.

BP observed in Water + Saline group did not change by the treatment. Nevertheless, as expected, the oral administration of WLPW (WLPW + Saline group) produced a significant decrease in both SPB and DBP (-28 ± 2.1 and -30 ± 8.02 mmHg, respectively; $p \leq 0.05$) respect to Water + Saline group (Figures 2, 3 and 4).

When animals were intraperitoneally treated with L-NAME (30 mg/kg bw), it caused an increase in SBP of water group. No changes were observed in DBP. Regarding WLPW + L-NAME group, SBP and DBP values were similar to those observed for Water + Saline group, showing a total loss of WLPW antihypertensive effect observed in WLPW + Saline group (Figure 2).

No SBP and DBP changes were observed after intraperitoneal administration of indomethacin (5 mg/kg bw) and sirtinol (1 mg/kg bw) in the two respective water groups (Water + Indomethacin and Water + Sirtinol, respectively) (Figures 3 and 4, respectively). SBP values of WLPW + Indomethacin and WLPW + Sirtinol groups were significantly lower than the one showed by Water + Saline group, showing an antihypertensive effect. However, their BP-lowering effects were less potent to those observed by WLPW + Saline group (Figures 3A and 4A, respectively). In the case of DBP, the values found in the WLPW + Indomethacin and WLPW + Sirtinol groups were similar to those found in their respective control groups (Water + Sirtinol and Water + Indomethacin, respectively) (Figures 3B and 4B).

In the second study, the expression of endothelial function-associated genes was studied at 6 h post-administration of water or WLPW to SHR (Figure 5). Administration of WLPW significantly upregulated the expression of e*Nos* and *Sirt1* (1.9 and 2.9 times higher, respectively) (Figure 5A), while that the

expression of the *Et1* and *Nox4,* involved in a vasoconstrictor effect, were significantly downregulated by WLPW intake (Figure 5B).

4. Discussion

Dietary patterns based on the consumption of fruits and vegetables are associated with lower risk of HTN and an improvement of endothelial function [19]. These beneficial effects are mainly related to the phenolic compounds present in these foods [20–22]. In this sense, a previous study carried out by our group in SHR demonstrated that WLPW administration at an acute dose of 125 mg/kg bw to SHR produced a potent decrease on BP, showing the maximum effect at 6 h post-administration. This effectiveness was attributed to its high content in phenolic compounds, mainly in flavanols and anthocyanins. In addition, it was evidenced that WLPW exerts its antihypertensive action via an improvement of oxidative stress since reduced levels of hepatic ROS and plasma malondialdehyde and increased levels of hepatic reduced glutathione (an endogenous antioxidant) were observed in the treated animals [15]. However, an improvement of endothelial function after WLPW administration should not be ruled out since it has been reported as one of the mechanisms involved in the antihypertensive effect of other winery by-products to hypertensive rats [17, 23, 24]. Therefore, the objective of this study was to elucidate the potential role of endothelial-derived factors and SIRT1 in the antihypertensive effect of WLPW. In order to achieve this goal, we treated SHR with Nw-nitro-L-arginine methyl ester (L-NAME), an inhibitor of eNOS, indomethacin, an inhibitor of COX and sirtinol, an inhibitor of sirtuins synthesis. Furthermore, the aortic expression levels of different genes involved in endothelial dysfunction were evaluated after WLPW administration in other additional experiment. Both studies were carried out at 6h post-administration since at this time point it is observed the maximum BP decrease caused by WLPW [15].

Initially, we focused in studying the role of NO in the antihypertensive effect of WL phenolic compounds. NO is the main vasodilator factor produced and

released by the endothelium via eNOS [4]. NO activates guanylyl cyclase that produces cyclic guanosine monophosphate. This molecule leads to relaxation of the muscle layer and vasodilation [4, 25]. Due to this activity, animals were intraperitoneal administered L-NAME, an inhibitor of eNOS, 4 h after oral administration of WLPW to inhibit the endothelial NO production. Its administration produced a total loss of the SBP and DBP-lowering effects showed by WLPW. Therefore, these results provide clear evidence that antihypertensive effect of WLPW is NO-mediated in SHR. According to this, human clinical studies have shown that moderate consumption of aged white wine produces a higher bioavailability of NO, which was linked to a reduction in BP [26]. Similar to our results, these effects were attributed to the presence of grape-derived compounds in the aged white wine, such as polyphenols. In addition, our results are also in concordance with previous results published by our group with other grape phenolic-rich extracts and flavanol-rich food [24, 27]. In this regard, Quiñones *et al*. and Pons *et al*. observed that the antihypertensive effect of an acute dose of 375 mg/kg bw of GSPE to SHR and to cafeteria diet-induced hypertensive rats, respectively, was totally abolished when animals were additionally treated with L-NAME [24, 27]. Same effect was also observed in SHR treated flavanol-rich cocoa in acute dose (300 mg/kg bw), when eNOS activity was inhibited [28].

Nevertheless, the BP-lowering effect of WLPW has been also associated with the high content of the phenol family of anthocyanins [14], which have also shown vascular benefits and their circulating metabolites have been directly related to these properties [29]. In this regard, the beneficial effect of anthocyanin rich foods or extracts on vascular health has been evidenced in a meta-analysis of randomised controlled trials [30]. Furthermore, the involvement of NO in the endothelium-dependent vascular relaxing effect of extracts rich in anthocyanins, and also in flavanols, has been also corroborated in *ex vivo* vascular reactivity studies carried out in presence of eNOS inhibitors [24] [31].

Studies conducted with grapes phenolic compounds using GSPE in a model of cafeteria diet-fed hypertensive rats and a red wine extract in SHR showed that these extracts increased aortic e*Nos* expression [16, 17, 23]. Our findings are in line of these studies since the expression of aortic *eNos* of WLPW-treated SHR was higher than those showed by untreated animals, suggesting that WL phenolic compounds could increase NO availability increasing *eNos* expression. Nevertheless, the increase of endothelial NO levels can be also due to changes in eNOS activity. SIRT1 promotes endothelium-dependent vasodilation by targeting eNOS for deacetylation, increasing the activity and/or expression of this enzyme [32]. It has been evidenced that SIRT1 is involved in the beneficial effects of some phenolic compounds present in grapes, wine and grape by-products such as proanthocyanidins, resveratrol or quercetin [33], [23]. Pons *et al*. reported that the antihypertensive effect of a grape seed proantocyanindin extract totally disappeared when SIRT1 activity was inhibited [23]. Specifically, the effect of the extract could be mediated by an upregulation of endothelial *Sirt1* expression since the aortic *Sirt1* expression was increased after its acute and chronic administration [17, 23]. In our study, the antihypertensive effect of WLPW was partially abolished by SBP and totally by DBP, when animals were treated with WLPW and sirtinol. Furthermore, WLPW produced an upregulation of endothelial *Sirt1* (1.5 times higher than water group). These results showed that SIRT1 is clearly involved in the BP-lowering process produced by WLPW. Flavanols present in WLPW, similarly to GSPE, could be the bioactive compounds responsible of this effect on SIRT1. However, it should not be ruled out that other phenolic compounds could be involved. Kitada *et al*. observed an upregulation of peripheral blood mononuclear *SIRT1* expression in volunteers consuming a non-alcoholic red wine extract during 8 weeks. The consumed daily dose contained 19.2 mg of resveratrol and 136 mg of other polyphenols including tanins, catechin, epicatechin or quercetin glucoside [34]. In addition, resveratrol and quercetin, also present in WLPW, were also reported to be activators of SIRT1 in other studies [33].

In addition to the effect of SIRT1 on eNOS, this deacetylase also can modulate the activity and expression of some vascular NOX such as NOX4, one of the main producers of ROS in endothelium [8], [35]. An overproduction of ROS linked to a oxidative stress state leads to reduces NO bioavailability since ROS scavenge NO to produce peroxynitrite [36], avoiding NO-induced vasodilation. There are evidences of the presence and possible involvement of oxidative stress in the antihypertensive process in SHR [37, 38]. In a previous study of our group, the antioxidant properties of WLPW were demonstrated in SHR after its acute administration. WLPW caused a decrease in ROS levels and an increase in reduced glutathione (GSH) in liver as well as a decrease in MDA and increase of NO in plasma [15]. In order to know if an antioxidant effect would be also produced in the endothelium, we studied the levels of *Nox4* expression in aorta of SHR treated with WLPW at 6 h post-administration. Aortic *Nox4* expression was drastically reduced, suggesting an important implication of this endothelial enzyme in the effect of WL phenolic compounds. Since SIRT1 can downregulate *Nox4* [8] and WLPW intake produced an upregulation of *Sirt1* expression in the present study, the increase of expression of *Sirt1* could be one of the mechanisms involved in the increase of NO availability. Our findings are in agreement with the effect observed for other antihypertensive grape flavanols, which produce a downregulation of *Nox4* [17, 23]. Furthermore, WLPW is rich in anthocyanins, which also may be involved in the downregulation of *Nox4*. Anthocyanins as malvidin, malvidin-6-glucoside or malvidin-6-galactoside from blueberry, also found in WLPW, have been demonstrated to reduce *NOX4* expression in high glucose-induced human umbilical cord vein endothelial cells (HUVECs) [39]. In addition, Galindo *et al*. have demonstrated that quercetin, also found in a great quantity in WLPW, reduces the aortic expression of *Nox4* after five weeks of administration [40].

Furthermore, it was studied the effect of WLPW in the production of endothelial ET-1. ET-1 is a potent vasoconstrictor and several studies have reported an overexpression of *Et1* in the endothelium in hypertensive state

[41]. In this sense, our results showed a reduction of aortic *Et1* gene expression 6 h after administration of WLPW, rich in flavanols and anthocyanins (**Figure 5**), suggesting their implication in the reduction of BP. Food by-products extracts rich in flavanols like GSPE have shown a reduction in aortic *Et1* mRNA expression levels in hypertensive rats [23, 24]. Furthermore, the administration of food extracts rich in anthocyanins reduced endothelial expression of *ET1* in HUVECs, which were induced endothelial dysfunction by hyperglycemia [42]. Specifically, in the same cells without induced endothelial dysfunction, the anthocyanins delphinidin and cyanidin exerted a reduction of ET-1 secretion and expression, where the higher effect was demonstrated by delphinidin [43]. In addition, a relationship between ET-1 and NOX has been reported. In this regard, it has been observed ET-1 increases the production of ROS in the vasculature through the activation of NOX [44, 45]. A reduction in *Et1* and *Nox4* gene expression has been found after the administration of WLPW. All these findings showed that WL phenolic compounds could improve the endothelial dysfunction linked to HTN, balancing the endothelium-derived vasodilator and vasoconstrictor factors.

In addition to NO, PGI_2 is another important endothelium-derived vasodilator involved in the regulation of BP, which is generated by the action of COX and prostacyclin synthase [11]. The administration of the COX inhibitor indomethacin to animals produces the inhibition of PGI_2 production. Our results showed that the antihypertensive effect of WLPW is also mediated by PGI_2 as animals treated WLPW and indomethacin showed a lower antihypertensive effect that the one observed in WLPW + Saline group. Similar results were founded with GSPE. The antihypertensive effect of that extract was partially mediated by prostacyclin in SHR and cafeteria diet-induced hypertensive rats [24, 27]. According to this, some studies have shown an increase in the release of PGI_2 in procyanidin-treated human aortic endothelial cells [46]. In addition, increased levels of prostacyclin have been observed in the plasma of rats [47, 48] and humans [46] after the consumption of flavanols.

5. Conclusions

In this study, we demonstrated the implication of NO and SIRT1 in the antihypertensive effect exerted by WLPW in SHR (Figure 6). WL phenolic compounds increase e*Nos* and *Sirt1* m-RNA levels in the endothelium and could increase the eNOS activity. Furthermore, WL phenolic compounds also reduce the endothelial expression of *Nox4* and *Et1*, which would reduce endothelial ROS production and therefore increase the availability of vascular NO as well as the vasoconstrictor ET-1. In addition, a partial involvement of PGI_2 in the antihypertensive effect of WLPW is also found, although more studies are needed to deep in this effect. Thus, WL phenolic compounds improve endothelium functionality and reduce BP in SHR. All these results suggest that WLPW could be a potential functional food ingredient with many benefits in the treatment of cardiovascular disease.

6. Patents

Patent application “Wine lees, derivatives thereof and their uses”: application number EP20382358.8 and PCT/EP2021/053051.

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Conflicts of Interest:

The authors declare no conflict of interest.

Bibliography

[1] Spieker, L. E.; Flammer, A. J.; Lüscher, T. F. The Vascular Endothelium in Hypertension. *Handb. Exp. Pharmacol.*, **2006**, *176* (PART2), 249–283. https://doi.org/10.1007/3-540-36028-x_8.

[2] Konukoglu, D.; Uzun, H. Endothelial Dysfunction and Hypertension. In *Advances in Experimental Medicine and Biology*; Springer New York LLC, 2016; Vol. 956, pp 511–540. https://doi.org/10.1007/5584_2016_90.

[3] Vallance, P.; Collier, J.; Moncada, S. Effects of Endothelium-Derived Nitric Oxide on Peripheral Arteriolar Tone in Man. *Lancet*, **1989**, *334* (8670), 997–1000. https://doi.org/10.1016/S0140-6736(89)91013-1.

[4] Förstermann, U.; Sessa, W. C. Nitric Oxide Synthases: Regulation and Function. *European Heart Journal*. 2012, pp 829–837. https://doi.org/10.1093/eurheartj/ehr304.

[5] Bucci, M.; Gratton, J.-P.; Rudic, R. D.; Acevedo, L.; Roviezzo, F.; Cirino, G.; Sessa, W. C. In Vivo Delivery of the Caveolin-1 Scaffolding Domain Inhibits Nitric Oxide Synthesis and Reduces Inflammation. *Nat. Med.*, **2000**, *6* (12), 1362–1367. https://doi.org/10.1038/82176.

[6] Mattagajasingh, I.; Kim, C. S.; Naqvi, A.; Yamamori, T.; Hoffman, T. A.; Jung, S. B.; DeRicco, J.; Kasuno, K.; Irani, K. SIRT1 Promotes Endothelium-Dependent Vascular Relaxation by Activating Endothelial Nitric Oxide Synthase. *Proc. Natl. Acad. Sci. U. S. A.*, **2007**, *104* (37), 14855–14860. https://doi.org/10.1073/pnas.0704329104.

[7] Man, A. W. C.; Li, H.; Xia, N. The Role of Sirtuin1 in Regulating Endothelial Function, Arterial Remodeling and Vascular Aging. *Frontiers in Physiology*. September 2019, p 1173. https://doi.org/10.3389/fphys.2019.01173.

[8] Zarzuelo, M. J.; López-Sepúlveda, R.; Sánchez, M.; Romero, M.; Gómez-Guzmán, M.; Ungvary, Z.; Pérez-Vizcaíno, F.; Jiménez, R.; Duarte, J. SIRT1 Inhibits NADPH Oxidase Activation and Protects Endothelial Function in the Rat Aorta: Implications for Vascular Aging. *Biochem. Pharmacol.*, **2013**, *85* (9), 1288–1296. https://doi.org/10.1016/j.bcp.2013.02.015.

[9] Qian, H.; Luo, N.; Chi, Y. Aging-Shifted Prostaglandin Profile in Endothelium as a Factor in Cardiovascular Disorders. *Journal of Aging Research*. 2012, pp 1–16. https://doi.org/10.1155/2012/121390.

[10] Sandoo, A.; Veldhuijzen van Zanten, J. J. C. .; Metsios, G. S.; Carroll, D.; Kitas, G. D. The Endothelium and Its Role in Regulating Vascular Tone. *Open Cardiovasc. Med. J.*, **2015**, *4* (1), 302–312. https://doi.org/10.2174/1874192401004010302.

[11] Mitchell, J. A.; Kirkby, N. S. Eicosanoids, Prostacyclin and Cyclooxygenase in the Cardiovascular System. *British Journal of Pharmacology*. John Wiley and Sons Inc. April 1, 2019, pp 1038–1050. https://doi.org/10.1111/bph.14167.

[12] Iglarz, M.; Schiffrin, E. L. Role of Endothelin-1 in Hypertension. *Curr. Hypertens. Rep.*, **2003**, *5* (2), 144–148. https://doi.org/10.1007/s11906-003-0071-4.

[13] Stauffer, B. L.; Westby, C. M.; DeSouza, C. A. Endothelin-1, Aging and Hypertension. *Current Opinion in Cardiology*. NIH Public Access July 2008, pp 350–355. https://doi.org/10.1097/HCO.0b013e328302f3c6.

[14] López-Fernández-Sobrino, R.; Soliz-Rueda, J. R.; Margalef, M.; Arola-Arnal, A.; Suárez, M.; Bravo, F. I.; Muguerza, B. ACE Inhibitory and Antihypertensive Activities of Wine Lees and Relationship among Bioactivity and Phenolic Profile. *Nutrients*, **2021**, *13* (2), 679. https://doi.org/10.3390/nu13020679.

[15] López-Fernández-Sobrino, R.; Soliz-Rueda, J. R.; Ávila-Román, J.; Arola-Arnal, A.; Suárez, M.; Muguerza, B.; Bravo, F. I. Blood Pressure-Lowering Effect of Wine Lees Phenolic Compounds Is Mediated by Endothelial-Derived Factors: Role of Sirtuin-1. *Nutrients*, **2021**, *13* (4), 1142. https://doi.org/10.3390/nu13041142.

[16] Kondrashov, A.; Vranková, S.; Dovinová, I.; Ševčík, R.; Parohová, J.; Barta, A.; Pecháňová, O.; Kovacsová, M. The Effects of New Alibernet Red Wine Extract on Nitric Oxide and Reactive Oxygen Species Production in Spontaneously Hypertensive Rats. *Oxid. Med. Cell. Longev.*, **2012**, *2012*, 1–8. https://doi.org/10.1155/2012/806285.

[17] Mas-capdevila, A.; Iglesias-carres, L.; Arola-arnal, A.; Suárez, M.; Francisca, I. Changes in Arterial Blood Pressure Caused by Long-Term Administration of Grape Seed Proanthocyanidins in Rats with Established Hypertension. *Food Funct.*, **2020**, *11* (10), 8735–8742. https://doi.org/10.1039/D0FO00981D.

[18] Quiñones, M.; Miguel, M.; Muguerza, B.; Aleixandre, A. Effect of a Cocoa Polyphenol Extract in Spontaneously Hypertensive Rats. *Food Funct.*, **2011**, *2* (11), 649. https://doi.org/10.1039/c1fo10119f.

[19] Rodríguez-Monforte, M.; Flores-Mateo, G.; Sánchez, E. Dietary Patterns and CVD: A Systematic Review and Meta-Analysis of Observational Studies. *Br. J. Nutr.*, **2013**, *114* (9), 1341–1359. https://doi.org/10.1017/S0007114515003177.

[20] Li, B.; Li, F.; Wang, L.; Zhang, D. Fruit and Vegetables Consumption and Risk of

Hypertension: A Meta-Analysis. *J. Clin. Hypertens.*, **2016**, *18* (5), 468–476. https://doi.org/10.1111/jch.12777.

[21] Fraga, C. G.; Croft, K. D.; Kennedy, D. O.; Tomás-Barberán, F. A. The Effects of Polyphenols and Other Bioactives on Human Health. *Food Funct.*, **2019**, *10* (2), 514–528. https://doi.org/10.1039/c8fo01997e.

[22] Zhao, C.-N.; Meng, X.; Li, Y.; Li, S.; Liu, Q.; Tang, G.-Y.; Li, H.-B. Fruits for Prevention and Treatment of Cardiovascular Diseases. *Nutrients*, **2017**, *9* (6), 598. https://doi.org/10.3390/nu9060598.

[23] Pons, Z.; Margalef, M.; Bravo, F. I.; Arola-Arnal, A.; Muguerza, B. Grape Seed Flavanols Decrease Blood Pressure via Sirt-1 and Confer a Vasoprotective Pattern in Rats. *J. Funct. Foods*, **2016**, *24*, 164–172. https://doi.org/10.1016/j.jff.2016.03.030.

[24] Quiñones, M.; Guerrero, L.; Fernández-Vallinas, S.; Pons, Z.; Arola, L.; Aleixandre, A.; Muguerza, B. Involvement of Nitric Oxide and Prostacyclin in the Antihypertensive Effect of Low-Molecular-Weight Procyanidin Rich Grape Seed Extract in Male Spontaneously Hypertensive Rats. *J. Funct. Foods*, **2014**, *6* (1), 419–427. https://doi.org/10.1016/j.jff.2013.11.008.

[25] Kang, K. T. Endothelium-Derived Relaxing Factors of Small Resistance Arteries in Hypertension. *Toxicol. Res.*, **2014**, *30* (3), 141–148. https://doi.org/10.5487/TR.2014.30.3.141.

[26] Roth, I.; Casas, R.; Ribó-Coll, M.; Estruch, R. Consumption of Aged White Wine under a Veil of Flor Reduces Blood Pressure-Increasing Plasma Nitric Oxide in Men at High Cardiovascular Risk. *Nutrients*, **2019**, *11* (6), 1266. https://doi.org/10.3390/nu11061266.

[27] Pons, Z.; Margalef, M.; Bravo, F. I.; Arola-Arnal, A.; Muguerza, B. Acute Administration of Single Oral Dose of Grape Seed Polyphenols Restores Blood Pressure in a Rat Model of Metabolic Syndrome: Role of Nitric Oxide and Prostacyclin. *Eur. J. Nutr.*, **2015**, *55* (2), 749–758. https://doi.org/10.1007/s00394-015-0895-0.

[28] Quiñones, M.; Muguerza, B.; Miguel, M.; Aleixandre, A. Evidence That Nitric Oxide Mediates the Blood Pressure Lowering Effect of a Polyphenol-Rich Cocoa Powder in Spontaneously Hypertensive Rats. *Pharmacol. Res.*, **2011**, *64* (5), 478–481. https://doi.org/10.1016/j.phrs.2011.06.005.

[29] Rodriguez-Mateos, A.; Istas, G.; Boschek, L.; Feliciano, R. P.; Mills, C. E.; Boby, C.; Gomez-Alonso, S.; Milenkovic, D.; Heiss, C. Circulating Anthocyanin Metabolites Mediate Vascular Benefits of Blueberries: Insights from Randomized Controlled Trials, Metabolomics, and Nutrigenomics. *Journals Gerontol. - Ser. A Biol. Sci. Med. Sci.*, **2019**, *74* (7), 967–976. https://doi.org/10.1093/gerona/glz047.

[30] Fairlie-Jones, L.; Davison, K.; Fromentin, E.; Hill, A. M. The Effect of Anthocyanin-Rich Foods or Extracts on Vascular Function in Adults: A Systematic Review and Meta-Analysis of Randomised Controlled Trials. *Nutrients*. August 20, 2017, p 908. https://doi.org/10.3390/nu9080908.

[31] Calfío, C.; Huidobro-Toro, J. P. Potent Vasodilator and Cellular Antioxidant Activity of Endemic Patagonian Calafate Berries (Berberis Microphylla) with Nutraceutical Potential. *Molecules*, **2019**, *24* (15), 2700. https://doi.org/10.3390/molecules24152700.

[32] Potente, M.; Dimmeler, S. NO Targets SIRT1. *Arterioscler. Thromb. Vasc. Biol.*, **2008**, *28* (9), 1577–1579. https://doi.org/10.1161/ATVBAHA.108.173682.

[33] Kratz, E. M.; Sołkiewicz, K.; Kubis-Kubiak, A.; Piwowar, A. Sirtuins as Important Factors in Pathological States and the Role of Their Molecular Activity Modulators. *Int. J. Mol. Sci.*, **2021**, *22* (2), 630. https://doi.org/10.3390/ijms22020630.

[34] Kitada, M.; Ogura, Y.; Monno, I.; Koya, D. Supplementation with Red Wine Extract Increases Insulin Sensitivity and Peripheral Blood Mononuclear Sirt1 Expression in Nondiabetic Humans. *Nutrients*, **2020**, *12* (10), 3108. https://doi.org/10.3390/nu12103108.

[35] Ray, R.; Murdoch, C. E.; Wang, M.; Santos, C. X.; Zhang, M.; Alom-Ruiz, S.; Anilkumar, N.; Ouattara, A.; Cave, A. C.; Walker, S. J.; et al. Endothelial Nox4 NADPH Oxidase Enhances Vasodilatation and Reduces Blood Pressure in Vivo. *Arterioscler. Thromb. Vasc. Biol.*, **2011**, *31* (6), 1368–1376. https://doi.org/10.1161/ATVBAHA.110.219238.

[36] Brown, D. I.; Griendling, K. K. Regulation of Signal Transduction by Reactive Oxygen Species in the Cardiovascular System. *Circulation Research*. Lippincott Williams and Wilkins January 29, 2015, pp 531–549. https://doi.org/10.1161/CIRCRESAHA.116.303584.

[37] Suzuki, H.; Swei, A.; Zweifach, B. W.; Schmid-Schönbein, G. W. In Vivo Evidence for Microvascular Oxidative Stress in Spontaneously Hypertensive Rats: Hydroethidine Microfluorography. *Hypertension*, **1995**, *25* (5), 1083–1089. https://doi.org/10.1161/01.HYP.25.5.1083.

[38] Negishi, H.; Njelekela, M.; Ikeda, K.; Sagara, M.; Noguchi, T.; Kuga, S.; Kanda, T.; Liu, L.; Nara, Y.; Tagami, M.; et al. Assessment of in Vivo Oxidative Stress in Hypertensive Rats and Hypertensive Subjects in Tanzania, Africa. In *Hypertension Research*; Japanese Society of Hypertension, 2000; Vol. 23, pp 285–289. https://doi.org/10.1291/hypres.23.285.

[39] Huang, W.; Hutabarat, R. P.; Chai, Z.; Zheng, T.; Zhang, W.; Li, D. Antioxidant Blueberry Anthocyanins Induce Vasodilation via PI3K/Akt Signaling Pathway in High-glucose-induced Human Umbilical Vein Endothelial Cells. *Int. J. Mol. Sci.*, **2020**, *21* (5), 1575. https://doi.org/10.3390/ijms21051575.

[40] Galindo, P.; González-Manzano, S.; Zarzuelo, M. J.; Gómez-Guzmán, M.; Quintela, A. M.; González-Paramás, A.; Santos-Buelga, C.; Pérez-Vizcaíno, F.; Duarte, J.; Jiménez, R. Different Cardiovascular Protective Effects of Quercetin Administered Orally or Intraperitoneally in Spontaneously Hypertensive Rats. *Food Funct.*, **2012**, *3* (6), 643–650. https://doi.org/10.1039/c2fo10268d.

[41] Schiffrin, E. L. Role of Endothelin-1 in Hypertension and Vascular Disease. In *American Journal of Hypertension*; Elsevier Inc., 2001; Vol. 14, pp 83S-89S. https://doi.org/10.1016/s0895-7061(01)02074-x.

[42] Markovics, A.; Biró, A.; Kun-Nemes, A.; Fazekas, M. É.; Rácz, A. A.; Paholcsek, M.; Lukács, J.; Stündl, L.; Remenyik, J. Effect of Anthocyanin-Rich Extract of Sour Cherry for Hyperglycemia-Induced Inflammatory Response and Impaired Endothelium-Dependent Vasodilation. *Nutrients*, **2020**, *12* (11), 1–12. https://doi.org/10.3390/nu12113373.

[43] Lazzè, M. C.; Pizzala, R.; Perucca, P.; Cazzalini, O.; Savio, M.; Forti, L.; Vannini, V.; Bianchi, L. Anthocyanidins Decrease Endothelin-1 Production and Increase Endothelial Nitric Oxide Synthase in Human Endothelial Cells. *Mol. Nutr. Food Res.*, **2006**, *50* (1), 44–51. https://doi.org/10.1002/mnfr.200500134.

[44] Jiménez, R.; López-Sepúlveda, R.; Kadmiri, M.; Romero, M.; Vera, R.; Sánchez, M.; Vargas, F.; O'Valle, F.; Zarzuelo, A.; Dueñas, M.; et al. Polyphenols Restore Endothelial Function in DOCA-Salt Hypertension: Role of Endothelin-1 and NADPH Oxidase. *Free Radic. Biol. Med.*, **2007**, *43* (3), 462–473. https://doi.org/10.1016/j.freeradbiomed.2007.05.007.

[45] López-Sepúlveda, R.; Jiménez, R.; Romero, M.; Zarzuelo, M. J.; Sánchez, M.; Gómez-Guzmán, M.; Vargas, F.; O'Valle, F.; Zarzuelo, A.; Pérez-Vizcaíno, F.; et al. Wine Polyphenols Improve Endothelial Function in Large Vessels of Female Spontaneously Hypertensive Rats. *Hypertension*, **2008**, *51* (4 PART 2 SUPPL.), 1088–1095. https://doi.org/10.1161/HYPERTENSIONAHA.107.107672.

[46] Schramm, D. D.; Wang, J. F.; Holt, R. R.; Ensunsa, J. L.; Gonsalves, J. L.; Lazarus, S. A.; Schmitz, H. H.; German, J. B.; Keen, C. L. Chocolate Procyanidins Decrease the Leukotriene-Prostacyclin Ratio in Humans and Human Aortic Endothelial Cells. *Am. J. Clin. Nutr.*, **2001**, *73* (1), 36–40. https://doi.org/10.1093/ajcn/73.1.36.

[47] Facino, R. M.; Carini, M.; Aldini, G.; Berti, F.; Rossoni, G.; Bombardelli, E.; Morazzoni, P. Diet Enriched with Procyanidins Enhances Antioxidant Activity and Reduces Myocardial Post-Ischaemic Damage in Rats. *Life Sci.*, **1999**, *64* (8), 627–642. https://doi.org/10.1016/S0024-3205(98)00605-5.

[48] Sacks, D.; Baxter, B.; Campbell, B. C. V.; Carpenter, J. S.; Cognard, C.; Dippel, D.; Eesa, M.; Fischer, U.; Hausegger, K.; Hirsch, J. A.; et al. Multisociety Consensus Quality Improvement Revised Consensus Statement for Endovascular Therapy of Acute Ischemic Stroke. *Int. J. Stroke*, **2018**, *13* (6), 612–632. https://doi.org/10.1177/1747493018778713.

TABLES

Table 1. Primer list characteristics.

Rat Primers	Sequence (5'.....3')	Size of Amplicon	Efficiency	GenBank Accesion No.
eNOS Fw	GGATTCTGGCAAGACCGATTAC	159	2.23 (111.5%)	NM_021838.2
eNOS Rv	GGTGAGGACTTGTCCAAACACT			
Sirt-1 Fw	TTGGCACCGATCCTCGAA	217	1.97 (98.5)	NM_001007684.1
Sirt-1 Rv	ACAGAAACCCCAGCTCCA			
NOX-4 Fw	GTGTCTGCATGGTGGTGGTA	150	1.86 (93%)	NM_053524.1
NOX-4 Rv	TCAACAAGCCACCCGAAACA			
ET-1 Fw	TGATTCTCTTGCCTCTTCTTG	110	2.23 (111.5%)	NM_012548.2
ET-1 Rv	TATGGAATCTCCTGGCTCTC			
PPIA Fw	CTTCGAGCTGTTTGCAGACAA	118	2.28 (114%)	NM_017101.1
PPIA Rv	AAGTCACCACCCTGGCACATG			

FIGURES

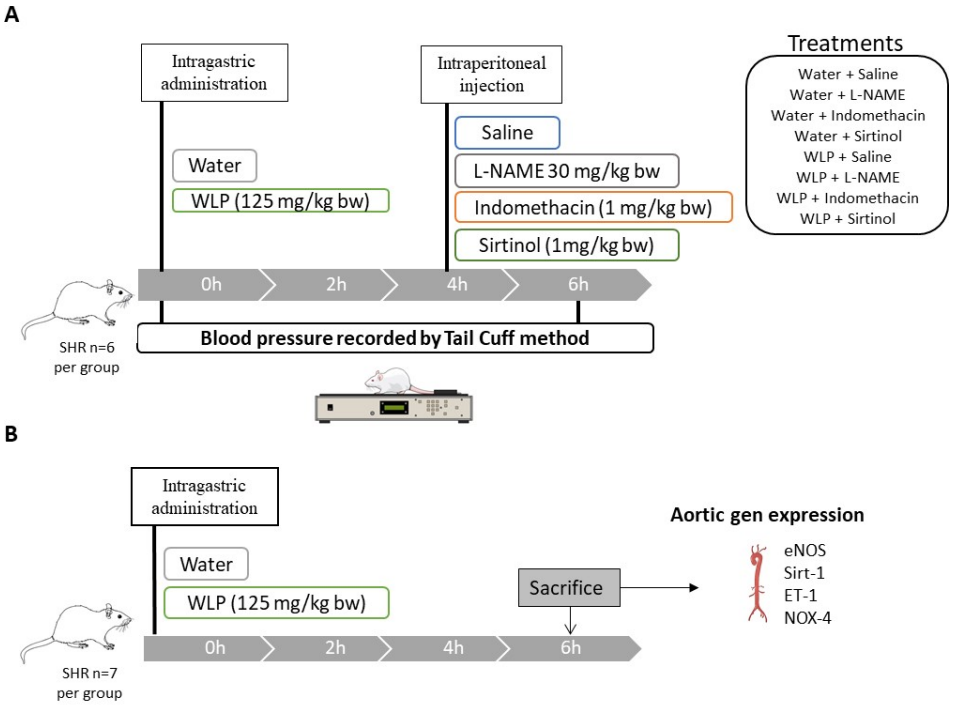

Figure 1. Graphical representation of the experimental design for Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME), Indomethacin and Sirtinol study in spontaneously hypertensive rats (SHR) (A), and graphical representation of the experimental design used to study the effects of wine lees powder (WLPW) on the endothelial function (B).

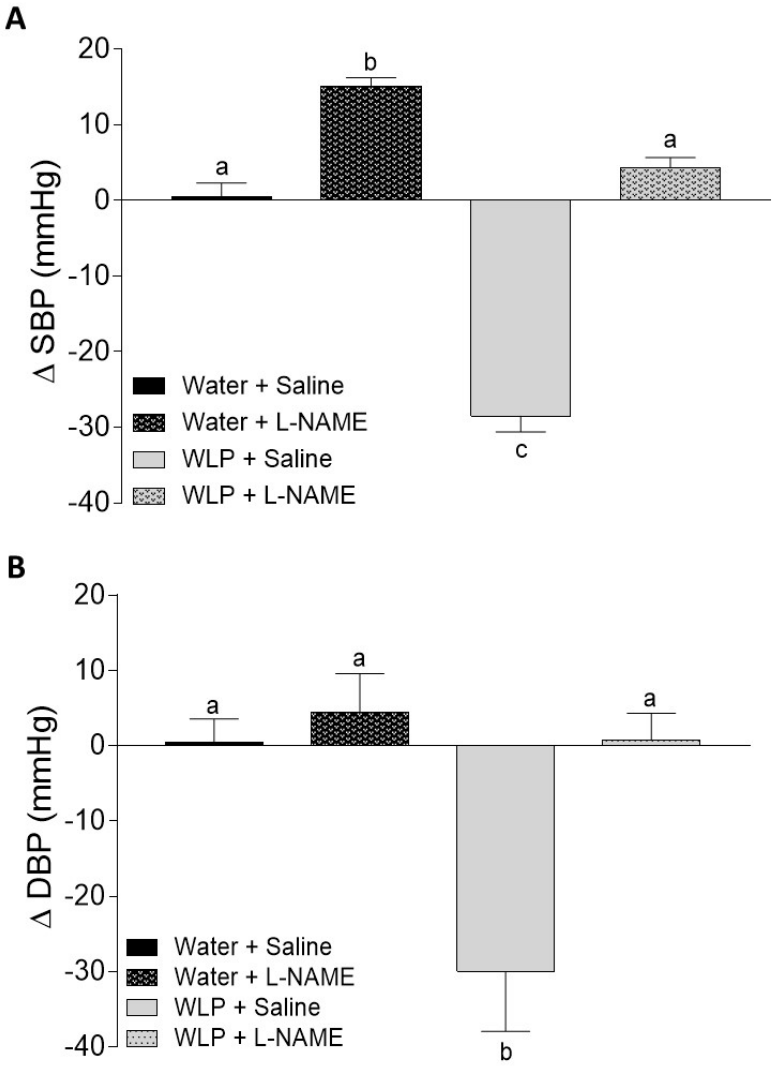

Figure 2. Changes in systolic blood pressure (SBP) (A) and diastolic blood pressure (DBP) (B) caused in spontaneously hypertensive rats 6 h post-administration to different treatments: oral administration of water or wine lees powder (WLP) and intraperitoneal injection of Saline or Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME). Significant differences ($p < 0.05$) are represented by different letters and p was estimated by one-way ANOVA.

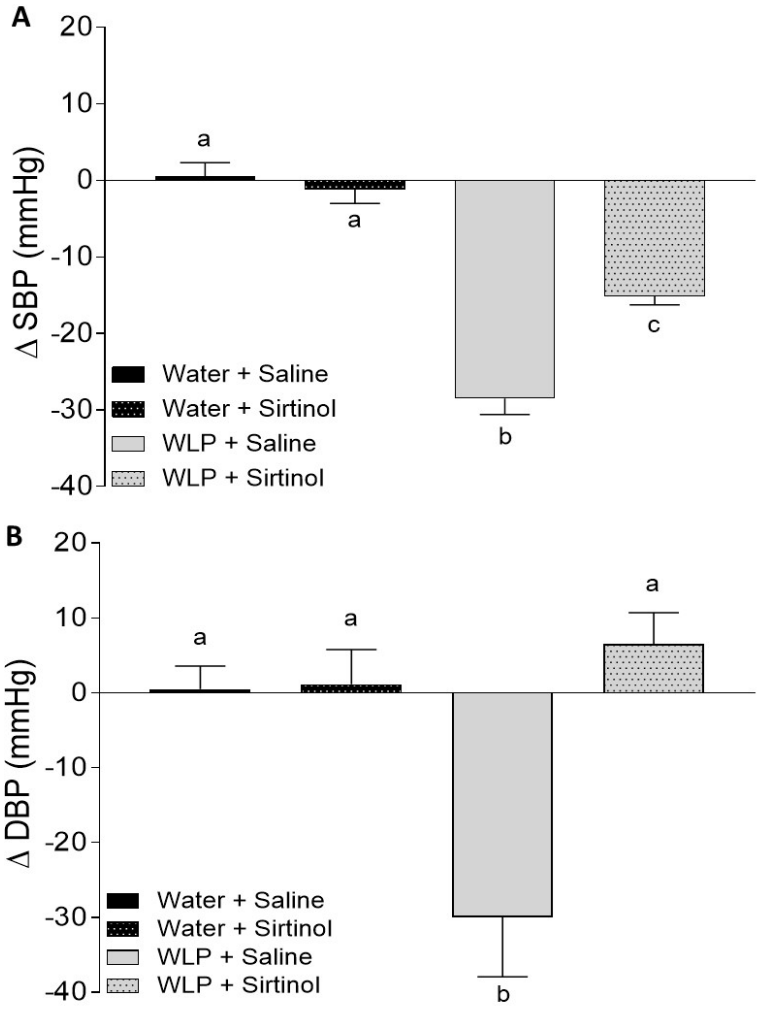

Figure 3. Changes in systolic blood pressure (SBP) (A) and diastolic blood pressure (DBP) (B) caused in spontaneously hypertensive rats 6 h post-administration to different treatments: oral administration of water or wine lees powder (WLP) and intraperitoneal injection of Saline or Sirtinol. Significant differences ($p < 0.05$) are represented by different letters and p was estimated by one-way ANOVA.

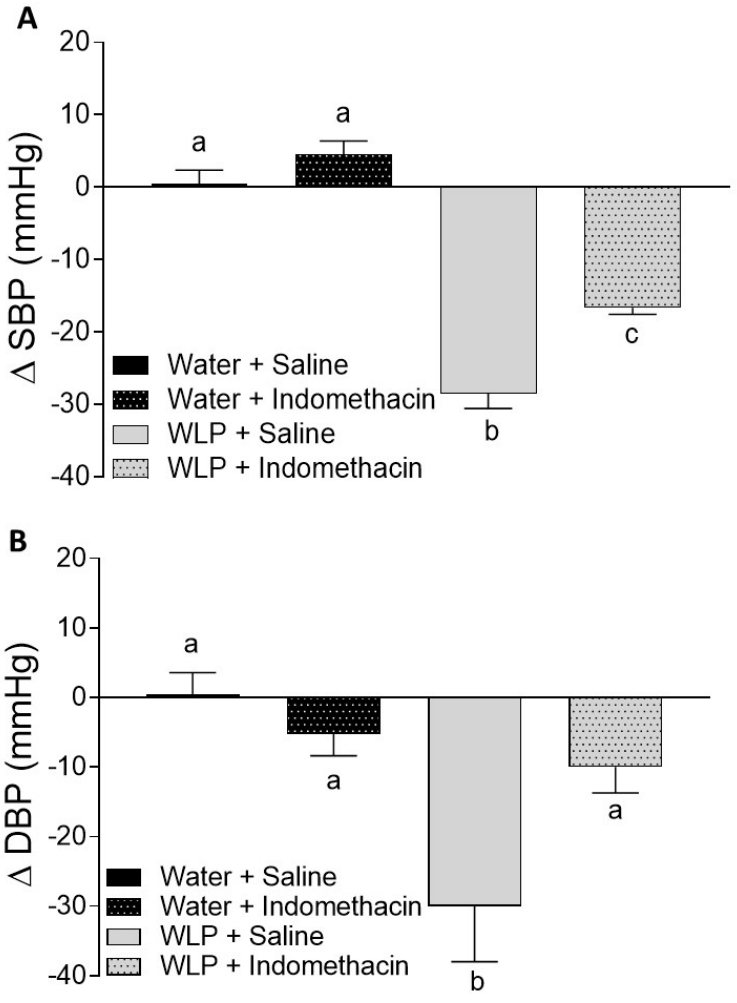

Figure 4. Changes in systolic blood pressure (SBP) (A) and diastolic blood pressure (DBP) (B) caused in spontaneously hypertensive rats 6 h post-administration to different treatments: oral administration of water or wine lees powder (WLP) and intraperitoneal injection of Saline or Indomethacin. Significant differences ($p < 0.05$) are represented by different letters and p was estimated by one-way ANOVA.

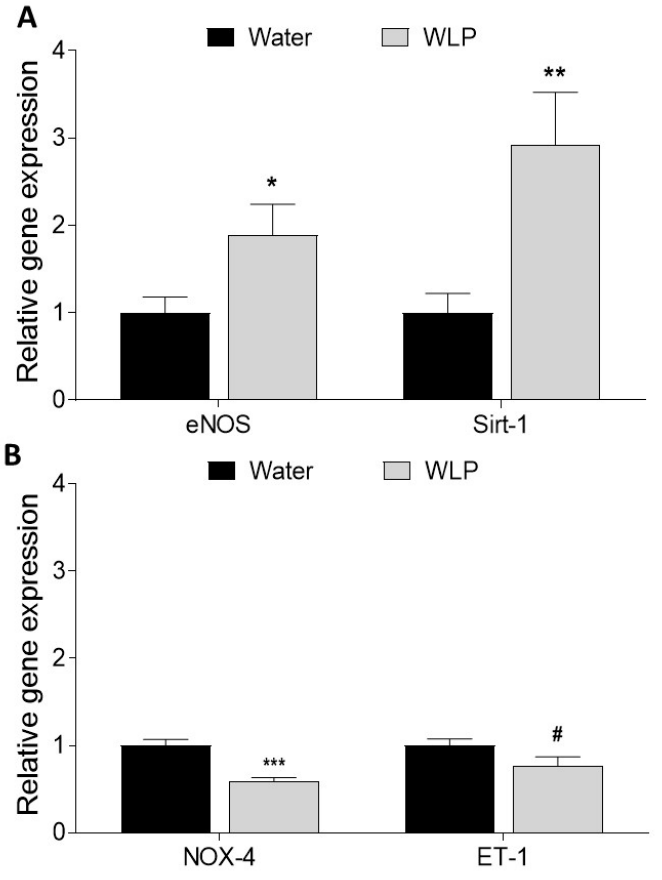

Figure 5. Aortic gene expression of *eNos* and *Sirt1* (A) and *Nox4* and *Et1* (B) in spontaneously hypertensive rats 6 h after administration with water or wine lees powder (WLPW). Statistical differences between treatments were carried out by Student's T-test when (*) $p < 0.05$, (**) $p < 0.01$ or (***) $p > 0.001$.

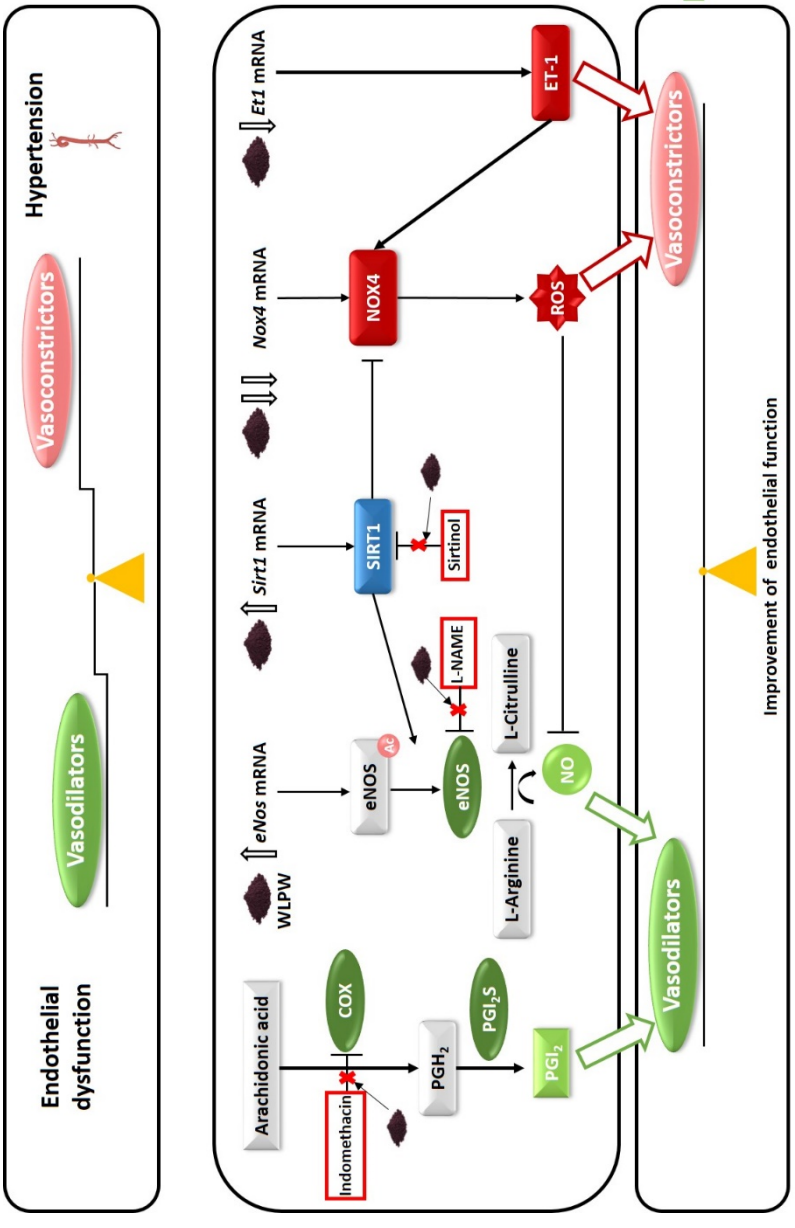

Figure 6. Schematic representation of the molecular mechanisms involved in the blood pressure (BP)-lowering effect of wine lees powder (WLPW) in endothelium. WLPW upregulates endothelial nitric oxide (eNos) mRNA levels, which would lead to higher production of nitric oxide (NO). Sirtuin-1 (Sirt1) mRNA levels were also upregulated by WLPW. The enzyme SIRT1, which mediates partially the antihypertensive

effect of WLPW, demonstrated after sirtinol administration, is able to increase eNos expression. In addition, SIRT1 deacetylates and activates eNOS and inhibits NADPH oxidase subunit 4 (NOX4). Furthermore, Nox4 mRNA levels were downregulated by WLPW. The decrease in the expression and activity of NOX4 would lead to a decrease in radical oxygen species (ROS) production and consequently, an increase in NO availability. All these events would lead to the increase of NO availability, which mediates the antihypertensive effect of WLPW, as it has been demonstrated after L-NAME administration. In addition, the indomethacin study has demonstrated that the endothelium-derived vasodilator factor prostaglandin I2 (PGI2) mediates partially the BP-lowering effect of WLPW. Moreover, WLPW also downregulates the expression of the vasoconstrictor endothelin-1 (Et1) mRNA levels, which would lead to lower production of NO. All of these factors lead to a restoration of the imbalance of endothelial-derived vasodilator and vasoconstrictor factors caused by hypertension, improving endothelial function and decreasing blood pressure. Arrows ending in points represent activation and those ending in lines represent inhibition.

SUPPLEMENTARY

Table S1. Non-anthocyanin composition of wine lees powder obtained by UHPLC-(ESI-)-Q-TOF-MS

Compound	Quantity (µg/g)
Flavanols	
Catechin	3,599.26 ± 17.70
Catechin gallate	29.49 ± 0.37
Epicatechin	1,602.95 ± 5.64
(Epi)catechin *O*-glucoside iso1	18.43 ± 0.01
(Epi)catechin *O*-glucoside iso2	12.17 ± 0.00
(Epi)catechin *O*-glucoside iso3	54.19 ± 1.08
Procyanidin dimer B2	1,275.58 ± 0.37
Procyanidin dimer iso1	2,367.19 ± 5.64
Procyanidin dimer iso2	525.71 ± 2.30
Procyanidin dimer iso3	109.12 ± 0.76
Procyanidin dimer iso4	471.89 ± 3.92
Procyanidin dimer iso5	159.26 ± 0.82
Procyanidin trimer iso1	600.18 ± 3.80
Procyanidin trimer iso2	529.03 ± 9.77
Procyanidin trimer iso3	225.25 ± 2.14
Procyanidin trimer iso4	118.71 ± 3.90
Procyanidin trimer iso5	508.39 ± 3.51
Flavonols	
Quercetin	1,355.94 ± 4.47
Quercetin-3-*O*-glucoside	60.09 ± 0.39
Quercetin-3-*O*-glucuronide	89.22 ± 0.76
Kaempferol	189.86 ± 1.50
kaempferol-3-*O*-glucuronide	17.70 ± 0.35
Isorhamnetin	411.43 ± 2.31
Phenolic acids	
Gallic acid	4,456.04 ± 89.03
Caffeic acid	120.55 ± 0.77
Caffeic acid *O*-glucoside iso1	20.28 ± 0.74
Caffeic acid *O*-glucoside iso2	24.33 ± 1.11
p-Coumaric acid	126.82 ± 0.68
4-Hydroxybenzoic acid	61.57 ± 2.14
Ferulic acid	27.65 ± 0.43
Vanillic acid	85.90 ± 2.41
Stilbenes	
trans-Resveratrol	169.59 ± 0.74
Resveratrol iso1	108.76 ± 0.37
Resveratrol *O*-glucoside iso1	9.95 ± 0.37

Resveratrol *O*-glucoside iso2	49.77 ± 1.47
Piceatannol	154.84 ± 2.00
Piceatannol 3-*O*-glucoside iso1	8.11 ± 0.00
Piceatannol 3-*O*-glucoside iso2	2.21 ± 0.00
Viniferin-iso1	9.95 ± 0.00
Viniferin-iso2	29.86 ± 0.41

Data are represented per g of wet weight.

Table S2. Anthocyanin composition of wine lees powder obtained by UHPLC-(ESI-)-Q-TOF-MS

Anthocyanins	Quantity (µg/g)
Gallocatechin-malvidin-3-glucoside dimer	9.09 ± 0.04
Malvidin-3-glucoside-(epi)catechin	40.95 ± 0.12
Delphinidin-3-glucoside	136.02 ± 1.68
Cyanidin-3-glucoside	8.34 ± 0.75
Petunidin-3-glucoside	185.45 ± 2.08
Petunidin-3-glucoside-pyruvic acid	3.28 ± 0.04
Peonidin-3-glucoside	100.31 ± 2.30
Malvidin-3-glucoside	2,236.82 ± 18.44
Peonidin-3-glucoside-pyruvic acid	1.50 ± 0.03
Delphinidin-(6-acetyl)-3-glucoside	33.48 ± 0.83
visitin A (malvidin-3-glucoside-pyruvic acid)	45.23 ± 0.12
Visitin B (malvidin-3-glucoside-acetaldehyde)	112.92 ± 0.54
Malvidin-3-glucoside-ethyl-(epi)catechin	13.47 ± 0.03
Cyanidin-(6-acetyl)-3-glucoside	7.50 ± 0.21
Acetylvisitin A	28.95 ± 0.41
Malvidin-3-glucoside-ethyl-(epi)catechin	50.75 ± 0.22
Petunidin-(6-acetyl)-3-glucoside	47.51 ± 1.72
Malvidin-3-glucoside-ethyl-(epi)catechin	75.36 ± 0.67
Acetylvisitin B	61.24 ± 0.41
Peonidin-(6-acetyl)-3-glucoside	48.65 ± 1.14
Delphinidin-(6-coumaroyl)-3-glucoside	16.10 ± 0.25
Malvidin-(6-acetyl)-3-glucoside	1,046.67 ± 0.77
Coumaroylvisitin A	7.38 ± 0.06
Malvidin-(6-caffeoyl)-3-glucoside	13.42 ± 0.25
Cyanidin-(6-coumaroyl)-3-glucoside	3.65 ± 0.15
Catechin-ethyl-Malvidin-3-acetylglucoside dimer	32.34 ± 0.29
Petunidin-(6-coumaroyl)-3-glucoside	27.46 ± 0.33
Pinotin A (malvidin-3-glucoside-vinylcatechol)	30.96 ± 0.47
Malvidin-glucoside-vinyl-catechin	5.64 ± 0.03
Coumaroylvisitin B	33.62 ± 0.26
Malvidin-3-glucoside-vinylguaiacol	21.92 ± 0.18
Catechin-ethyl-malvidin-3-coumaroylglucoside dimer	25.24 ± 0.10
Catechin-ethyl-malvidin-3-acetylglucoside dimer	5.33 ± 0.06
Peonidin-(6-coumaroyl)-3-glucoside	34.82 ± 1.00
Malvidin-(6-coumaroyl)-3-glucoside	396.97 ± 0.55
Malvidin-glucoside-vinyl-catechin	5.99 ± 0.02
Acetyl-pinotin A	0.26 ± 0.00
Malvidin 3-*O*-glucoside 4-vinylphenol (Pigment A)	23.58 ± 0.07
Catechin-ethyl-malvidin-3-coumaroylglucoside dimer	4.37 ± 0.01
Malvidin acetyl 3-*O*-glucoside 4-vinylphenol (Acetyl-pigment A)	14.12 ± 0.16

Data are represented per g of wet weight.

CHAPTER 3:

To assay the effect of dealcoholized wine lees after a long-term administration in hypertensive rats

Manuscript 6:

Objective:

To investigate the effect of WL after long-term administration to SHR on BP and other cardiovascular parameters using a telemetry system.

Effect of dealcoholized wine lees on blood pressure, heart rate and locomotor activity in a long-term treatment in spontaneously hypertensive rats

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Manuscript in preparation for Hypertension

Effect of dealcoholized wine lees on blood pressure, heart rate and locomotor activity in a long-term treatment in spontaneously hypertensive rats

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Keywords: cardioprotection, darkness, dipper, heart rate, hypertension, light, non-dipper, phenolic compounds

Abbreviations: CVD, cardiovascular disease; BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; SHR, spontaneously hypertensive rats; WLPW, wine lees powder

ABSTRACT

The antihypertensive effect of long-term intake of a wine lees powder (WLPW) with high concentration of phenolic compounds was evaluated in spontaneously hypertensive rats (SHR). Additionally, the effects of the phenolic-rich powder on heart rate (HR), locomotor activity and body temperature were investigated. Weight gain, as well as food and liquid intakes were recorded throughout the experimental period. Blood pressure (BP) was measure through a telemetry system one week before and after administration of WLPW (125 mg/kg bw) or vehicle (VH) for five weeks. WLPW intake attenuated the development of HTN compared to VH, decreasing systolic and diastolic BP (SBP and DBP) in light and dark periods. The marker of cardiac status heart rate (HR) was also lower in WLPW respect to VH group. This reduction was specific of the light period. In addition, HR is modified along the time changing from dipper to non-dipper pattern in the VH group, however, the administration of WLPW kept the dipper pattern Moreover, WLPW increased the locomotor activity levels in SHR in the darkness period. No changes were observed respect to VH rats in body weight gain, solid and liquid intakes, and body temperature. SBP, DBP, HR and locomotor activity values returned to control values upon WLPW administration interruption, confirming the beneficial properties of WLPW. Therefore, WLPW could be used as a functional ingredient in the management of HTN.

1. Introduction

Hypertension (HTN) is the leading preventable risk factor for cardiovascular disease (CVD) and the main cause of premature death worldwide [1]. HTN is defined as systolic blood pressure (SBP) ≥140 mmHg and/or diastolic blood pressure (DBP) ≥90 mmHg [2] and it usually requires lifelong treatments. Some of the used antihypertensive drugs can exert unwanted side effects in some patients. Due to this fact, the search of new compounds to manage HTN has arisen, mainly in natural sources [3]. In this regard, the study of natural compounds with antihypertensive properties is considered as a field of great interest especially for prehypertensive subjects since this population is not usually clinically treated.

HTN development has been related with an elevated heart rate (HR) and a reduction of the locomotor activity [4]–[6]. In addition, the variability along the day in parameters such as BP or HR rhythms have demonstrated to be a key factor in the prediction of future cardiovascular events [7]–[9]. Specifically, a typical nocturnal decline in BP and HR (period of inactivity) versus the day (period of activity) between 10-20% (dipper pattern) of these parameters is related to a healthier cardiovascular state and a lower risk of CVD. However, a reduction of less than 10% (non-dipper pattern) at night is related to an increased risk of CVD, stroke and death [8], [10]–[12].

In addition, low consumption of fruits and vegetables has been related with a higher risk to suffer CVD or HTN [13]. Thus, lifestyle changes, including a diet rich in fruits and vegetables, in combination or without pharmacological treatments are always recommended since they are effective in the treatment or prevention of HTN [14]. Fruits and vegetables contain bioactive compounds with different chemical structures, such as phenolic compounds, peptides, oligosaccharides, vitamins and fatty acids [15]. Among them, phenolic compounds stand out for their wide range of functional effects including cardioprotective, antioxidant or anti-inflammatory agents [16]. It is worth mentioning that these compounds are also contained in by-products generated

during fruit and vegetable processing [17]. This fact is of great interest since these by-products can be used to obtain bioactive phenolic-rich products or extracts and through this way also revalue these wastes [18], [19].

Winery by-products have been successfully used to obtain phenolic-rich extracts with antihypertensive effects in animals and humans [20], [21]. In this regard, our group has recently evidenced the acute antihypertensive effect of wine lees (WL) from Cabernet grape variety. Spontaneously Hypertensive rats (SHR) were administered the soluble fraction of WL obtained by centrifugation. The BP-lowering effect were related to the high presence of phenolic compounds in these WL, mainly anthocyanins an flavanols [22]. The administration of the most effective dose (125 mg/kg bw) of the dealcoholized WL powder (WLPW) caused a decrease in BP up to -48.0 ± 4.9 mmHg and -44.6 ± 8.8 mmHg, for SBP and BDP respectively, at 6 h post-administration [23]. Consequently, WLPW could be of great interest as functional ingredient for food and nutraceutical industries. However, since HTN is a chronic disease, the effects of the long-term antihypertensive activity of WLPW have to be demonstrated. Thus, the objective of this study was to evaluate the antihypertensive effect of long-term administration of WLPW to SHR using a telemetry system. In addition, their effect over HR and locomotor activity and its relationship with BP were determined.

2. Material and methods

2.1. Obtaining of wine lees powder

WL were provided by the cellar Grandes Vinos y Viñedos in the Cariñena P.O.D. area (Spain). They were collected immediately after the racking of wine (first-fermentation WL), elaborated with Cabernet grapes. WL was centrifuged at 4 ºC for 15 min at 2950 × g to obtain the supernatant. It was further lyophilized and stored at -20 ºC until its analysis. Tables S1 and S2 show the phenolic profile of WLPW. Individual phenolic compounds were characterised by using a UHPLC-ESI-Q-TOF-MS system using 1290 UHPLC Infinity II series

coupled to a Q-TOF/MS 6550 (Agilent Technologies, Palo Alto, CA, USA). Both negative and positive ionization ([M-H]- or [M-H]+) were used to identify parental ions and fragmentation patterns [23].

2.2. Animal study

Ten male SHR with 14-15 week-old and weighing 280–330 g were obtained from Charles River Laboratories España S.A. (Barcelona, Spain). Animals were kept in a well-ventilated animal housing room with a 12-hour light/dark cycle (8:00 am – 8:00 pm) and at a temperature of 23 ºC and humidity of 50%. The animals had free access to standard diet (A04 Panlab, Barcelona, Spain) and tap water during all the experiment.

After a 10-days adaptation period, rats were surgically implanted with telemetry transmitters (model HD-S10, Data Sciences International, Minneapolis, USA). Briefly, the analgesic buprenorphine (0.05 mg/kg bw) was administered to rats via intramuscular injection. Then, animals were anesthetized with a mixture of ketamine and xylazine (43 and 8.7 mg/kg bw, respectively). Ketamine 100 mg/mL, Xylazine 20 mg/mL and Buprenorphine 0.3 mg/mL were provided by Proyma Ganadera S.L. (Alcázar de San Juan, Spain). A midline abdominal incision was made to insert the catheter of the HD-S10 transmitter into the abdominal aorta. The transmitter was secured in the abdominal cavity and the abdomen closed. As post-operative analgesia, the same dose of buprenorphine was administered every 8 h for 2 days. Rats were left to recover from surgery for 10 days before the onset of the experimental protocol. **Figure 1** shows a graphical representation of the experimental designs used in this study.

Animals were orally administered vehicle (VH) (sweetened milk containing 5.5 % sucrose) or 125 mg/kg bw WLPW dissolved in VH (n=5 per group). Treatments were daily administered in a volume of 1.5 mL between 08:00 and 08:15 h for 5 weeks, allowing animal to drink it directly from a syringe. Animals did not receive any treatment at the sixth week of the experiment (follow-up period).

Body weight as well as food and water intake were measured once a week during the experiment. SBP, DBP, HR, activity and core body were monitored by radiotelemetry using a Data Sciences International (DSI; St. Paul, MN) system. It consisted in the following components: MX2: 2.0, RPC-1 receptor for cages; HD-S10 sensor (catheter measurements: 8 cm x 6 mm), APR-2 ambient pressure detector, data acquisition and analysis system Powerlab 16/35 and LabChart Pro 8. Data were obtained with the LabChart Pro 8 Software and were recorded during all day in three different days during the week. The average of all data by weeks was represented. In addition, the average of the week was also made by period of light (8:00 a.m. to 8:00 p.m.) and period of darkness (8:00 p.m. to 8:00 a.m.).

Dipper and non-dipper patterns were calculated as the percentage of difference between light and night periods as follows:

$$\% = \frac{\sum darkness\ period\ (12h) - \sum light\ period\ (12h)}{\sum darkness\ period\ (12h)}$$

All animal protocols were approved by the Animal Ethics Review Committee for Animal Experimentation of the Universitat Rovira i Virgili and further approved by Generalitat de Catalunya (permission number 10780).

2.3. Statistical analysis

Differences produced by the long-term administration of VH or WLPW were analysed by two-way ANOVA with Tukey's test as post hoc. Student t-test was used to analyse the differences between groups in the same week. All the analyses were performed using GraphPad Prism 7.04 for Windows (GraphPad Software, San Diego, California). Grubbs test was used to discard possible outliers. Differences between groups were considered significant when $p < 0.05$.

3. Results

3.1. Effect of the wine lees powder on body weight and food and water intake

Body weight and food and water intake were measured during the six weeks of study in the two animal groups (**Figure 2**). Before the study, animals showed a weight of 307.7 ± 19.0 g. During the 6 weeks, body weight of all animals increased progressively (**Figure 2A**). No differences were found in body weight or body weight gain between VH and WLPW groups. In addition, no differences were observed in food and water intake of animals neither across the time nor between groups of treatments.

3.2. Effect of the wine lees powder on blood pressure

SBP and DBP levels in SHR were constantly recorded during 24 h on the day before treatment and three times per week during VH and WLPW (125 mg/kg bw) treatment period (5 weeks). Initial values of SBP and DBP were 185.6 ± 3.5 and 119.9 ± 4.4 mmHg, respectively (**Figures 3 A and D**, week 0). During treatment period, the control group (VH) showed a progressive increase in SBP, reaching values up to 204.8 ± 3.2 mmHg (mean of the values found during all the day) in the last week of treatment (**Figure 3A**). However, SBP of the WLPW-treated group was maintained showing values of 188.86 ± 3.42 mmHg in the 5th week. Significant differences in SBP levels were found between VH and WLPW groups from the 3rd to the 5th week of treatment. The higher differences were found in the last week of treatment. In addition, these SBP differences between groups were also observed in both dark and lightness periods (**Figures 3B and 3C**). Similar pattern was showed in DBP levels. Thus, considering DBP as the daily mean of the dark and the light periods, a progressive increase of this parameter was observed up to the 5th week (149.37 ± 4.41 mmHg) in the VH-group while it did not vary in the WLPW-group (**Figure 3D-F**). DBP values of animals treated with WLPW was also lowest than that observed in the control group in the last 3 weeks of treatment.

In the 6th week, animals were not treated. **Figure 3** shows that SBP and DBP of the WLPW-group increased after one week without treatment, observing BP values similar to that observed in the VH-group.

Additionally, the dipper or non-dipper patterns were also studied in SBP and DBP (**Figure 4 and Table 1**). **Figure 4** shows the SBP in the different weeks in a period of 24 h. Before starting the study, all animal groups presented a non-dipper pattern showing less than 4% difference between light and darkness periods in SBP and DBP (**Figures 4A and 4B and Table 1**). No differences were found between treatments during the experiment, showing both groups a non-dipper pattern. SBP was higher throughout the day in the VH group compared to the WLPW group across the 5 weeks of treatment, with the maximum difference between groups in the week 5th (**Figure 4C**). Regarding DBP (**Figures 4B, C** and **F**), the variation in DBP followed the same pattern as SBP with the maximum difference in DBP between groups at week 5th. Finally, SBP and DBP were not different between VH and WLPW groups during all day in the week without treatment.

3.3. Effect of the wine lees powder on heart rate, temperature and activity

Initial values of HR in the animals were 325.75 ± 4.92 bpm (**Figure 6A**). During the period of treatment, VH-group kept the HR at the same values, while the animals treated with WLPW showed a reduction in HR in the last week of treatment, reaching values of 322.22 ± 1.80 bpm and 304.62 ± 4.56 bpm, respectively. In the follow-up period without treatment (6th week), HR was increased in the animals treated with WLPW and was maintained in the control group, showing no differences between treatments. When we analysed the pulse for periods of light and dark, no differences were found between treatments in the darkness period (**Figure 6B**). Nevertheless, in the light period differences between groups were found with an important reduction of HR in WLPW-group in the last week of treatment (**Figure 6C**). After treatment interruption (6th week), WLPW-group recovered their baseline HR values showing no differences between groups.

The study of dipper and non-dipper patterns in the HR are shown in **Figure 7** and **Table 1**. A clear dipper pattern is showed before starting the experiment in

both groups (**Figure 7A**) with a percentage of difference between light and darkness periods of 11.95 and 11.71 % in the VH and WLPW groups respectively (**Table 1**). During the treatment period, the HR difference between light and darkness period in the animals treated with VH was reduced along the weeks reaching a non-dipper pattern in week 5th with a value of 9.87% in the VH-group. However, animals treated with WLPW showed an increase of the difference between light-dark HR reaching a value of 13.31 % in the week 5th (**Figure 7B, Table 1**), showing a dipper pattern. After a week without treatment (**Figure 7C**), the non-dipper effect in the rats treated with VH was more evident and the group treated with WLPW recovered values similar to that observed in VH group, also showing a non-dipper pattern.

Activity of SHR was measured to identify if WLPW modify this parameter. In the week before treatment, the activity was 232.47 ± 14.81 in AUC (area under the curve). The results showed a reduction in the activity higher in WLPW group respect to control group (**Figure 8A**). After treatment interruption, activity values were the same in both groups. Thus, WLPW increased the general activity of SHR. In addition, this increased activity produced by WLPW is specific to the period of darkness since during the period of light no changes were observed between treatments (**Figures 8B and 8C**). The activity of the control group is gradually reduced until the last week in the dark period, which does not occur in the group treated with WLPW. In the second week of treatment with WLPW, the activity of rats increased while it was decreased in the control group. In addition, after a week without treatment, both groups showed equal level of activity in the dark period with a reduction in activity in the WLPW group.

Finally, temperature was recorded in all animals and no changes were found across the time and between groups (**Figure 8D**). The temperature was different between darkness and light period with 37.96 ± 0.07 and 37.06 ± 0.06 ºC respectively (**Figures 8E and 8F**).

4. Discussion

HTN is one of the main risk factors for CVD [1]. Indeed, the control of BP is considered the main objective in the different therapies used for the treatment of CVD [2]. Nevertheless, HTN is a chronic disease, which usually requires lifelong treatments. The side effects generated by some antihypertensive drugs have promoted the research for other alternatives in the management of HTN [3], [14], [23]. In this regard, natural bioactive compounds have emerged as potential interesting option due to their ability to reduce BP avoiding side effects [3]. Recently, we have demonstrated the antihypertensive properties of liquid WL from Cabernet grape variety [22] and WL extract enriched in phenolic compounds [24]. However, in adition to phenolic compounds, WL also contain ethanol, which disappears in the drying process. Therefore the antihypertensive activity of the WLPW obtained after the WL drying process was investigated. WLPW acute administration at the most effective dose of 125 mg/kg bw lowered BP levels, showing antihypertensive properties more potent than that observed for liquid WL [23]. Their vasoprotective and antioxidant effects were confirmed as the mechanisms involved in these BP-lowering properties. Therefore, in the current study we focused on evaluating the long-term effect of WLPW on BP and other markers of cardiovascular status in SHR. Furthermore, light and darkness periods were studied separately allowing to elucidate the effects of the intervention study on dipper and non-dipper patterns of BP and HR.

SHR are considered one of the best experimental models to evaluate the antihypertensive effect of antihypertensive compounds since the development of HTN in these rats is very similar to that observed in humans [25]. Several studies have reported that SHR show an initial constant increase in BP levels until it reaches a period of stability [26], [27], [28]. Thus, SBP reaches approximately 200 mmHg SBP at 17–20 weeks of age [26]. In this study, rats were 15 weeks old when the study started and their SBP values were 185.56 ± 3.52. Across the experimental period, when rats from the VH-group were 21

weeks old, SBP reached values of 202.68 ± 3.55 mmHg. WLPW administration attenuated the development of HTN in SHR, maintaining the initial BP values throughout the treatment, which only raised their values in the WLPW-group animals when the administration of the phenolic rich powder was stopped at week 6th.

Phenolic extracts obtained from other by-products as grape seeds has also shown long-term antihypertensive effects in a model of diet-induced hypertensive rats [29]. Notably, the BP-lowering effect produced by WLPW, could be a promising result because small reductions in BP may have an important impact on cardiovascular event in the hypertensive population [30]. In this regard, a reduction in 5 and 10 mmHg for DBP and SBP, respectively, in humans has been reported to be enough to produce a significant reduction in the risk of suffering or worsening cardiovascular disease [31], [32].

It is well known that BP is modified along the day (24 h) with a higher mean value of BP during the day-time (light period) compared to night-time (dark period) [7], [9]. This is due to different factors such as the period of activity [33]. The use of different drugs has been shown to be effective in treating HTN, but not all showed the same effect throughout the day. In the chronic treatment of HTN, beta-blockers and ACE inhibitors have been proven to be effective in reducing both day-time and night-time BP [11], [34], [35]. In these regard, WL were previously selected by their capacity as ACE inhibitor, exhibiting a great ACE inhibitor activity *in vitro* with a great acute antihypertensive effect in SHR [22], [23]. The present study has also demonstrated its long-term antihypertensive effect. Furthermore, when we analysed its effect in the periods of light and darkness, it was observed that the antihypertensive effect was maintained throughout the day, exhibiting antihypertensive properties in both periods. Similar results were found with the ACE inhibitor Lisinopril in SHR, where the BP was reduced in the light and darkness periods [36]. These results demonstrated the efficacy of WLPW throughout the day during long-term treatment.

Abnormalities in cardiac autonomic regulation have been shown to contribute to an increased risk of CVD, in addition to high BP [6], [37]. The most important of these parameters is HR where high HR values are related to an increase in BP [38]. In this sense, therapy against the increase in BP can favour a reduction in high levels of HR. The use of natural compounds has demonstrated to be effective in the treatment of HTN. In addition, by-products such as grape seed have shown to reduce BP and HR in pre- and mild hypertensive subjects [39]. Furthermore, the long-term administration of flavonoids such as quercetin reduced BP and HR in SHR [40]. Our results are in concordance with these findings, showing a reduction in HR with following long-term administration with the phenolic rich powder. A study conducted with SHR and Wistar-Kyoto rats (their normotensive control), have demonstrated that HR is reduced with age in both animals models [4]. This could explain the decreasing of HR throughout the experiment in both SHR groups. In addition, HR also show a circadian rhythm being higher in the period of activity (darkness period in rats) and lower in the non-activity period (light period in rats). Our findings are in the same line showing the higher HR levels in the darkness period and the lower levels in the light periods. High HR in the non-activity period (light period in rats) has been related with a higher chance of developing CVD in humans [41]. WLPW did not modify the HR in the darkness period. Nevertheless, WLPW reduced HR in light period, which could lead to an improvement of cardiovascular function. In this regard, some phenolic extracts have also shown to improve cardiac function through the heart rate (HR) [39], [42], [43].

Variations between day-night in BP and HR has been described, exhibiting different ways of HTN depending on their circadian patterns [11]. Dipper and non-dipper patterns are the most common, where dipper is the nightly drop in BP or HR in more than 10 %, generally produced in normotensive and primary HTN, and non-dipper is the fall or not of the BP or HR at night with a difference of less than 10 % compared to the day, which are mainly produced in secondary HTN [10], [11]. Non-dipper pattern has been related with an increased risk of

organ damage, CVD and death [8], [12]. Our study showed a non-dipper BP patterns in the animals before starting the experiment and during all the experiment regardless of treatment.

Regarding the HR, a dipper pattern was showed before starting the experiment and changed to non-dipper (9.87 %) in the VH-group at the last week of treatment (week 5^{th}). However, the animals treated with WLPW maintained their dipper pattern throughout the experiment, even increasing the dipper percentage from 11.71 to 13.31 %. An increase in 2.4 times of CVD has been related with a non-dipper HR [8]. Thus, WLPW have demonstrated that could modulate the circadian rhythm of HR by reducing the risk of organ damage and death related with a high and non-dipper HR [8]. In addition, the intake of phenolic compounds has been related with the manage or modulation of circadian rhythms [44]. Thus, the intake of WLPW could modulate the circadian rhythm in the HR avoiding loss of circadian rhythm of HR in HTN stage.

Higher activity or exercise has been related with a lower risk of suffer HTN in humans [45], [46]. It has been shown that the increase of activity or exercise training decrease sympathetic nervous (SNS) activity, which has an important role in the development and maintenance of HTN. Thus, the inhibition of SNS could be attributed to the reduction in BP after exercise [47]. In animal models, studies conducted in SHR have shown that exercise in early ages in rats with pre-hypertensive or very early hypertensive state reduce the BP, but not in older rats [5]. The use of fruit juices or extracts rich in phenolic compounds increase locomotor activity [42], [43], [48]. *Aronia melanocarpa* juice (AMJ), which is rich in phenolic compounds, especially in anthocyanins, have demonstrated to increase the activity in aged rats [48]. Furthermore, green tea extract rich in phenolic compounds has shown antihypertensive capacity in addition to increasing the activity in rats to which HTN has been induced by administration of olanzapine which decreases locomotor activity [43]. The results of this study with the powder enriched in phenolic compounds are in accordance with these findings, demonstrating that WLPW increased the

activity and controls the rise of BP in SHR. Muscle contraction and the increment muscle blood flow due to the local release of nitric oxide (NO), prostaglandins and ATP are one of the mechanisms involved in the BP reduction with higher locomotor activity [47]. In this sense, it has been previously shown that the antihypertensive effect of WLPW is mainly mediated by NO and partially by prostaglandins [Manuscript 5], suggesting that these molecules could be responsible for the increased activity mediated by long-term WLPW administration. In addition, the locomotor activity of VH-group decreased across the time (Figure 5A). This is due to the fact that locomotor capacity is lost with age, which depends on muscle strength, balance and motor coordination [47]. However, no modifications were found in the activity of animals treated with WLPW during treatment period. Additionally, this increment of activity was specific for darkness-period, the period of activity of the animal, without finding differences in the period of light. Thus, WLPW could be involved in the regulation of locomotor activity increasing it during the dark period (active period). However, more studies are necessaries to elucidate their possible mechanism involved in that process. Despite the higher activity in the group treated with WLPW, weight as well as food and water intake were not modified compared to the control. Finally, no changes were shown in the body temperature with the intake of WLPW.

5. Conclusion

This study shows the long-term antihypertensive effect of WLPW, which attenuates the development of HTN in SHR. Additionally, the effect of WLPW showed a cardioprotector effect with the reduction of HR. According to this, WLPW elevated the locomotor activity of SHR. Additionally, the intake of WLPW avoid the change from dipper to non-dipper pattern in HR observed in the control SHR, which has been related with a high risk to suffer CVD and death. These findings suggest that WLPW could be used as a functional food to manage HTN in early stage with an improvement of the cardiac function and an increase of locomotor activity. Nevertheless, before the commercial use of WLPW, it

would be necessary to carry out clinical studies to demonstrate their long-term antihypertensive efficiency in humans.

6. Patents

Patent application “Wine lees, derivatives thereof and their uses”: application number EP20382358.8 and PCT/EP2021/053051.

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Conflicts of Interest:

The authors declare no conflict of interest

Bibliography

[1] K. T. Mills, A. Stefanescu, and J. He, "The global epidemiology of hypertension," *Nature Reviews Nephrology*, vol. 16, no. 4. Nature Research, pp. 223–237, 01-Apr-2020.

[2] T. Unger *et al.*, "2020 International Society of Hypertension Global Hypertension Practice Guidelines," *Hypertension*, vol. 75, no. 6, pp. 1334–1357, May 2020.

[3] M. Margalef, F. I. Bravo, A. Arola-Arnal, and B. Muguerza, "Natural Angiotensin Converting Enzyme (ACE) Inhibitors with Antihypertensive Properties," in *Natural Products Targeting Clinically Relevant Enzymes*, Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA, 2017, pp. 45–67.

[4] I. Kohno, H. Honma, T. Nakamura, and K. Tamura, "Comparison of blood pressure, heart rate and activity between normotensive and spontaneously-hypertensive rats," *Chronobiologia*, vol. 21, no. 1–2, pp. 45–56, 1994.

[5] K. D. Schlüter, R. Schreckenberg, and R. M. Da Costa Rebelo, "Interaction between exercise and hypertension in spontaneously hypertensive rats: A meta-analysis of experimental studies," *Hypertens. Res.*, vol. 33, no. 11, pp. 1155–1161, Nov. 2010.

[6] J. F. Thayer, S. S. Yamamoto, and J. F. Brosschot, "The relationship of autonomic imbalance, heart rate variability and cardiovascular disease risk factors," *International Journal of Cardiology*, vol. 141, no. 2. Elsevier, pp. 122–131, 28-May-2010.

[7] M. W. Millar-Craig, C. N. Bishop, and E. B. Raftery, "CIRCADIAN VARIATION OF BLOOD-PRESSURE," *Lancet*, vol. 311, no. 8068, pp. 795–797, Apr. 1978.

[8] K. Eguchi *et al.*, "Nocturnal nondipping of heart rate predicts cardiovascular events in hypertensive patients," *J. Hypertens.*, vol. 27, no. 11, pp. 2265–2270, 2009.

[9] M. Van Den Buuse, "Circadian rhythms of blood pressure, heart rate, and locomotor activity in spontaneously hypertensive rats as measured with radio-telemetry," *Physiol. Behav.*, vol. 55, no. 4, pp. 783–787, Apr. 1994.

[10] K. Kario, "Perfect 24-h management of hypertension: Clinical relevance and perspectives," *Journal of Human Hypertension*, vol. 31, no. 4. Nature Publishing Group, pp. 231–243, 01-Apr-2017.

[11] B. Lemmer, "The importance of circadian rhythms on drug response in hypertension and coronary heart disease-from mice and man," *Pharmacology and Therapeutics*, vol. 111, no. 3. Pergamon, pp. 629–651, 01-Sep-2006.

[12] Y. Chen *et al.*, "Assessment of left ventricular function and peripheral vascular arterial stiffness in patients with dipper and non-dipper hypertension," *J.*

Investig. Med., vol. 66, no. 2, pp. 319–324, Feb. 2018.

[13] E. M. Alissa and G. A. Ferns, "Dietary fruits and vegetables and cardiovascular diseases risk," *Critical Reviews in Food Science and Nutrition*, vol. 57, no. 9. Taylor and Francis Inc., pp. 1950–1962, 13-Jun-2017.

[14] C. Ozemek, S. Tiwari, A. Sabbahi, S. Carbone, and C. J. Lavie, "Impact of therapeutic lifestyle changes in resistant hypertension," *Progress in Cardiovascular Diseases*, vol. 63, no. 1. W.B. Saunders, pp. 4–9, 01-Jan-2020.

[15] M. Bencze, A. Vavřínová, J. Zicha, and M. Behuliak, "Pharmacological suppression of endogenous glucocorticoid synthesis attenuated blood pressure and heart rate response to acute restraint in wistar rats," *Physiol. Res.*, vol. 69, no. 3, pp. 415–426, 2020.

[16] C. G. Fraga, K. D. Croft, D. O. Kennedy, and F. A. Tomás-Barberán, "The effects of polyphenols and other bioactives on human health," *Food Funct.*, vol. 10, no. 2, pp. 514–528, Feb. 2019.

[17] J. P. Trigo, E. M. C. Alexandre, J. A. Saraiva, and M. E. Pintado, "High value-added compounds from fruit and vegetable by-products–Characterization, bioactivities, and application in the development of novel food products," *Critical Reviews in Food Science and Nutrition*, vol. 60, no. 8. Taylor and Francis Inc., pp. 1388–1416, 27-Apr-2020.

[18] M. López-Pedrouso, J. M. Lorenzo, J. Cantalapiedra, C. Zapata, J. M. Franco, and D. Franco, "Aquaculture and by-products: Challenges and opportunities in the use of alternative protein sources and bioactive compounds," in *Advances in Food and Nutrition Research*, vol. 92, Academic Press Inc., 2020, pp. 127–185.

[19] S. Pimentel-Moral, M. de la L. Cádiz-Gurrea, C. Rodríguez-Pérez, and A. Segura-Carretero, "Recent advances in extraction technologies of phytochemicals applied for the revaluation of agri-food by-products," in *Functional and Preservative Properties of Phytochemicals*, Elsevier, 2020, pp. 209–239.

[20] A. Teixeira *et al.*, "Natural Bioactive Compounds from Winery By-Products as Health Promoters: A Review," *Int. J. Mol. Sci.*, vol. 15, no. 9, pp. 15638–15678, Sep. 2014.

[21] M. M. Dohadwala and J. A. Vita, "Grapes and cardiovascular disease," in *Journal of Nutrition*, 2009, vol. 139, no. 9.

[22] R. López-Fernández-Sobrino *et al.*, "ACE Inhibitory and Antihypertensive Activities of Wine Lees and Relationship among Bioactivity and Phenolic Profile," *Nutrients*, vol. 13, no. 2, p. 679, Feb. 2021.

[23] R. López-Fernández-Sobrino *et al.*, "Blood Pressure-Lowering Effect of Wine Lees: Dose-Response Study, Effect of Dealcoholization and Possible Mechanisms of Action," *Nutrients*, vol. 13, no. 4, p. 1142, Mar. 2021.

[24] R. López-Fernández-Sobrino *et al.*, "Enzyme-Assisted Extraction to Obtain Phenolic-Enriched Wine Lees with Enhanced Bioactivity in Hypertensive Rats," *Antioxidants*, vol. 10, no. 4, p. 517, Mar. 2021.

[25] K. OKAMOTO and K. AOKI, "Development of a Strain of Spontaneously Hypertensive Rats," *Jpn. Circ. J.*, vol. 27, no. 3, pp. 282–293, Mar. 1963.

[26] M. Quiñones *et al.*, "Long-term intake of CocoanOX attenuates the development of hypertension in spontaneously hypertensive rats," *Food Chem.*, vol. 122, no. 4, pp. 1013–1019, Oct. 2010.

[27] M. Miguel *et al.*, " Changes in arterial blood pressure in hypertensive rats caused by long-term intake of milk fermented by Enterococcus faecalis CECT 5728 ," *Br. J. Nutr.*, vol. 94, no. 1, pp. 36–43, Jul. 2005.

[28] A. Cabassi, S. Vinci, M. Calzolari, G. Bruschi, and A. Borghetti, "Regional sympathetic activity in pre-hypertensive phase of spontaneously hypertensive rats," *Life Sci.*, vol. 62, no. 12, pp. 1111–1118, Feb. 1998.

[29] A. Mas-capdevila, L. Iglesias-carres, A. Arola-arnal, M. Suárez, and I. Francisca, "Changes in arterial blood pressure caused by long-term administration of grape seed proanthocyanidins in rats with established hypertension," *Food Funct.*, vol. 11, no. 10, pp. 8735–8742, 2020.

[30] F. Turnbull *et al.*, "Effects of different blood-pressure-lowering regimens on major cardiovascular events: Results of prospectively-designed overviews of randomised trials," *Lancet*, vol. 362, no. 9395, pp. 1527–1535, Nov. 2003.

[31] A. Zanchetti, "Hypertension: Lower or higher blood-pressure targets for high-risk patients?," *Nature Reviews Cardiology*, vol. 13, no. 11. Nature Publishing Group, pp. 637–638, 01-Nov-2016.

[32] D. Ettehad *et al.*, "Blood pressure lowering for prevention of cardiovascular disease and death: A systematic review and meta-analysis," *Lancet*, vol. 387, no. 10022, pp. 957–967, Mar. 2016.

[33] C. J. Morris *et al.*, "Day/night variability in blood pressure: Influence of posture and physical activity," *Am. J. Hypertens.*, vol. 26, no. 6, pp. 822–828, Jun. 2013.

[34] B. Lemmer, "Cardiovascular Chronobiology and Chronopharmacology," in *Biologic Rhythms in Clinical and Laboratory Medicine*, Springer Berlin Heidelberg, 1992, pp. 418–427.

[35] B. Lemmer, "Signal Transduction and Chronopharmacology of Regulation of Circadian Cardiovascular Rhythms in Animal Models of Human Hypertension," *Heart Failure Clinics*, vol. 13, no. 4. Elsevier Inc., pp. 739–757, 01-Oct-2017.

[36] S. Albarwani, S. Al-Siyabi, and M. O. Tanira, "Lisinopril Indifferently Improves Heart Rate Variability During Day and Night Periods in Spontaneously Hypertensive Rats," *Physiol. Res*, vol. 62, pp. 237–245, 2013.

[37] A. P. Hallstrom, P. K. Stein, R. Schneider, M. Hodges, G. Schmidt, and K. Ulm, "Characteristics of heart beat intervals and prediction of death," *Int. J. Cardiol.*, vol. 100, no. 1, pp. 37–45, Apr. 2005.

[38] S. Reule and P. E. Drawz, "Heart rate and blood pressure: Any possible implications for management of hypertension," *Curr. Hypertens. Rep.*, vol. 14, no. 6, pp. 478–484, Dec. 2012.

[39] G. Belcaro, A. Ledda, S. Hu, M. R. Cesarone, B. Feragalli, and M. Dugall, "Grape seed procyanidins in pre- and mild hypertension: A registry study," *Evidence-based Complement. Altern. Med.*, vol. 2013, 2013.

[40] J. Duarte *et al.*, "Antihypertensive effects of the flavonoid quercetin in

spontaneously hypertensive rats," *Br. J. Pharmacol.*, vol. 133, no. 1, pp. 117–124, May 2001.

[41] C. D. Johansen *et al.*, "Resting, night-time, and 24 h heart rate as markers of cardiovascular risk in middle-aged and elderly men and women with no apparent heart disease," *Eur. Heart J.*, vol. 34, no. 23, pp. 1732–1739, Jun. 2013.

[42] J. S. S. Reis *et al.*, "Antidepressant- and anxiolytic-like activities of an oil extract of propolis in rats," *Phytomedicine*, vol. 21, no. 11, pp. 1466–1472, Sep. 2014.

[43] B. M. Razavi, F. Lookian, and H. Hosseinzadeh, "Protective effects of green tea on olanzapine-induced-metabolic syndrome in rats," *Biomed. Pharmacother.*, vol. 92, pp. 726–731, Aug. 2017.

[44] A. Arola-Arnal *et al.*, "Chrononutrition and polyphenols: Roles and diseases," *Nutrients*, vol. 11, no. 11. MDPI AG, p. 2602, 01-Nov-2019.

[45] E. A. Bakker, X. Sui, A. G. Brellenthin, and D. C. Lee, "Physical activity and fitness for the prevention of hypertension," *Current Opinion in Cardiology*, vol. 33, no. 4. Lippincott Williams and Wilkins, pp. 394–401, 01-Jul-2018.

[46] J. E. Sharman, A. La Gerche, and J. S. Coombes, "Exercise and cardiovascular risk in patients with hypertension," *American Journal of Hypertension*, vol. 28, no. 2. Oxford University Press, pp. 147–158, 01-Feb-2015.

[47] N. Sumien, M. N. Sims, H. J. Taylor, and M. J. Forster, "Profiling psychomotor and cognitive aging in four-way cross mice," *Age (Omaha).*, vol. 28, no. 3, pp. 265–282, Sep. 2006.

[48] E. Daskalova *et al.*, "Aronia melanocarpa (Michx.) Elliot fruit juice reveals neuroprotective effect and improves cognitive and locomotor functions of aged rats," *Food Chem. Toxicol.*, vol. 132, p. 110674, Oct. 2019.

TABLES

Table 1. Dipper and non-dipper patterns showed as the percentage of difference between light-night periods in systolic blood pressure, diastolic blood pressure and heart rate. Dipper when the difference is > 10 % and non-dipper when is < 10 %.

Difference between light-night periods							
Systolic blood pressure							
Weeks	0	1	2	3	4	5	6
VH	2.71 %	4.19 %	4.43 %	3.68 %	2.12 %	4.48%	2.76 %
WLPW	2.92 %	3.65 %	5.31 %	3.29 %	4.46 %	4.34 %	2.46 %
Diastolic blood pressure							
VH	3.83 %	4.56 %	5.14 %	4.45 %	4.10 %	3.48%	4.42 %
WLPW	2.58 %	5.26 %	6.17 %	4.86 %	5.43 %	5.42 %	3.21 %
Heart rate							
VH	11.95 %	10.38 %	11.39 %	11.28 %	11.05 %	9.87 %	8.47 %
WLPW	11.71 %	13.19 %	13.92 %	12.83 %	12.90 %	13.31 %	8.08 %

FIGURES

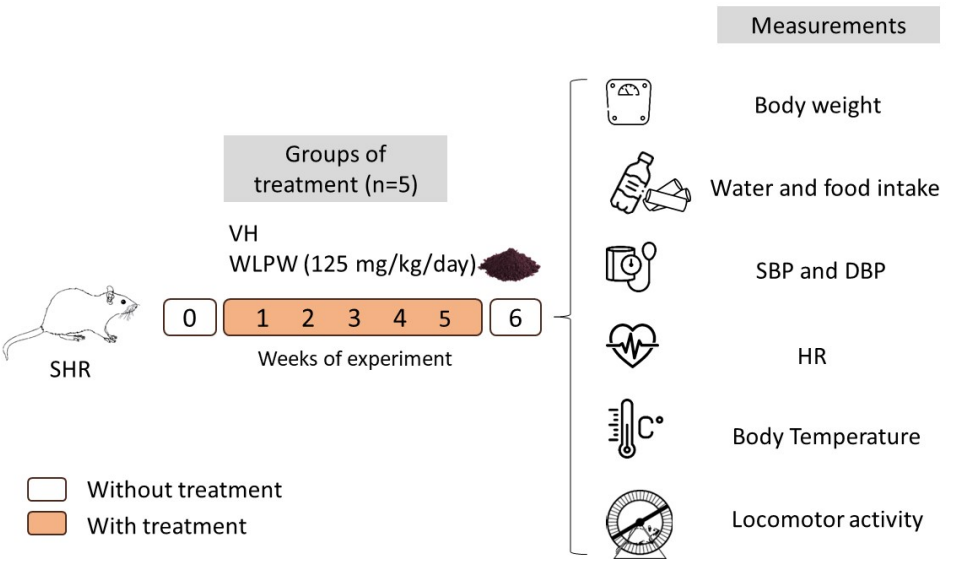

Figure 1. Experimental design. Measurement by radiotelemetry of blood pressure (BP), heart rate (HR), locomotor activity and body temperature during treatment period (weeks 1-5) with 125 mg/kg/day of WLPW or VH and during the follow-up period (week 6). Additionally, body weight, food and water intake were measured during all experiment.

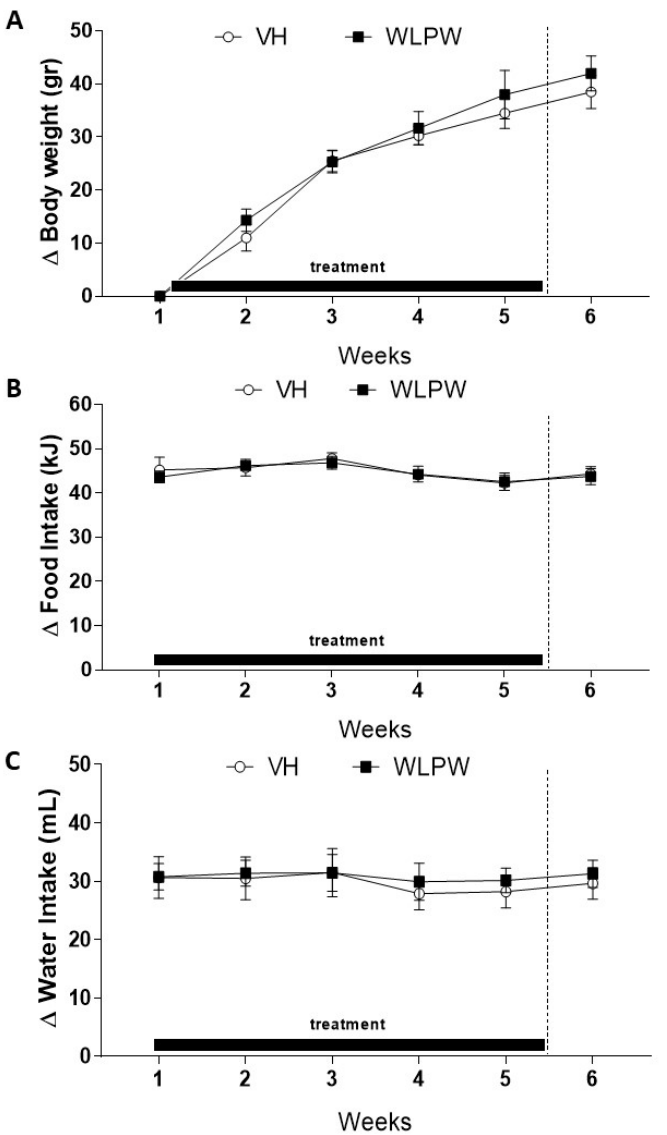

Figure 2. Measurements of: **A)** body weight (g), **B)** food intake (kJ) and **C)** water intake (mL) in spontaneously hypertensive rats during five weeks of the treatment period with vehicle (VH) or wine lees powder (WLPW) and in the week after removing treatment. Significant differences ($p < 0.05$) between treatments are represented by different letters in the legend. p value was estimated by two-way ANOVA and Tukey test was used as post hoc.

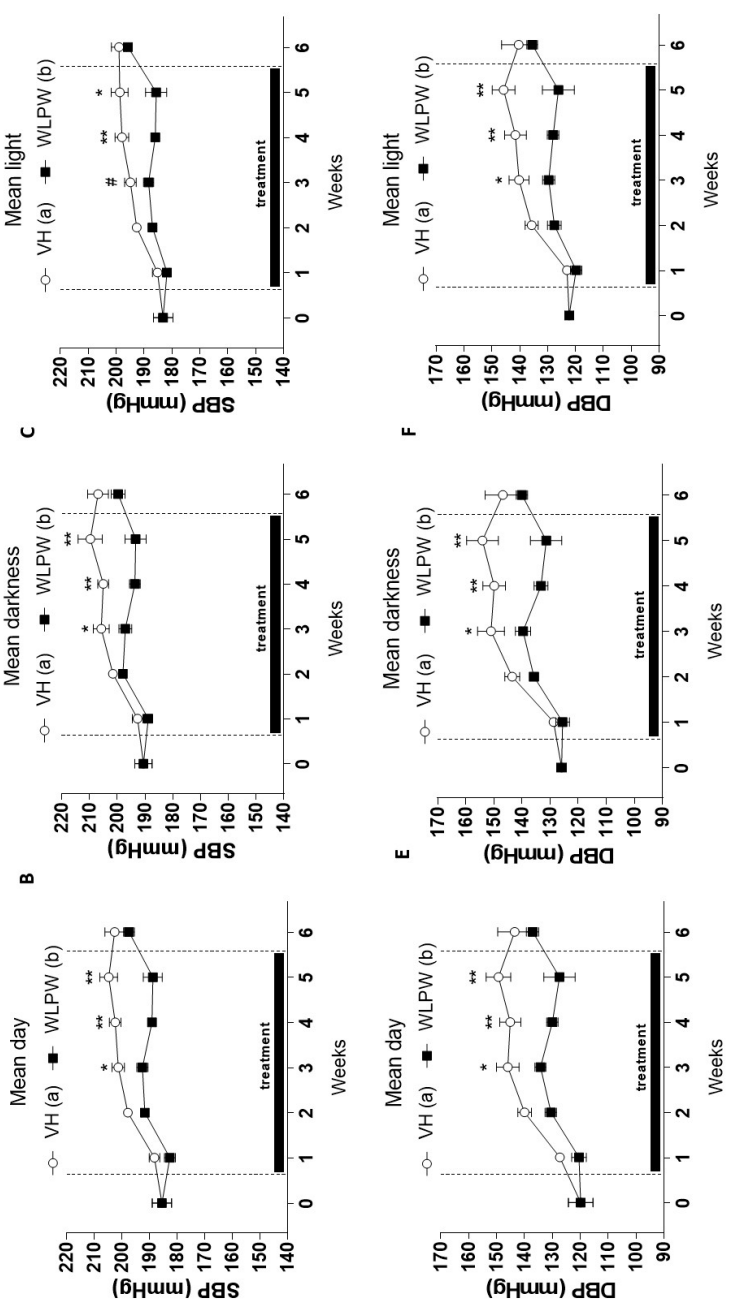

Figure 3. A) Systolic blood pressure (SBP) during long-term of treatment (weeks 1-5) with vehicle (VH) or wine lees powder (WLPW) and before and after removing treatment in spontaneously hypertensive rats (SHR). **Figures B and C** represent the mean SBP in darkness and light periods respectively. **D)** Diastolic blood pressure (DBP) during long-term of treatment (weeks 1-5) with VH or WLPW and before and

after removing treatment in SHR. **Figures E and F** represent the mean DBP in darkness and light periods respectively. Significant differences ($p < 0.05$) between treatments are represented by different letters in the legend. p value was estimated by two-way ANOVA and Tukey test was used as post hoc. (*) and (**) were used to identify significant differences between groups in the same week for $p < 0.05$ or $p < 0.01$, respectively. p was estimated by Student's T-test.

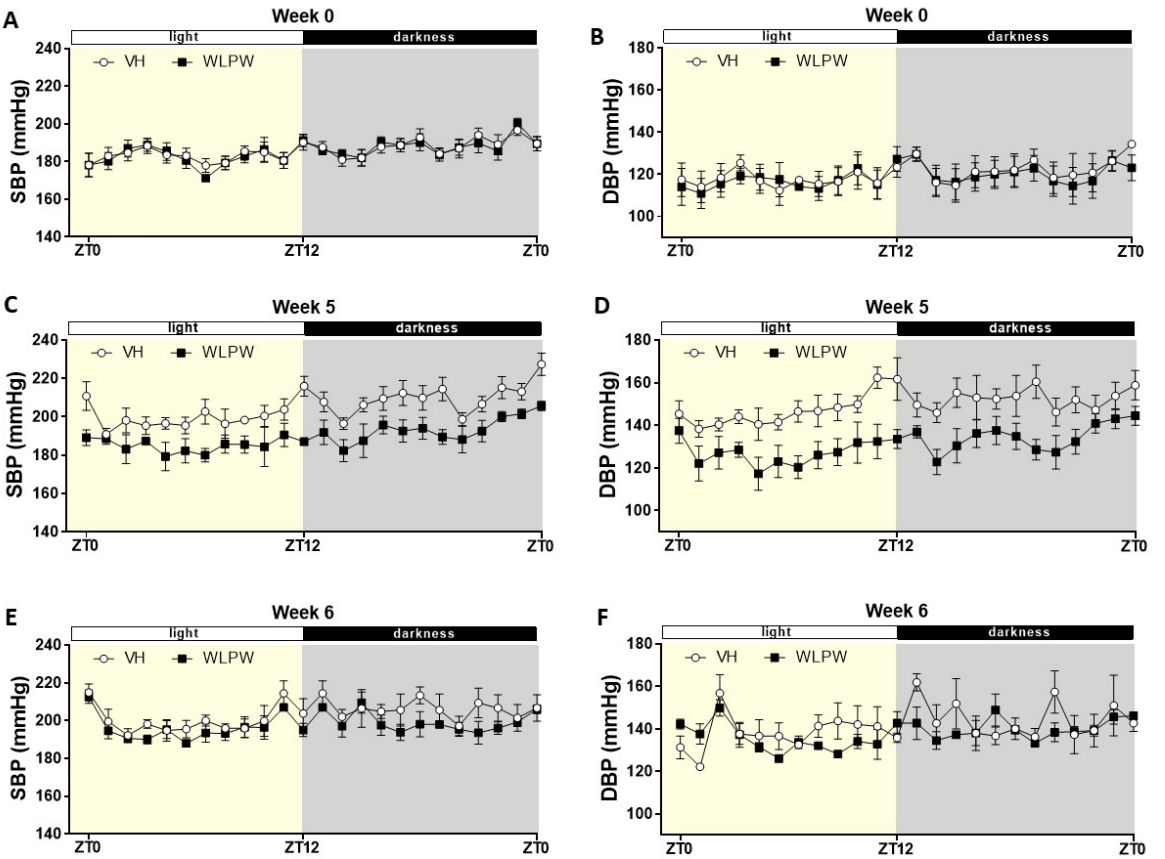

Figure 4. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) recorded during 24 h. ZT0 and ZT12 is when the light period and the darkness period initiate respectively. **A and B** represent the initial 24-h SBP and DBP after start the treatment. **C and D** represent the SBP and DBP respectively during 24h in the last week of treatment (week 5) with 125 mg/kg/day bw of wine lees powder (WLPW) or vehicle (VH). **E and F** show the variability of SBP and DBP respectively during 24h in the follow-up period without treatment.

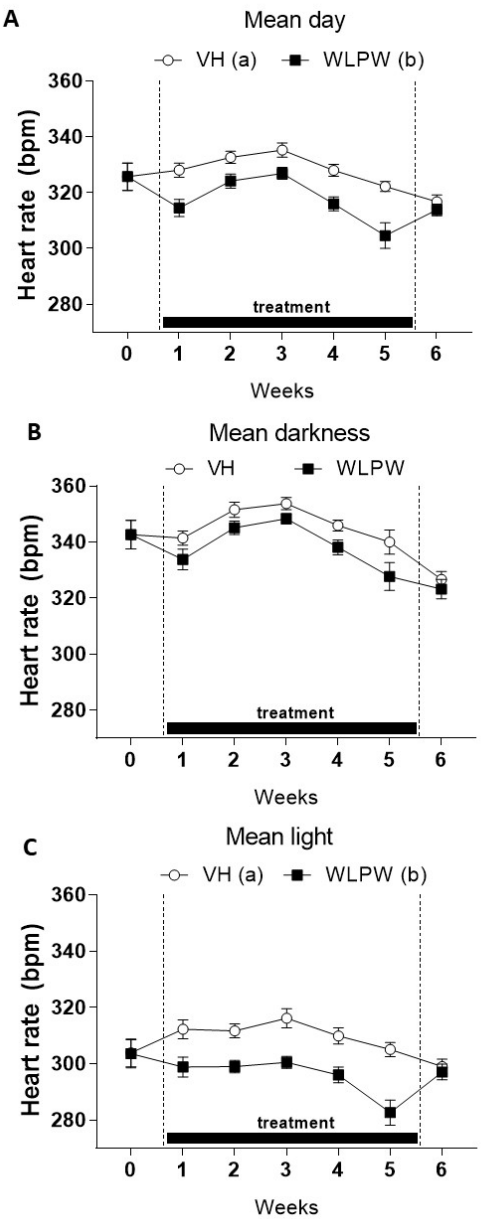

Figure 6. A) Heart rate (HR) during long-term of treatment (weeks 1-5) with vehicle (VH) or wine lees powder (WLPW) and before and after stopping treatment in spontaneously hypertensive rats (SHR). **Figures B and C** represent the mean HR in darkness and light periods respectively. Significant differences ($p < 0.05$) between treatments are represented by different letters in the legend. p value was estimated by two-way ANOVA and Tukey test was used as post hoc.

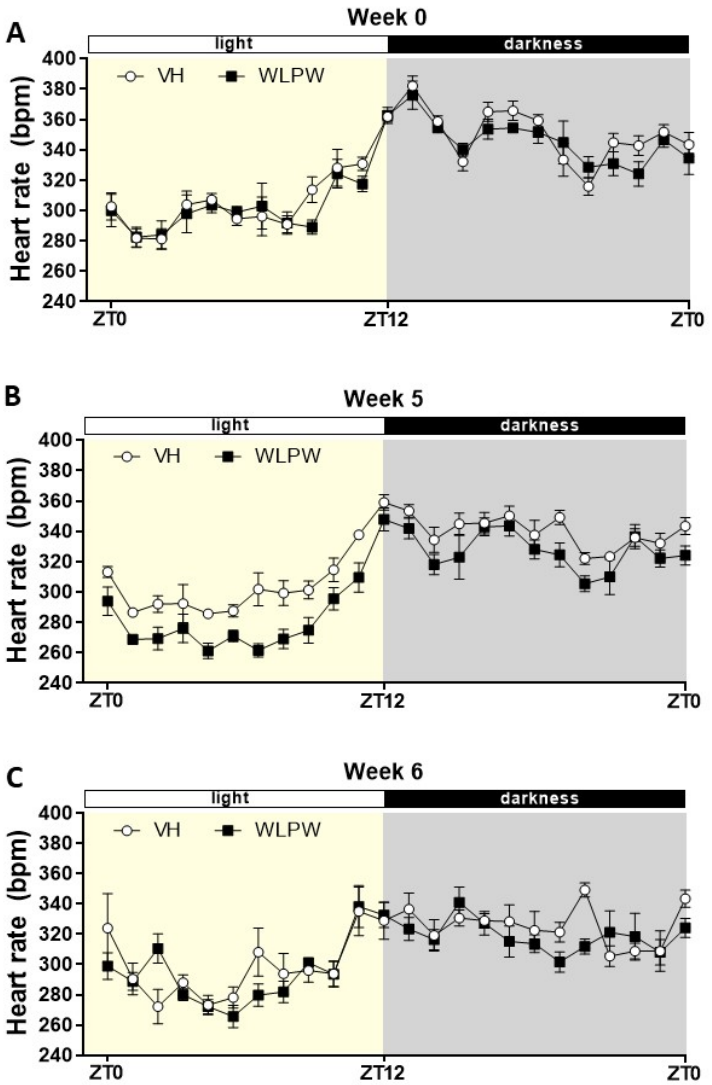

Figure 7. Heart rate (HR) recording during 24 h. ZT0 is the initiation of light period and ZT12 the initiation of darkness period. **A)** Represent the initial 24-h HR after start the treatment. **B** represent the variability of HR during 24h in the last week of treatment (week 5) with 125 mg/kg/day bw of wine lees powder (WLPW) or vehicle (VH). **C)** Variability of HR during 24h in the follow-up period without treatment.

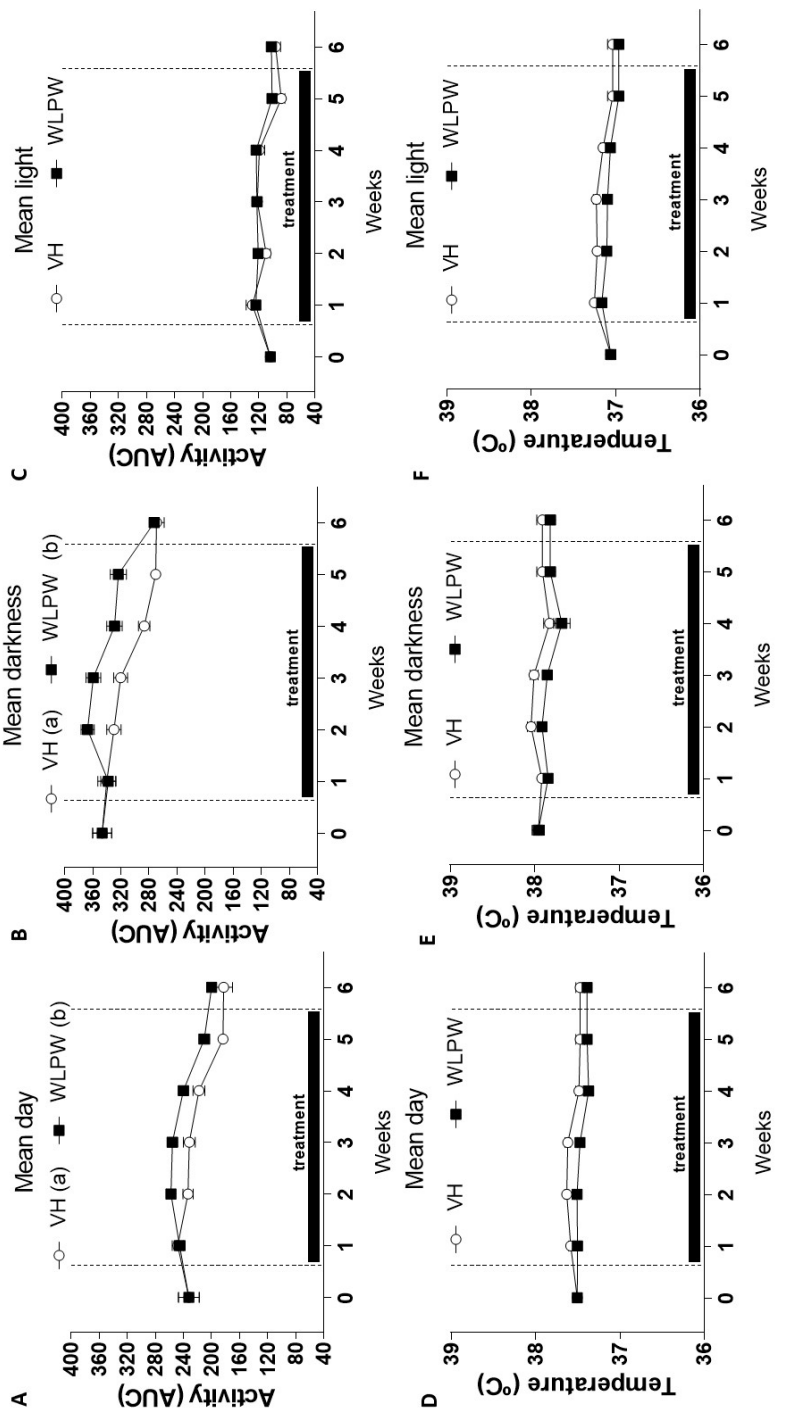

Figure 8. A) Activity during long-term of treatment (weeks 1-5) with vehicle (VH) or wine lees powder (WLPW) and before and after removing treatment in spontaneously hypertensive rats (SHR). **Figures B and C** represent the mean of activity in darkness and light periods

respectively. **D)** Body temperature during long-term of treatment (weeks 1-5) with VH or WLPW and before and after removing treatment in SHR. **Figures E and F** represent the mean of body temperature in darkness and light periods respectively. Significant differences ($p < 0.05$) between treatments are represented by different letters in the legend. p value was estimated by two-way ANOVA and Tukey test was used as post hoc.

SUPPLEMENTARY

S1. Non-anthocyanin composition of wine lees powder obtained by UHPLC-(ESI-)-Q-TOF-MS

Compound	µg/g wet matter
Catechin	3,599.26 ± 17.70
Catechin gallate	29.49 ± 0.37
Epicatechin	1,602.95 ± 5.64
(Epi)catechin O-glucoside iso1	18.43 ± 0.01
(Epi)catechin O-glucoside iso2	12.17 ± 0.00
(Epi)catechin O-glucoside iso3	54.19 ± 1.08
Procyanidin dimer B2	1,275.58 ± 0.37
Procyanidin dimer iso1	2,367.19 ± 5.64
Procyanidin dimer iso2	525.71 ± 2.30
Procyanidin dimer iso3	109.12 ± 0.76
Procyanidin dimer iso4	471.89 ± 3.92
Procyanidin dimer iso5	159.26 ± 0.82
Procyanidin trimer iso1	600.18 ± 3.80
Procyanidin trimer iso2	529.03 ± 9.77
Procyanidin trimer iso3	225.25 ± 2.14
Procyanidin trimer iso4	118.71 ± 3.90
Procyanidin trimer iso5	508.39 ± 3.51
Total Flavanols	**12,206.80**
Quercetin	1,355.94 ± 4.47
Quercetin-3-O-glucoside	60.09 ± 0.39
Quercetin-3-O-glucuronide	89.22 ± 0.76
Kaempferol	189.86 ± 1.50
kaempferol-3-O-glucuronide	17.70 ± 0.35
Isorhamnetin	411.43 ± 2.31
Total Flavonols	**2,124.24**
Gallic acid	4,456.04 ± 89.03
Caffeic acid	120.55 ± 0.77
Caffeic acid O-glucoside iso1	20.28 ± 0.74
Caffeic acid O-glucoside iso2	24.33 ± 1.11
p-Coumaric acid	126.82 ± 0.68
4-Hydroxybenzoic acid	61.57 ± 2.14
Ferulic acid	27.65 ± 0.43
Vanillic acid	85.90 ± 2.41
Total Phenolic acids	**4,923.14**
trans-Resveratrol	169.59 ± 0.74

Resveratrol iso1	108.76 ± 0.37
Resveratrol O-glucoside iso1	9.95 ± 0.37
Resveratrol O-glucoside iso2	49.77 ± 1.47
Piceatannol	154.84 ± 2.00
Piceatannol 3-O-glucoside iso1	8.11 ± 0.00
Piceatannol 3-O-glucoside iso2	2.21 ± 0.00
Viniferin-iso1	9.95 ± 0.00
Viniferin-iso2	29.86 ± 0.41
Total Stilbenes	**543.04**

Data are represented per g of wet weight

S2. Anthocyanin composition of wine lees powder obtained by UHPLC-(ESI-)-Q-TOF-MS

Compound	Quantity (µg/g)
Gallocatechin-Malvidin-3-glucoside dimer	9.09 ± 0.04
Malvidin-3-glucoside-(epi)catechin	40.95 ± 0.12
Delphinidin-3-glucoside	136.02 ± 1.68
Cyanidin-3-glucoside	8.34 ± 0.75
Petunidin-3-glucoside	185.45 ± 2.08
Petunidin-3-glucoside-pyruvic acid	3.28 ± 0.04
Peonidin-3-glucoside	100.31 ± 2.30
Malvidin-3-glucoside	2,236.82 ± 18.44
Peonidin-3-glucoside-pyruvic acid	1.50 ± 0.03
Delphinidin-(6-acetyl)-3-glucoside	33.48 ± 0.83
visitin A (malvidin-3-glucoside-pyruvic acid)	45.23 ± 0.12
Visitin B (malvidin-3-glucoside-acetaldehyde)	112.92 ± 0.54
Malvidin-3-glucoside-ethyl-(epi)catechin	13.47 ± 0.03
Cyanidin-(6-acetyl)-3-glucoside	7.50 ± 0.21
Acetylvisitin A	28.95 ± 0.41
Malvidin-3-glucoside-ethyl-(epi)catechin	50.75 ± 0.22
Petunidin-(6-acetyl)-3-glucoside	47.51 ± 1.72
Malvidin-3-glucoside-ethyl-(epi)catechin	75.36 ± 0.67
Acetylvisitin B	61.24 ± 0.41
Peonidin-(6-acetyl)-3-glucoside	48.65 ± 1.14
Delphinidin-(6-coumaroyl)-3-glucoside	16.10 ± 0.25
Malvidin-(6-acetyl)-3-glucoside	1,046.67 ± 0.77
Coumaroylvisitin A	7.38 ± 0.06
Malvidin-(6-caffeoyl)-3-glucoside	13.42 ± 0.25
Cyanidin-(6-coumaroyl)-3-glucoside	3.65 ± 0.15
Catechin-ethyl-Malvidin-3-acetylglucoside dimer	32.34 ± 0.29
Petunidin-(6-coumaroyl)-3-glucoside	27.46 ± 0.33
Pinotin A (malvidin-3-glucoside-vinylcatechol)	30.96 ± 0.47
Malvidin-glucoside-vinyl-catechin	5.64 ± 0.03
Coumaroylvisitin B	33.62 ± 0.26
Malvidin-3-glucoside-vinylguaiacol	21.92 ± 0.18
Catechin-ethyl-malvidin-3-coumaroylglucoside dimer	25.24 ± 0.10
Catechin-ethyl-malvidin-3-acetylglucoside dimer	5.33 ± 0.06
Peonidin-(6-coumaroyl)-3-glucoside	34.82 ± 1.00
Malvidin-(6-coumaroyl)-3-glucoside	396.97 ± 0.55
Malvidin-glucoside-vinyl-catechin	5.99 ± 0.02
Acetyl-pinotin A	0.26 ± 0.00
Malvidin 3-*O*-glucoside 4-vinylphenol (Pigment A)	23.58 ± 0.07
Catechin-ethyl-malvidin-3-coumaroylglucoside dimer	4.37 ± 0.01
Malvidin acetyl 3-O-glucoside 4-vinylphenol (Acetyl-pigment A)	14.12 ± 0.16
Total Anthocyanins	**4,996.66**

Data are represented per g of wet weight

GENERAL DISCUSSION

GENERAL DISCUSSION

Raised BP is the leading cause of death globally, with 10.4 million deaths a year [1]. Existing treatments for HTN are effective reducing BP in the majority of hypertensive patients. However, some of these drugs can produce side effects in some patients. Thus, currently there is an increased interest in the use of natural products to reduce BP. In this regard, the study of new bioactive compounds is considered as a good strategy in decreasing the risk of HTN, especially in prehypertensive subjects since this population is not usually clinically treated [2].

Food-derived compounds have emerged as source of natural compounds to manage HTN, being phenolic compounds and bioactive peptides some of the most studied [2]. They can modulate BP through different mechanisms such as inhibiting ACE, improving endothelial function or reducing reactive oxygen species (ROS) [3], [4]. In addition to foods, other interesting source of these functional compounds are agri-food industry by-products. These allow enhancing the valorization of these wastes and making the agri-food industries more environmentally friendly [5], [6]. Annually, large amount of agri-food by-products are generated during the food processing and, their valorization has attracted a great deal of attention over the past few years [7]. Grapes are one of the world's largest fruit crops and the winery industry generates large amounts of by-products [8]. In this regard, our group has a range experience in evaluating the beneficial properties, of a grape seed proanthocyanidins extract [9]. However, the wine production process generates many other by-products. All of them rich in grape phenolic compounds and many of these compounds have evidenced ACE inhibitory and antihypertensive effects [2]. In fact, some of the winery by-products such as grape pomace, skin or stems have been used to obtain phenolic-enriched extracts able to reduce BP [10]–[12]. However, the potential antihypertensive effect of other winery by-products such as WL as source of antihypertensive

compounds remains unexplored. Thus, in this Thesis the potential of WL as a source of antihypertensive compounds to elaborate functional ingredients, nutraceuticals or food supplements after their oral administration was investigated.

Therefore, the first objective was to explore the BP lowering properties of WL **[Manuscript 1]**. It is well known that phenolic composition of fruits and vegetables is modified by different factors including variety or plan growth conditions [13]. Consequently, winery by-products could have different grape phenolic composition depending of these factors. Thus, the soluble fraction of five WL, obtained in the elaboration of wine with a single grape variety (red grapes varieties: Cabernet, Garnacha, Mazuela, Merlot, and white grape variety: Macabeo), were selected to determine their ability to inhibit ACE. ACEi activity is commonly used as a screening tool in the search for natural antihypertensive compounds. The results showed that ACEi activity of the red grape varieties exerted higher activity than those obtained from the white grape variety. Similar results were reported by Pozo-Bayón et al. and Alcaide-Hidalgo et al., who studied the ACEi activity of red (Tempranillo) and white (Airén, Verdejo and Sauvignon Blanc) wines, respectively [14], [15]. Phenolic compounds are present in red grapes in larger quantities than in white grapes [16]. Therefore, these compounds could be responsible for the ACEi effects, since *in vitro* studies have demonstrated inhibitory properties of phenolic compounds on ACE [2]. It is worthy to mention that the ACEi potencies of the studied red WL are higher to those reported by other authors in milk fermented with *Enterococcus faecalis* and *Lactobacillus helveticus* [17], [18]. However, high *in vitro* ACEi activity does not always correlate to *in vivo* antihypertensive activity since bioactive compounds can be modified during gastrointestinal digestion. In addition, phenolic compounds, are widely metabolized, since the absorbed molecules, are recognized as xenobiotics and undergo Phase-II enzymatic detoxification. Moreover, many of them reach the colon, where they are subjected to microbial metabolism and can be further absorbed and

undergo Phase-II reactions [13]. Therefore, *in vivo* studies are necessary to demonstrate the antihypertensive effect of ACE inhibitors. Cabernet, Garnacha and Mazuela WL were selected by their ACEi activity and were evaluated in SHR after a single oral dose of 5.0 mL/kg bw. SHR are considered one of the best experimental models to evaluate the antihypertensive effect of drugs since the development of HTN in these rats is in fact very similar to humans [19]. The results show that the antihypertensive properties of WL depended on the grape used in the wine elaboration, since only WL obtained in the elaboration of wine with Cabernet grapes showed an antihypertensive effect. Furthermore, the antihypertensive effect of these was significantly similar to that observed for the antihypertensive drug Captopril. In addition, a possible undesirable hypotensive effect of WL from the Cabernet grape variety was rule out when administered to WKY rats, the normotensive control of SHR. These results indicate that the antihypertensive effect of these WL is specific to the hypertensive condition.

Phenolic compounds have been widely investigated due to their large number of beneficial properties, such as their cardioprotective effect [20], in which antihypertensive activity is included [21]. Since WL are rich in these compounds, the phenolic profile of the three WL tested in SHR was studied in order to understand their different BP-lowering behavior. Results revealed that the WL from Cabernet grape contained twice the amount of total phenolic compounds when compare to Mazuela or Garnacha WL, with high levels of families' flavanol and anthocyanin. Therefore, the BP-lowering effect of these WL was related to these two families. Specifically, the flavanols catechin, epicatechin, procyanidins and the anthocyanins: malvidin-3-glucoside, malvidin-(6-acetyl)-3-glucoside and malvidin-(6-coumaroyl)-3-glucoside were the compounds whose concentration was higher in the WL from Cabernet grape variety compared to the Mazuela and Garnacha WL. According to these findings, the intake of food or extracts rich in flavanols and anthocyanins or the consumption of the phenolic compounds whose levels were increased in WL

from Cabernet grapes have been associated with a BP reduction or cardioprotective action [22]–[28]. Nevertheless, as was aforementioned, fruit phenolic composition can be modified by different factors including plant growth conditions, which can vary from year to year. Therefore, the ACEi and antihypertensive activities of WL from Cabernet grape variety coming from a different harvest were also studied. However, no significant differences were founded in their properties, indicating a good reproducibility of the beneficial effects of these WL and opening the door to their commercial use within the wine industry.

However, biological functionalities of phenolic compounds not always act in a dose-dependent manner [29]. Therefore, the establishment of the most effective dose was considered as essential to optimize the effectivity and manufacturing of this product. Therefore, the most effective antihypertensive dose of WL from the Cabernet grapes (2.5, 5.0 and 7.5 mg/kg bw) was determined in SHR **[Manuscript 4]**. All doses of WL exhibited antihypertensive effect, reducing both SBP and DBP values, with the maximum BP decreases at 6 h post-administration. BP lowering effect was dose-dependently up to 5 mL/kg bw, and no differences were found after the administration of 5.0 and 7.5 mL/kg bw. Thus, 5 mL/kg bw (equivalent to 125 mg/kg bw) was considered as the most effective dose. It has reported that a reduction in 10 mmHg for SBP and 5 mmHg for DBP produces a significant reduction in the risk of suffering or worsening CVD [50,51]. Thus, these evidences point to the great potential of the WL from the Cabernet grape variety to control BP, more considering that the dose tested in rats corresponds to only 73 mL for a 70 kg person [30].

Phenolic compounds can be in the food matrix in free form or covalently bound to soluble or insoluble substances [31]. For example, tannins, phenolic compounds present in winery by-products, form complexes with proteins [32]. However, the antihypertensive WL tested in the animals only contained soluble phenolic compounds, since only the soluble fraction of WL was administered to SHR. Therefore, a high amount of the bioactive compounds can remain in the

non-soluble fraction of WL. Thus, the extraction of these compounds could improve the potential of WL, increasing their phenolic content and potentially enhancing their functionality **[Manuscript 2]**. Different methodologies have been described to facilitate the release of phenolic compounds from the matrix [33]. Enzyme-assisted extraction has emerged as a more eco-friendly alternative to conventional solvent-based extraction methods [34]. This technique is based on the use of enzymes to break down different components of the cell, which enhances the release of phenolic compounds [35]. WL were submitted to a hydrolysis with the enzymatic commercial preparation Flavourzyme. The hydrolysis caused an increase of 57.20 % of the phenolic content compared to the original WL. In this regard, anthocyanins and flavonols were the phenolic families that increased more after enzymatic extraction, being the resulting hydrolyzed WL rich in flavanols, anthocyanins, phenolic acids and flavonols. Moreover, this hydrolyzed WL also showed enhanced functionality, since a higher antioxidant, ACEi and antihypertensive activities were exhibited by the hydrolizated compared to the control lees. The improvement in their antioxidant effects is related to the higher content of phenolic compounds, specially to the increment in the anthocyanin content. In fact, a good correlation between anthocyanin compounds and antioxidant activity has been reported for different WL extracts [36]. In relation to the antihypertensive activity of the hydrolysate, a single dose of 5 mL/kg bw of it displayed a more powerful BP-lowering effect than the one showed by the control WL. Furthermore, the antihypertensive effect was more prolonged than the one shown by the drug Captopril, remaining until 48 h post administration. This improvement of the antihypertensive effect was also linked to the release of phenolic compounds, mainly flavanols and anthocianins, after the enzymatic hydrolisis.

In addition to the higher content of phenolic compounds, the WL hydrolizated present also a higher content in protein and the amino acid residues Pro, Ile, Leu, Val and Trp than the control WL. This fact indicates the

release of peptides during the WL hydrolysis. Therefore, the next goal was to identify potential ACE inhibitors and/or antihypertensive peptides released during the hydrolysis of WL **[Manuscript 3]**. Thus, the peptides of the hydrolysate were subjected to separation according to their molecular size and hydrophobicity by ultrafiltration and subsequently, RP-HPLC. The posterior selection according to the exerted ACEi activity allowed to identify the hydrolysate fractions presenting the highest ACEi activity. These fractions were further analyzed by HPLC-MS, resulting in the identification of six amino acid sequences (FKTTDQQTRTTVA, NPKLVTIV, TVTNPARIA, PAGELHP, LDSPSEGRAPG and LDSPSEGRAPGAD) in the hydrolysate. All of these peptides were released during the WL protein hydrolysis since these peptides were not found in the WL without hydrolyzing. TVTNPARIA, PAGELHP and LDSPSEGRAPG exhibited the highest ACEi activity, showing an IC_{50} value between 0.5 and 18 μM. Additionally, the antihypertensive effect of the six peptides were also evaluated at a dose of 10 mg/kg bw in SHR. All peptides showed an antihypertensive effect, decreasing both SBP and DBP, except the peptide PAGELHP that did not show this bioactivity. Similar decrements on BP to the obtained with the identified peptides have been founded in other peptides obtained from dietary food [37]–[39]. An additional *in silico* study revealed that all the identified peptides are susceptible to gastrointestinal hydrolysis by trypsin, chymotrypsin or pepsin. Thus, probably fragments of these peptides, more than the complete sequences, could be the responsible of the peptide bioactivity. Nevertheless, future studies are required to demonstrate which amino acid sequences could be responsible of the antihypertensive effect of these peptides since the BP-lowering effect of the resultant fragments have not been previously reported. All these results demonstrate that enzymatic extraction is a useful methodology to release phenolic compounds and bioactive peptides from WL that can be also be marketed.

Nevertheless, in addition to phenolic compounds, WL contain other components such as ethanol, which will disappear in the drying process.

Different studies carried out testing the effects of alcoholic drinks showed that alcohol can modify BP depending on the dose and intake duration [40]. Therefore, the drying of WL, and their consequent dealcoholization, could modify their antihypertensive properties. Thus, taking into account that a drying process is necessary to the commercial use of the WL, the next goal was to investigate the antihypertensive activity of the WL powder (WLPW) obtained from the Cabernet grapes **[Manuscript 4]**. WL from the Cabernet grape variety were dried, removing alcohol in the process, and the most effective dose (125 mg/kg bw) of these WLPW were administered to SHR. WLPW demonstrated a greater and more prolonged antihypertensive effect than WL. In concordance with our results, studies conducted with dealcoholized red wine exerted a potent antihypertensive effect in subjects with HTN in comparison with red wine [41]. Nevertheless, the dose of WLPW was lower than those presented in other studies, where they administered 375 mg/kg bw of flavanol-rich grape seed extract in SHR with similar BP lowering effects [18]. This could be explained by the participation in their antihypertensive effect of other phenolic compounds in addition to flavanols, such as anthocyanins. Furthermore, since the BP-lowering effect of the WLPW was even more potent than the drug captopril, it was considered essential to rule out a potential hypotensive effect on normotensive animals. However, as it has been previously reported for other products rich in phenolic compounds [26], [42]–[44], the antihypertensive effect of the dealcoholized WL were specific to the hypertensive state, since no BP-lowering effects were observed in the normotensive rats. These results open the door to the use to commercialize the WLPW as functional ingredient, nutraceutical and food supplements with potent BP-lowering effects.

Different mechanisms may be involved in the BP lowering effect produced by the WLPW **[Manuscript 5]** and **[Manuscript 6]**. Since WL from the Cabernet grapes were selected according their high ACEi activity, inhibition of ACE was considered as potential mechanism involved in their antihypertensive effect **[Manuscript 5]**. However, no changes in the plasma ACE activity were

found in SHR after 6 h post-administration of 125 mg/kg bw of dried WL respect to control group. These results could seem contradictory since the *in vitro* ACE inhibitory properties of WL have been reported [45]. However, the lack of correspondence between *in vitro* ACEi activity and its plasma ACE activity has been showed for other phenolic-rich extracts such as a grape seed proanthocyanidin extract in SHR rats or quercetin-rich onion skin extract in pre-hypertensive subjects [26], [46]. Nevertheless, the results of this study do not rule out the involvement of ACE, acting before 6 h post-administration in the antihypertensive effect of the WLPW.

Oxidative stress is involved in the development of HTN [47]. Since WLPW showed a potent *in vitro* antioxidant effect, WL could exhibit their antihypertensive effect improving the oxidative stress of the hypertensive rats. In fact, SHR treated WLPW showed an increase of the hepatic GSH and an increase of plasma NO metabolites respect to non-treated animals. Furthermore, hepatic ROS levels were also found reduced in these WLPW-treated animals, which could be associated with the increased NO levels found in that animals. It is known that ROS reduce endothelial NO availability [44]. Additionally, administration of WLPW reduced plasma MDA, a marker of tissue damage and failure of the antioxidant defense mechanisms [47], in addition to play an important role in the endothelial dysfunction through inhibition of eNOS activity and expression. Therefore, the WLPW exert their antihypertensive effect through the regulation of redox system **[Manuscript 5]**.

On the other hand, the improvement of the redox system by the WLPW seems to be related to an increase in the availability of NO and the expression of *eNos*. Thus, in the following study the role of NO, PGI_2 and Sirtuins in the antihypertensive effect of WLPW was also evaluated **[Manuscript 6]**. A clear implication of NO and a partial involvement of PGI_2 and Sirtuins were found in the antihypertensive effect of WLPW, administrating the WLPW with the inhibitors of eNOS (L-NAME), prostacyclin (indomethacin) and sirtuin (sirtinol) to SHR. In addition, this was confirmed with the increment in the aortic levels

expression of *eNos* and *Sirt1*. Sirt-1 plays an important role in eNOS deacetylation, their active form, therefore, the increase of Sirt-1 promotes the activation of eNOS and increase NO levels with improve the HTN stage [48]. WLPW also exert their antioxidant effect through endothelium with the reduction of *Nox-4* m-RNA expression, one of the main NADPH oxidases responsible of the overproduction of ROS in HTN [49], [50]. Furthermore, a lower ROS production favors a greater amount of free NO, claiming the very important role of NO in the lowering of BP produced by WLPW. Finally, the vasoconstrictor *Et-1* was also reduced, in the animals administered the WLPW.

All these results confirm the vasoprotective and antioxidant effect of WLPW, however, HTN is a chronic disease. Therefore, other objective of this thesis was to evaluate the effect of the WLPW in a long-term administration in SHR **[Manuscript 7]**. Hemodynamic parameters such as BP and HR, locomotor activity and temperature were monitored by telemetry system before (1 week), during (5 weeks) and after (1 week) the administration of the most effective dose of WLPW (125 mg/kg/day bw). Additionally, body weight, food and water intake were measure during all the experiment. Body weight, food and water intake not showed differences between the animals administered WLPW and the control group administered vehicle. Several studies have reported that until reaching the period of stability of BP in SHR, there is an initial period where this variable increases constantly [51], [52]. In this animal model, the values of SBP reported reach approximately 200 mmHg SBP at 17–20 weeks of age [53]. Thus, the animal experiment started when SHR had 15 weeks old. SBP and DBP showed an increase in the control group along the experiment; nevertheless, WLPW attenuated the increase of BP. It is well known that BP is modify along day, therefore, the different periods, light and darkness related in turn to the periods of inactivity and activity [54], [55], respectively, were studied separately. The powerful attenuating effect of WLPW was maintained in both phases and finally, after one week without treatment, BP increased in the group treated with WLPW until it equaled the control. Furthermore, a reduction

between 10-20% (dipper) in BP at night is related to a better risk of CVD, however, a reduction of less than 10% (non-dipper) is related to a greater risk of stroke and death [56]. SHR showed a non-dipper pattern BP before treatment which was improved by the administration of WLPW, nevertheless, the pattern did no change to dipper.

Abnormalities in the HR such as high HR, has been related with an increase in the BP [57]. Moreover, high HR in the non-activity period (light period in rats) has been related with a higher chance of developing CVD in humans [58]. Our results showed a reduction in HR which was specific of the light period (inactive period) showing the cardioprotective effect of long-term administration of the WLPW. In the same way as BP, a reduction in HR by more than 10% (dipper) is related to a cardiac improvement and a lower risk of death from CVD [59]. Thus, SHR started with a dipper HR which, in the VH-group, changed to non-dipper in the last week of treatment, showing a lower HR-variability between the light and dark period with age. However, the animals treated with WLPW maintained or increased the HR-variability between light and dark presenting a dipper pattern throughout the experiment. Additionally, an increase of activity or exercise training decrease sympathetic nervous (SNS) activity, which has an important role in the development and maintenance of HTN. Thus, the inhibition of SNS could be attributed to the reduction in BP after exercise [60]. WLPW increase the activity in SHR during treatment, specifically, during the peak period (light period). That results suggesting that WLPW could reduce BP through SNS-mediated locomotor activity. Finally, all the parameters were equal to those of the control after one week without treatment. Furthermore, no changes were observed in the body temperature.

REFERENCES

[1] "Hypertension." [Online]. Available: https://www.who.int/news-room/fact-sheets/detail/hypertension. [Accessed: 12-Oct-2020].

[2] M. Margalef, F. I. Bravo, A. Arola-Arnal, and B. Muguerza, "Natural Angiotensin Converting Enzyme (ACE) Inhibitors with Antihypertensive Properties," in *Natural Products Targeting Clinically Relevant Enzymes*, Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA, 2017, pp. 45–67.

[3] C. G. Fraga, K. D. Croft, D. O. Kennedy, and F. A. Tomás-Barberán, "The effects of polyphenols and other bioactives on human health," *Food Funct.*, vol. 10, no. 2, pp. 514–528, Feb. 2019.

[4] B. Hernández-Ledesma, M. Del Mar Contreras, and I. Recio, "Antihypertensive peptides: Production, bioavailability and incorporation into foods," *Adv. Colloid Interface Sci.*, vol. 165, no. 1, pp. 23–35, 2011.

[5] S. Ben-Othman, I. Jõudu, and R. Bhat, "Bioactives from Agri-Food Wastes: Present Insights and Future Challenges," *Molecules*, vol. 25, no. 3, p. 510, Jan. 2020.

[6] J. Banerjee, R. Singh, R. Vijayaraghavan, D. MacFarlane, A. F. Patti, and A. Arora, "Bioactives from fruit processing wastes: Green approaches to valuable chemicals," *Food Chemistry*, vol. 225. Elsevier Ltd, pp. 10–22, 15-Jun-2017.

[7] S. Ben-Othman, I. Jõudu, and R. Bhat, "Bioactives from agri-food wastes: Present insights and future challenges," *Molecules*, vol. 25, no. 3. MDPI AG, p. 510, 24-Jan-2020.

[8] N. Musee, L. Lorenzen, and C. Aldrich, "Cellar waste minimization in the wine industry: a systems approach," *J. Clean. Prod.*, vol. 15, no. 5, pp. 417–431, Jan. 2007.

[9] C. Bladé *et al.*, "Proanthocyanidins in health and disease," *BioFactors*, vol. 42, no. 1, pp. 5–12, Jan. 2016.

[10] R. S. de Moura *et al.*, "Antihypertensive, vasodilator and antioxidant effects of a vinifera grape skin extract," *J. Pharm. Pharmacol.*, vol. 54, no. 11, pp. 1515–1520, Nov. 2002.

[11] Z. Rasines-Perea, I. Ky, G. Cros, A. Crozier, and P.-L. Teissedre, "Grape Pomace: Antioxidant Activity, Potential Effect Against Hypertension and Metabolites Characterization after Intake," *Diseases*, vol. 6, no. 3, p. 60, Jul. 2018.

[12] N. Goutzourelas *et al.*, "Polyphenolic composition of grape stem extracts affects antioxidant activity in endothelial and muscle cells," *Mol. Med. Rep.*, vol. 12, no. 4, pp. 5846–5856, Oct. 2015.

[13] S. Maqsood, O. Adiamo, M. Ahmad, and P. Mudgil, "Bioactive compounds from

date fruit and seed as potential nutraceutical and functional food ingredients," *Food Chemistry*, vol. 308. Elsevier Ltd, p. 125522, 05-Mar-2020.

[14] J. M. Alcaide-Hidalgo, A. J. Martínez-Rodríguez, P. J. Martín-Álvarez, and E. Pueyo, "Influence of the elaboration process on the peptide fraction with angiotensin I-converting enzyme inhibitor activity in sparkling wines and red wines aged on lees," *Food Chem.*, vol. 111, no. 4, pp. 965–969, Dec. 2008.

[15] M. Á. Pozo-Bayón, J. M. Alcaíde, M. C. Polo, and E. Pueyo, "Angiotensin I-converting enzyme inhibitory compounds in white and red wines," *Food Chem.*, vol. 100, no. 1, pp. 43–47, Jan. 2007.

[16] F. Mattivi, R. Guzzon, U. Vrhovsek, M. Stefanini, and R. Velasco, "Metabolite profiling of grape: Flavonols and anthocyanins," *J. Agric. Food Chem.*, vol. 54, no. 20, pp. 7692–7702, Oct. 2006.

[17] B. Muguerza *et al.*, "Antihypertensive activity of milk fermented by Enterococcus faecalis strains isolated from raw milk," *Int. Dairy J.*, vol. 16, no. 1, pp. 61–69, Jan. 2006.

[18] A. Fuglsang, D. Nilsson, and N. C. B. Nyborg, "Cardiovascular effects of fermented milk containing angiotensin-converting enzyme inhibitors evaluated in permanently catheterized, spontaneously hypertensive rats," *Appl. Environ. Microbiol.*, vol. 68, no. 7, pp. 3566–3569, Jul. 2002.

[19] K. OKAMOTO and K. AOKI, "Development of a Strain of Spontaneously Hypertensive Rats," *Jpn. Circ. J.*, vol. 27, no. 3, pp. 282–293, Mar. 1963.

[20] M. Dell'Agli, A. Buscialà, and E. Bosisio, "Vascular effects of wine polyphenols," *Cardiovascular Research*, vol. 63, no. 4. Oxford Academic, pp. 593–602, 01-Sep-2004.

[21] A. Durazzo *et al.*, "Polyphenols: A concise overview on the chemistry, occurrence, and human health," *Phytotherapy Research*, vol. 33, no. 9. John Wiley and Sons Ltd, pp. 2221–2243, 01-Sep-2019.

[22] T. C. Wallace, "Anthocyanins in cardiovascular disease," *Advances in Nutrition*, vol. 2, no. 1. Adv Nutr, pp. 1–7, Jan-2011.

[23] E. O. Igwe, K. E. Charlton, S. Roodenrys, K. Kent, K. Fanning, and M. E. Netzel, "Anthocyanin-rich plum juice reduces ambulatory blood pressure but not acute cognitive function in younger and older adults: a pilot crossover dose-timing study," *Nutr. Res.*, vol. 47, pp. 28–43, Nov. 2017.

[24] C. Heiss *et al.*, "Improvement of endothelial function with dietary flavanols is associated with mobilization of circulating angiogenic cells in patients with coronary artery disease," *J. Am. Coll. Cardiol.*, vol. 56, no. 3, pp. 218–224, Jul. 2010.

[25] L. Fairlie-Jones, K. Davison, E. Fromentin, and A. M. Hill, "The effect of

anthocyanin-rich foods or extracts on vascular function in adults: A systematic review and meta-analysis of randomised controlled trials," *Nutrients*, vol. 9, no. 8. MDPI AG, 20-Aug-2017.

[26] M. Quiñones *et al.*, "Low-molecular procyanidin rich grape seed extract exerts antihypertensive effect in males spontaneously hypertensive rats," *Food Res. Int.*, vol. 51, no. 2, pp. 587–595, May 2013.

[27] Z. Pons, M. Margalef, F. I. Bravo, A. Arola-Arnal, and B. Muguerza, "Grape seed flavanols decrease blood pressure via Sirt-1 and confer a vasoprotective pattern in rats," *J. Funct. Foods*, vol. 24, pp. 164–172, Jun. 2016.

[28] N. P. Bondonno *et al.*, "Flavonoid-Rich Apple Improves Endothelial Function in Individuals at Risk for Cardiovascular Disease: A Randomized Controlled Clinical Trial," *Mol. Nutr. Food Res.*, vol. 62, no. 3, Feb. 2018.

[29] V. Calabrese *et al.*, "The Hormetic Role of Dietary Antioxidants in Free Radical-Related Diseases," *Curr. Pharm. Des.*, vol. 16, no. 7, pp. 877–883, Mar. 2010.

[30] S. Reagan-Shaw, M. Nihal, and N. Ahmad, "Dose translation from animal to human studies revisited," *FASEB J.*, vol. 22, no. 3, pp. 659–661, Mar. 2008.

[31] F. Shahidi and J. D. Yeo, "Insoluble-bound phenolics in food," *Molecules*, vol. 21, no. 9. MDPI AG, 01-Sep-2016.

[32] T. Ozdal, E. Capanoglu, and F. Altay, "A review on protein-phenolic interactions and associated changes," *Food Research International*, vol. 51, no. 2. Elsevier, pp. 954–970, 01-May-2013.

[33] J. C. Hogervorst, U. Miljić, and V. Puškaš, "Extraction of Bioactive Compounds from Grape Processing By-Products," in *Handbook of Grape Processing By-Products*, Elsevier, 2017, pp. 105–135.

[34] P. Panja, "Green extraction methods of food polyphenols from vegetable materials," *Current Opinion in Food Science*, vol. 23. Elsevier Ltd, pp. 173–182, 01-Oct-2018.

[35] A. K. Landbo and A. S. Meyer, "Enzyme-assisted extraction of antioxidative phenols from black currant juice press residues (Ribes nigrum)," *J. Agric. Food Chem.*, vol. 49, no. 7, pp. 3169–3177, 2001.

[36] R. Romero-Díez, S. Rodríguez-Rojo, M. J. Cocero, C. M. M. Duarte, A. A. Matias, and M. R. Bronze, "Phenolic characterization of aging wine lees: Correlation with antioxidant activities," *Food Chem.*, vol. 259, pp. 188–195, Sep. 2018.

[37] J. M. Alcaide-Hidalgo, M. Romero, J. Duarte, and E. López-Huertas, "Antihypertensive Effects of Virgin Olive Oil (Unfiltered) Low Molecular Weight Peptides with ACE Inhibitory Activity in Spontaneously Hypertensive Rats," *Nutrients*, vol. 12, no. 1, p. 271, Jan. 2020.

[38] N. Shobako *et al.*, "A Novel Antihypertensive Peptide Identified in Thermolysin-

Digested Rice Bran," *Mol. Nutr. Food Res.*, vol. 62, no. 4, p. 1700732, Feb. 2018.

[39] Y. Xia, J. Yu, W. Xu, and Q. Shuang, "Purification and characterization of angiotensin-I-converting enzyme inhibitory peptides isolated from whey proteins of milk fermented with Lactobacillus plantarum QS670," *J. Dairy Sci.*, vol. 103, no. 6, pp. 4919–4928, Jun. 2020.

[40] S. Arranz, G. Chiva-Blanch, P. Valderas-Martínez, A. Medina-Remón, R. M. Lamuela-Raventós, and R. Estruch, "Wine, beer, alcohol and polyphenols on cardiovascular disease and cancer," *Nutrients*, vol. 4, no. 7. MDPI AG, pp. 759–781, 2012.

[41] G. Chiva-Blanch *et al.*, "Dealcoholized red wine decreases systolic and diastolic blood pressure and increases plasma nitric oxide: Short communication," *Circ. Res.*, vol. 111, no. 8, pp. 1065–1068, Sep. 2012.

[42] X. Lin, T. Han, Y. Fan, S. Wu, F. Wang, and C. Wang, "Quercetin improves vascular endothelial function through promotion of autophagy in hypertensive rats," *Life Sci.*, vol. 258, p. 118106, Oct. 2020.

[43] M. Tanito *et al.*, "Enhanced Oxidative Stress and Impaired Thioredoxin Expression in Spontaneously Hypertensive Rats," *Antioxidants Redox Signal.*, vol. 6, no. 1, pp. 89–97, Jul. 2004.

[44] R. Del Pino-García, M. D. Rivero-Pérez, M. L. González-Sanjosé, K. D. Croft, and P. Muñiz, "Antihypertensive and antioxidant effects of supplementation with red wine pomace in spontaneously hypertensive rats," *Food Funct.*, vol. 8, no. 7, pp. 2444–2454, 2017.

[45] R. López-Fernández-Sobrino *et al.*, "ACE Inhibitory and Antihypertensive Activities of Wine Lees and Relationship among Bioactivity and Phenolic Profile," *Nutrients*, vol. 13, no. 2, p. 679, Feb. 2021.

[46] V. Brüll *et al.*, "Effects of a quercetin-rich onion skin extract on 24 h ambulatory blood pressure and endothelial function in overweight-to-obese patients with (pre-)hypertension: A randomised double-blinded placebo-controlled cross-over trial," *Br. J. Nutr.*, vol. 114, no. 8, pp. 1263–1277, Aug. 2015.

[47] H. Uzun *et al.*, "Oxidative stress in white coat hypertension; role of paraoxonase," *J. Hum. Hypertens.*, vol. 18, no. 7, pp. 523–528, Jul. 2004.

[48] A. J. Donato, K. A. Magerko, B. R. Lawson, J. R. Durrant, L. A. Lesniewski, and D. R. Seals, "SIRT-1 and vascular endothelial dysfunction with ageing in mice and humans," *J. Physiol.*, vol. 589, no. 18, pp. 4545–4554, Sep. 2011.

[49] R. Ray *et al.*, "Endothelial Nox4 NADPH oxidase enhances vasodilatation and reduces blood pressure in vivo," *Arterioscler. Thromb. Vasc. Biol.*, vol. 31, no. 6, pp. 1368–1376, Jun. 2011.

[50] A. Konior, A. Schramm, M. Czesnikiewicz-Guzik, and T. J. Guzik, "NADPH

oxidases in vascular pathology," *Antioxidants and Redox Signaling*, vol. 20, no. 17. Mary Ann Liebert Inc., pp. 2794–2814, 10-Jun-2014.

[51] M. Miguel *et al.*, " Changes in arterial blood pressure in hypertensive rats caused by long-term intake of milk fermented by Enterococcus faecalis CECT 5728 ," *Br. J. Nutr.*, vol. 94, no. 1, pp. 36–43, Jul. 2005.

[52] A. Cabassi, S. Vinci, M. Calzolari, G. Bruschi, and A. Borghetti, "Regional sympathetic activity in pre-hypertensive phase of spontaneously hypertensive rats," *Life Sci.*, vol. 62, no. 12, pp. 1111–1118, Feb. 1998.

[53] M. Quiñones *et al.*, "Long-term intake of CocoanOX attenuates the development of hypertension in spontaneously hypertensive rats," *Food Chem.*, vol. 122, no. 4, pp. 1013–1019, Oct. 2010.

[54] M. W. Millar-Craig, C. N. Bishop, and E. B. Raftery, "CIRCADIAN VARIATION OF BLOOD-PRESSURE," *Lancet*, vol. 311, no. 8068, pp. 795–797, Apr. 1978.

[55] M. Van Den Buuse, "Circadian rhythms of blood pressure, heart rate, and locomotor activity in spontaneously hypertensive rats as measured with radio-telemetry," *Physiol. Behav.*, vol. 55, no. 4, pp. 783–787, Apr. 1994.

[56] K. Kario, "Perfect 24-h management of hypertension: Clinical relevance and perspectives," *Journal of Human Hypertension*, vol. 31, no. 4. Nature Publishing Group, pp. 231–243, 01-Apr-2017.

[57] S. Reule and P. E. Drawz, "Heart rate and blood pressure: Any possible implications for management of hypertension," *Curr. Hypertens. Rep.*, vol. 14, no. 6, pp. 478–484, Dec. 2012.

[58] C. D. Johansen *et al.*, "Resting, night-time, and 24 h heart rate as markers of cardiovascular risk in middle-aged and elderly men and women with no apparent heart disease," *Eur. Heart J.*, vol. 34, no. 23, pp. 1732–1739, Jun. 2013.

[59] K. Eguchi *et al.*, "Nocturnal nondipping of heart rate predicts cardiovascular events in hypertensive patients," *J. Hypertens.*, vol. 27, no. 11, pp. 2265–2270, 2009.

[60] N. Sumien, M. N. Sims, H. J. Taylor, and M. J. Forster, "Profiling psychomotor and cognitive aging in four-way cross mice," *Age (Omaha).*, vol. 28, no. 3, pp. 265–282, Sep. 2006.

CONCLUSIONS

CONCLUSIONS

1. WL obtained from the elaboration of wine with red grapes presented a potent *in vitro* ACEi activity, but only the WL from grapes of the Cabernet variety exhibited BP-lowering effects. The beneficial antihypertensive effects were reproducible in these WL from different harvests.

2. The BP-lowering effect of the WL was related to the high amount of anthocyanins and flavanols present in these lees.

3. WL did not reduced BP in WKY, the normotensive control of the SHR. Therefore, WL effect is linked to hypertension condition.

4. The most effective dose of WL was 5 mL/kg bw, showing the maximum antihypertensive effect 6 h post-administration to SHR. The BP decrease pattern observed in rats administered WL was similar than the observed in the group administered the antihypertensive drug Captopril.

5. Enzyme-assisted extraction using Flavourzyme was a useful methodology to obtain WL rich in phenolic compounds.

6. The WL obtained via enzyme-assisted showed more antioxidant, ACEi and antihypertensive activities than the control WL. The BP-lowering effect of an acute dose of 5 mL/kg bw the hydrolyzed WL to SHR was higher than the one observed in WL-treated rats.

7. The main phenolic compounds released via hydrolysis were anthocyanins and flavanols, being the resulting WL rich in flavanols, anthocyanins, phenolic acids and flavonols.

8. In addition to phenolic compounds, antihypertensive peptides were released via enzyme-assisted extraction, indicating that WL can be a good source of bioactive peptides. FKTTDQQTRTTVA, NPKLVTIV,

TVTNPARIA and LDSPSEGRAPG, LDSPSEGRAPGAD were the antihypertensive peptides identified in the hydrolyzed WL.

9. The drying and consequent dealcoholization of WL leads to an enhancement of their antihypertensive effects. The administration of 125 mg/kg bw caused a maximum antihypertensive effect 6 h post-administration to SHR. The BP decrease pattern observed in SHR administered WL powder was higher than the observed in the group administered the antihypertensive drug Captopril.

10. The dried WL did not reduced BP in WKY, the normotensive control of SHR. Therefore, the BP-lowering effect of the dried WL is linked to hypertension condition.

11. The mechanisms operating in the BP-lowering effect of WLPW at 125 mg/kg bw and 6 h post-administration in SHR is mediated by an antioxidant effect and improving endothelial function. The involvement of SIRT1, NO and PGI_2 pathways, the enhancing of the expression of *Sirt1* and *eNOS* and decreasing of the expression of *Nox4* are demonstrated.

12. Long-term administration of WLPW (125 mg/kg bw) attenuate the increase of BP and reduce heart rate in hypertensive rats. WLPW also increased the locomotor activity in hypertensive rats.

13. Chronic administration of WLPW modulate the HR circadian rhythm maintaining a dipper pattern.

Cabernet Wine lees are a good source of antihypertensive compounds. Wine lees from grapes of the Cabernet variety exhibit an antihypertensive effect related to their high content of flavanols and antohcyanins. These results highlight the potential of these WL for controlling arterial BP, opening the door to commercial use of these lees within the wine industry. In addition, the use of

enzyme-assisted extraction allows to obtain WL rich in phenolic compounds. This technology opens the door to the winery industry to maximize the extraction of phenolic compounds from WL and obtaining hydrolyzed WL with enhanced antioxidant and antihypertensive properties. In addition to the high content of flavanols, anthocyanins, phenolic acids and flavonols, antihypertensive peptides were identified in the hydrolyzed WL. The identified peptides FKTTDQQTRTTVA, NPKLVTIV, TVTNPARIA, LDSPSEGRAPG and LDSPSEGRAPGAD could contribute to these WL functionalities. Finally, the drying and consequent dealcoholization of the WL leaded to an enhancement of their antihypertensive properties, opening the doors to commercialize the dried WL as functional ingredient, nutraceutical and food supplements with potent BP-lowering effects.

Thus, the WL, the hydrolyzed WL, the identified peptides and the dried WL could be good candidates to be used as nutraceuticals or to be included in functional food, nutraceutical and food supplement for the mitigation and the prevention of HTN.

ANNEX

Manuscript 7:

Objective:

To discuss the potential of grapes and winery by-products as sources of antihypertensive compounds, including less-studied by-products such as wine lees and grapes stems.

Grapes, Wine and Winery by-products: source of phenolic compounds with antihypertensive properties

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Keywords: Antioxidant activity, blood pressure, hypertension, ACEinhibitory activity, wine by-product, grape by-product

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ABSTRACT

Background. Hypertension is one of the leading causes of death in the world. Available antihypertensive pharmacological treatments may have several side effects and are not suitable for pre-hypertensive subjects. Therefore, there is an increasing interest in the use of natural products to reduce blood pressure (BP) and the risk of hypertension. In this regard, agri-food by-products, have emerged as a novel source of natural antihypertensive agents allowing for their valorization and making food and agricultural industries more environmentally friendly.

Scope and approach. In this review, the potential of grape and winery industry by-products as a source of antihypertensive phenolic compounds is discussed. Moreover, the impact of winery making process and the use of extraction techniques to increase the phenolic compounds content in these by-products are also considered. *Key Findings and Conclusions*. Grapes are one of the world's largest fruit crops and over 57 % is destined to produce wine. Thus, winery industry generates large amounts of by-products that have been shown to be a source of bioactive compounds, especially phenolic compounds, with several beneficial effects including antihypertensive properties. Among these, grape seed, skin and wine lees have shown capability to reduce BP in both human and animal studies. Therefore, these by-products may lead to the development of new antihypertensive therapeutics alternatives with reduced side effects, allowing for their reuse and contributing to a more sustainable system. Moreover, extraction procedures such as enzymatic hydrolysis can be applied to obtain products with enriched phenolic compounds content and enhanced functionality.

Keywords: Antioxidant activity, blood pressure, hypertension, ACE-inhibitory activity, wine by-product, grape by-product

1. Introduction

Hypertension (HTN), defined as systolic blood pressure (SBP) ≥ 140 mmHg and/or diastolic blood pressure (DBP) ≥ 90 mmHg, is a common risk factor for cardiovascular disease (CVD) and a major global public health challenge (Unger et al., 2020). Thus, HTN is the leading cause of death globally and increases the risk of developing brain, hear, kidney or other diseases (Mills et al., 2019; Roth et al., 2018). In the last years, there have been an increase in HTN prevalence attributed to unhealthy diets (i.e. high sodium and low potassium intake) and lack of physical activity (Manson et al., 2004; Mills et al., 2019). According to the World Health Organization (WHO), 1.13 billion of people worldwide suffer HTN and less than 20 % are under control (WHO, 2019). HTN is also associated with other diseases. Thus, more than 50% of hypertensive people have other common cardiovascular risk factors such as diabetes, lipid disorders, overweight-obesity or metabolic syndrome (Unger et al., 2020). Antihypertensive therapy has been shown to reduce the risk of CVD (Lonn et al., 2016). Hence, It has been suggested that the reduction of both diastolic blood pressure (DBP) and/or systolic blood pressure (SBP) by only 5 mmHg and10 mmHg respectively, reduce the risk of suffering cardiovascular events (Ettehad et al., 2016; Thomopoulos et al., 2014; Zanchetti, 2016). Therefore, treatments and prevention strategies for lowering blood pressure (BP) and slowing the progression of HTN are crucial.

In patients with HTN or a pre-existing cardiovascular risk of 10% or higher, both lifestyle change and medication are recommended (Muntner & Whelton, 2017). Current available treatments include five classes of drugs according to their targets: angiotensin II receptor blockers and angiotensin-converting enzyme (ACE) inhibitors, beta-blockers, diuretics and calcium-channel blockers, (James et al., 2014; Mancia & Zanchetti, 2008). However, they may present several side effects such as hypotension, dizziness, hyperkalaemia or increase creatinine levels among others (Bœuf-Gibot et al., 2020). Moreover, these drugs are not

suitable for treating pre-hypertensive or normotensive individuals at risk of cardiovascular diseases as they may have different metabolic side effects. Thus, implementation of lifestyle modifications is the most recurrent method in these patients (Collier & Landram, 2012; Samadian et al., 2016), (Margalef et al., 2017). However, it is important to act at this pre-hypertensive stage with the aim to avoid the development of HTN (Margalef et al., 2017). Therefore, there is a need to develop new therapeutic antihypertensive treatments with reduced side effects and suitable for pre-hypertensive patients. In this regard, natural food products are of high interest for both scientific and food industry communities as they are a great source of bioactive compounds with several beneficial effects including antihypertensive (Jakubczyk et al., 2020; Maqsood et al., 2020). Indeed, new natural treatments aiming to reduce BP have emerged in the last years, specially ACE inhibitors (ACEi) (Sturrock et al., 2019). ACEi can exert their inhibition on the RAAS system preventing the change from Ang I to Ang II, a potent vasoconstrictor implicated in the development of HTN (Weir, 1999). Reduction of Ang II production mediated by ACE inhibition lowers BP thereby controlling HTN status (Margalef et al., 2017). The use of angiotensin-receptor inhibitors and some calcium channels inhibitors are the only drugs capable of treating prehypertension in a safer way, avoiding possible side effects (Messerli et al., 2007). Therefore, new natural therapeutic antihypertensive treatments are focused in the inhibition of ACE to manage HTN (Margalef et al., 2017).

In addition to foods, an interesting source of functional compounds are agri-food industry by-products (Teixeira et al., 2014; Trigo et al., 2020). Indeed, according to the Food and Agriculture Organization (FAO) of the United Nations, about 1.3 billion tonnes of foods are lost or wasted globally (FAO, 2020). Therefore, the possibility to reuse them to obtain bioactive compounds allows for their valorisation. Moreover, the shortage of raw materials around the world is an economic, social and environmental problem, making food products more expensive, exploiting natural resources and increasing levels of poverty,

malnutrition and new diseases (Tomiyama et al., 2020). The current linear economic model is based on the constant need for short shelf-life products, forcing greater production to alleviate the high demand from the consumer and generating an environmental and economic crisis due to the limitation of natural resources (Garza-Reyes et al., 2019; Subramanian, 2018). Thus, a new (circular) economy model has emerged in the last decade in order to respond to high demands by generating fewer waste products and reusing them, contributing to a more sustainable and environmentally friendly economy (Del Borghi et al., 2020). Therefore, agri-food by-products have emerged as a novel source to obtain natural bioactive compounds with a wide range of beneficial activities, including antihypertensive properties, that can be used as functional ingredients or nutraceuticals, (Ben-Othman et al., 2020; Di Lorenzo et al., 2015; Margalef et al., 2017; Pons et al., 2016a; Rasines-Perea et al., 2018; Shrikhande, 2000; Teixeira et al., 2014) (Banerjee et al., 2017) (Iriondo-Dehond et al., 2018).

Fruits and vegetables and their derived by-products are of particular interest as they are rich in bioactive compounds, mainly phenolic compounds and peptides (Margalef et al., 2017). These bioactive compounds present similar or even more potent antihypertensive effects than pharmacological therapies, inspiring many researchers to explore new therapies for HTN (Zhao et al., 2017). Among these plant-based products, grapes are one of the most important as they are the world's largest fruit crops. The 57 % of its production is destined to make wine generating large amounts of by-products (Musee et al., 2007; OIV & International Organisation of Vine and Wine, 2019). Several studies have shown different beneficial effects of grapes and wine on BP, attributed to their bioactive compound content, mainly phenolic compounds (Dohadwala & Vita, 2009; Wightman & Heuberger, 2015; Ye et al., 2019).

Therefore, the objective of this review was to discuss the potential of grapes and winery by-products as sources of antihypertensive compounds, including less-studied by-products such as wine lees and grapes stems.

2. Grapes, wine, and derived by-products as a source of bioactive phenolic compounds

2.1. Grapes

Grapes are one of the most common and important fruits worldwide, and they are often consumed raw or after being converted to juice, wine, or jam (Dohadwala & Vita, 2009; Sovak, 2001). Grape production in 2018 was 77.8 mt worldwide but only a 36 % of this production is destined to be consumed as a fruit. The rest is destined to produce wine or make other grape products using dried grape (57% and 7%, respectively) (OIV & International Organisation of Vine and Wine, 2019). Grape berries beneficial effects have been mainly associated to their content in phenolic compounds, as they are their main constituents (Rasines-Perea & Teissedre, 2017). This fact, together with their high production and consumption in the world, has made them and its derivatives one of the most studied products to evaluate their beneficial effects.

The phenolic composition of grapes may vary depending on different factors such as soil, climate, degree of maturity or grape variety (Garrido & Borges, 2013; Josep et al., 2010). Focusing on grape variety, the red ones have a higher total amount of phenolic compounds than the white varieties. This is due to the pigments contained in the red grape responsible for the different shade of the grape. These pigments are mainly anthocyanins that are absent in white grapes. **Table 1** shows an example of the phenolic composition found in the most studied grape varieties. Phenolic compounds in grapes are mostly distributed in grape seeds at 60%–70%, followed by skins at 28%–35% and pulp with less than 10% (Trigo et al., 2020). Anthocyanins are mainly found in red grapes since they are responsible for their red colour. Indeed, they also contribute with other pigmentations (blue or purple) of many flowers, fruits and vegetables (Khoo et al., 2017). There are five main types of anthocyanins found in red grapes: malvidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside,

petunidin-3-*O*-glucoside, and peonidin-3-*O*-glucoside. Malvidin-3-*O*-glucoside was the most predominant (Iglesias-Carres et al., 2018, 2019; Zhao et al., 2017).

Regarding flavanols, (+)-catechin and its isomer (-)-epicatechin are the most abundant in grapes. Frequently, flavanols are conjugated with gallic acid giving rise to the gallate flavanols which include epicatechin gallate, catechin gallate, epigallocatechin gallate and gallocatechin gallate (Braicu et al., 2011). Condensed tannins, also known as proanthocyanidins, are oligomeric and polymeric compounds which consist in couple flavanol units. Within proanthocyanidins, procyanidins are the most abundant, which contain exclusively (epi)catechin units (D. Del Rio et al., 2013).

Flavonols are less distributed in grapes than anthocyanins and are typically found as glycosides. The main flavonols found in the grapes are quercetin, kaempferol and isorhamnetin glucosides (Larson et al., 2012; Y. Li et al., 2016; Marunaka et al., 2017). The disaccharide quercetin-3-O-rutinoside is a common dietary component (D. Del Rio et al., 2013). Moreover, grapes also contain non-flavonoids, composed by two groups: phenolic acids and stilbenes. Stilbenes are compounds presenting two aromatic rings connected by ethylene bridge. Resveratrol and its structural analogue, pterostilbene, are the main stilbenes found in grapes (Peng et al., 2018). Gallic acid (GA) is the main phenolic acid found in grapes (Dludla et al., 2019; Jin et al., 2017; Mirvish et al., 1975).

2.2. Wine

The main grape-derived product is wine, being around 57 % of grape production used to this purpose (OIV & International Organisation of Vine and Wine, 2019). According to 2019 statistical report from the International Organization of Vine and Wine, Spain is the third wine-producing country in the world with 44.4 million hectolitres, behind Italy and France (54.8 and 48.6 million hectolitres, respectively). However, Spain is the country with the highest number of hectares of vineyard (Jones & Webb, 2010; OIV & International Organisation of Vine and Wine, 2019).

Wine is composed mainly of water, carbohydrates, organic acids, minerals, alcohol, polyphenols and aromatics (Snopek et al., 2018). Wine may be classified in red, white, and rosé wines being the red and white wines the most consumed. Wine contains a large quantity of phenolic compounds responsible of its beneficial effects. Among the two main types of wine, phenolic compounds content ranges from around 189-554 mg/L in white wine and from 1,538-3,406 mg/L in red wine. This difference is mainly due to the high anthocyanins content of red wine (>700 mg/L), which are absent in white wine (Visioli et al., 2020). Thus, red wine is the most studied by its higher content in phenolic compounds.

Flavanols and anthocyanins are the most abundant phenolic compounds in wine, followed by phenolic acids, flavonols and stilbenes. Among these, the main compounds are catechin, epicatechin and proanthocyanidins in flavanols group; cyanidin, delphinidin, malvidin, peodinin, petunidin and their derivatives (commonly linked to sugar molecules, aliphatic acids or acyl groups) in anthocyanin group; caffeic acid, ferulic acid, *p*-coumaric acid, *o*-coumaric acid, 2,3-dihydroxybenzoic acid, 2-hydroxybenzoic acid, 4-hydroxybenzoic acid, gallic acid and vanillic acid in the phenolic acids group; kaempferol, myricetin, quercetin and their respective glycosides in the flavonols group; and resveratrol and piceatannol in stilbene group (Visioli et al., 2020).

Resveratrol is one of the phenolic compounds found in great quantity in wine and of great importance in research. Thus, resveratrol is the most studied phenolic compound in wine, and have been related with several healthy properties including: amelioration of renal injury, decrease of oxidative stress, improvement of cardiac function, reduction of lipogenesis and anti-inflammatory and antihypertensive properties (Izdebska et al., 2018; E. N. Kim et al., 2018; Matsumura et al., 2018; Rafe et al., 2019; S. Rio et al., 2018).

2.3. Wine derived by-products

Winery industries produce millions of tons of waste. Winemaking process is based on an ancestral procedure so that the winemaking process has been closely linked to artisan techniques or practices, limiting progress technology to those applications aimed at minimizing waste. Thus, the valorisation of these by-products is of great interest. The main residues of this activity are, in order of importance, organic residues (bagasse, seeds, pulp, skins, scrapes and leaves), sewage, emission of greenhouse gases (CO_2, volatile organic compounds, etc.) and inorganic residues (diatomaceous earth, perlite, clays, bentonite) (Teixeira et al., 2014).

The characteristics and composition of these by-products are very related to the winemaking techniques used. Thus, the bioactive compounds such as phenolic compounds may vary in the same by-product depending on the winemaking process. The general procedure to make wine starts with the harvest of grapes and, depending on the desired wine composition, stems can be removed (**Figure 1**). Grapes are crushed and, in the case of red wine, fermentation and maceration are carried out with the juice and pomace providing pigments, mainly anthocyanins, which are responsible of its red colour. Afterwards, the generated grape juice is pressed to remove grape bagasse, also called grape pomace, grape skin and grape seed. Then, the fermentation can either continue in the tanks or finish. After the fermentation process, wine is separated from solid residues called lees (lees of first decanting step) by transferring it to another container, a process called racking. The obtained wine may continue with the malolactic fermentation in the tanks if it is desired. Lees can also be obtained from this second fermentation after sedimentation decanting. Finally, wine suffers a process of maturation in barrels and natural clarification and stabilization before bottling. In white winemaking process, the process is the same but with the exception of the first fermentation, which is not carried out (Beres et al., 2017).

Winery-derived by-products include grape pomace, grape seed, grape skin, grape stem and wine lees (WL) (Di Lorenzo et al., 2015; Giacobbo et al., 2019; Musee et al., 2007; Shrikhande, 2000).

2.3.1. Grape pomace. Bagasse or commonly called grape pomace (GP) is an abundant by-product from the wine industry, resulting from the pressing and/or fermentation processes. GP consists of the remaining skin, seeds and stalks and represents around 25% of total grape weight used in the winemaking process (Beres et al., 2017). It has a high content in dietary fibre with values between 172.8-887.0 mg/g of dry weight following by carbohydrates (122.0-405.3 mg/g) and protein (35.7-141.7 mg/g) (Antonić et al., 2020). In red wine the first fermentation before generation of pomace is the only step that does not occurs in white wine, however, there are not large changes in their bioactive compounds content. Therefore, significant amounts of bioactive compounds such as phenolic compounds are retained in red and white grape pomace (Beres et al., 2017). GP phenolic composition may vary depending on the variety and maturity of grape and climatological conditions (Antonić et al., 2020). It has a wide range of phenolic compounds as it is made up of polyphenol-rich grape skin and seed. Thus, we found (+)-catechin and (-)-epicatechin like the most abundant flavonoids, followed by gallic acid, quercetin and quercetin-3-glucoside. The stilbene compounds are low with trans-resveratrol and piceatannol. Anthocyanins, the main group in red grapes, are mainly concentrated in the skin and flavanols in the seed. Malvidin 3-*O*-glucoside is the anthocyanin predominant followed by peonidin, delphinidin-3-glucoside or petunidin (García-Lomillo & González-SanJosé, 2017).

2.3.2. Grape seed. Grape seeds are another by-product from the grape/wine industry that has been widely studied. They represent 13% of the grape's weight and from 38% to 52% of GP in dry weight. They are composed of around 40% dietary fibre, 16% oil, 11% proteins and 7% phenolic compounds and other compounds (Pasini et al., 2019). Regarding phenolic composition, flavanols are

the major group found in grape seeds, being proanthocyanidins the most abundant. These dimeric compounds are composed by flavanols units of (+)-catechin and (−)-epicatechin linked together through interflavanoid bonds and by gallate esters (Rockenbach et al., 2012). In addition, quercetin and their derivatives are other flavonols found in high quantities in grapes seeds (Kadri et al., 2019). Moreover, these seeds contain phenolic acids, standing out the gallic acid found as gallic acid and gallic acid ethyl ester (M. M. Pantelić et al., 2016; Yilmaz & Toledo, 2004). Therefore, due to its high phenolic content, grape seeds have been extensively used to generate different proanthocyanidin-rich extracts (Gupta et al., 2020; Katiyar, 2015; Liang et al., 2016). As an example, Quiñones et al. 2013 studied a grape seed proanthocyanidin extract (GSPE) comprised of 52% of phenolic compounds. The analysis of the individual phenolic compounds in GSPE by reverse-phase LC-MS revealed that the most abundant phenols included GA, monomeric flavanols (-)-epicatechin and (+)-catechin, and dimer flavanols, both in their free and linked to gallate forms (M. Quiñones et al., 2013).

2.3.3. Grape skin. Grape skins can be obtained from pomaces generated in the vinification. Their phenolic content represents between 28%–35% of the total phenolic content in grapes (Trigo et al., 2020). However, the quantity of phenolic compounds depends on different factors such as the winemaking process used. Grape skins obtained in white winemaking preserve nearly all their phenolic compounds while those obtained from red winemaking are less interesting. They loss an important part of their content in these compounds (mainly anthocyanins) in the maceration process during alcoholic fermentation (Luo et al., 2016). Anthocyanins are the main group of phenolic compounds found in white grape skin, with malvidin 3-*O*-glucoside as the predominant compound mostly followed by peonidin 3-*O*-glucoside. This is due to the lack of alcoholic fermentation and maceration steps in white winemaking (Kammerer et al., 2004). Flavanols are the second group found in the major quantities in white grape skin and the first in red grape skin, being (+)-catechin the main

monomer present in grape skin, followed by (-)-epicatechin and procyanidins (B1 and B3) (Rodríguez Montealegre et al., 2006). High levels of flavonols, specially quercetin and kaempferol derivatives glycosides (myricetin-3-O-glucoside, quercetin-3-O-galactoside, quercetin-3-O-glucoside and kaempferol-3-O-glucoside) (Pea-Neira et al., 2007). Regarding phenolic acid group, gallic acid, protocatechuic acid, caftaric acid, coutaric acid, p-hydroxybenzoic acid and caffeic acid are identified although their content is small in comparison to the other groups (Kammerer et al., 2004; Rodríguez Montealegre et al., 2006). Trans-resveratrol and trans-polydatin are the main stilbenes found in grape skin. Many studies use the skin together with the seed of the grapes, since both by-products are extracted at the same time in the winemaking (fermentation or pressing) (Mahmoudi et al., 2018; Sochorova et al., 2020; Yilmaz & Toledo, 2004).

2.3.4. Grape stem. The grape stem represents 5% of the grapes processed by dry weight, being approximately 25% of the total by-products generated by the wine industry. As mentioned earlier, like other the wine industry by-products, the grape stem is rich in phenolic compounds with amounts between 187-378 mg/gallic acid by dry matter (Goutzourelas, Stagos, Spanidis, et al., 2015; Nieto et al., 2020). Forty-two phenolic compounds have been identified in grape stem extract, including phenolic acids, stilbenes, flavonols and flavanols, being flavanols the predominant in grape stem. Gallic and caftarc acids are the principal phenolic acids in grape stem extract. Trans-resveratrol, ε-viniferin, trans-resveratrol-glucoside (piceid), along with different dimmers and trimers of trans- and cis-resveratrol, are the main stilbenes identified. Catechin followed by epicatechin, was the main monomeric compound and procyanidin dimer B1 the highest dimer compound in the flavanols group. The most remarkable flavonols are quercetin-3-*O*-glucuronide, followed by quercetin-3-*O*-glucoside, and malvidin-3-*O*-glucoside is the most abundant anthocyanin (Nieto et al., 2020).

2.3.5. Wine lees. According to the Council Regulation (EEC) No. 337/79, wine lees (WL) are "the residue that forms at the bottom of recipients containing wine, after fermentation, during storage or after authorized treatments, as well as the residue obtained following the filtration or centrifugation of this product" (Pérez-Serradilla & de Castro, 2008). Wine lees (WL) represent between 14-25% of waste wineries (Giacobbo et al., 2019). They are mainly composed of yeasts, tartaric acid, inorganic matter and phenolic compounds (Pérez-Serradilla & Luque de Castro, 2011). WL has two phases, a solid and a liquid. The solid phase is rich in protein and essential free amino acids. However, the high amounts of phenolic compounds associated with these proteins make this solid fraction not assimilable (Beres et al., 2017; Bustamante et al., 2008; De Iseppi et al., 2020). Phenolic profile of WL has been characterized in phenolic extracts obtained from the liquid and solid fractions (**Table 2**). The most common technique for extraction of phenolic compounds from WL is solid-liquid extraction. Most of the studies have been carried out on the solid fraction of WL after a centrifugation process and have shown a high content of phenolic compounds (Jara-Palacios, 2019). However, phenolic compounds are not totally extracted by centrifugation, remaining in the solid fraction bound to yeast cell walls. The presence of phenolic compounds in WL is due to the great adsorption capacity of the yeast cell wall used in the winemaking process. Thus, the use of extraction methods are required to extract all the phenolic potential contained in this by-product (Morata et al., 2005). The phenolic compound profile of WL varies depending on the type of grape used and the winemaking process (Pérez-Serradilla & Luque de Castro, 2011). Total phenolic content shows a high level of phenolic compounds with higher quantity of non-flavonoids vs. flavonoids. Within flavonoids, anthocyanins are the most abundant in WL (Giacobbo et al., 2019; Landeka Jurčević et al., 2017). Rutin and quercetin followed by ellagic, gallic, caffeic and p-coumaric acids have been described as the most abundant phenolic compounds in WL (Caro et al., 2017a; Landeka Jurčević et al., 2017). Moreover, we have recently shown that the phenolic compounds content and

profile may vary dependeing on the grape variety. Thus, WL from Cabernet, Mazuela, and Garnacha varieties showed different total phenolic compounds content, being almost doubled in WL from cabernet (CWL) compared to WL from Mazuela and Garnacha (690.6, 395.3, and 379.6 mg/L, respectively). In addition, CWL showed higher content of flavanols and anthocyanins than WL from Mazuela and Garnacha varieties (López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021).

3. Phenolic compounds and their antihypertensive potential

As mentioned above, phenolic compounds are the main bioactive constituents in grape, wine and their derived by-products. These compounds involve a large group of bioactive phytochemicals that present at least one aromatic ring with one or more hydroxyl group in their main structure (D. Del Rio et al., 2013). These can be classified into flavonoids (flavanols, flavonols and anthocyanins), stilbenes, phenolic acids and lignans (Fraga et al., 2019). Different studies have shown several health properties of phenolic compounds, including antihypertensive effects (Dabeek & Marra, 2019; Durazzo et al., 2019; Guerrero et al., 2012; Maaliki et al., 2019; Schini-Kerth et al., 2010). Moreover, consumption of phenol-rich foods has been associated with a range of health benefits, including the reduction of cardiometabolic risk factors in humans (Fraga et al., 2019). Figure 2 shows the different mechanisms through which phenolic compounds manage HTN in the endothelium. Within flavonoids, flavanols of different sources such as cocoa have been proved to improve CVD by reducing BP (Martin & Ramos, 2021; X. Wang et al., 2014). Furthermore, consumption of foods and extracts rich in flavanols and proanthocyanidins has been shown to improve the CVD through the reduction of BP in hypertensive and pre-hypertensive subjects (Heiss et al., 2010; Ren et al., 2021). In addition, flavonols have shown ACE inhibitory potential. This enzyme acts at the renin-angiotensin-aldosterone system (RAAS) level, one of the main mechanisms of BP regulation (Hall et al., 1990). It cleaves the decapeptide angiotensin I (Ang I)

releasing the octapeptide angiotensin II (Ang II), which can act as vasoconstrictor through its binding to Ang-II type 1 receptor (AT1R). Moreover, Ang II induces the production of aldosterone in the zona glomerulosa of adrenal glands, leading to the retention of Na^+ and water and increasing BP (Cachofeiro et al., 2008; Reaux et al., 1999). Therefore, its inhibition is one of the main targets when looking for compounds with BP-lowering effects. In an *in vitro* study, seventeen different flavonoids showed ACE inhibitory potencies ranging from 17-95% at a concentration of 500 μM. Their activity was related with sub-structures on the flavonoid skeleton, mainly the catechol group in the B-ring, the double bond between C2 and C3 at the C-ring and the ketone group in C4 at the C-ring (Guerrero et al., 2012). Furthermore, administration of individual flavanols, such as catechin and epicatechin, to hypertensive animals also led to BP reduction (Mar Quiñones et al., 2015). These beneficial effects has been linked to increased production in endothelial cells of nitric oxide (NO), the main endothelium-derived vasodilator factor (M. Quiñones et al., 2014; Schini-Kerth et al., 2010). This is also related to the strong antioxidant potential of phenolic compounds. Thus, overproduction of reactive oxygen species (ROS) contributes to the development of CVD and endothelial dysfunction (Cheng et al., 2017). In the blood vessels, high levels of ROS are related with low levels of vasodilators generating endothelial dysfunction. NO is the main vasodilator affected as it interacts with oxygen free radicals to generate peroxynitrite (ONOO-). This leads to a reduced NO availability and increased ROS levels promoting HTN stage (Rodrigo et al., 2015) (Incalza et al., 2018). Furthermore, the increment of ROS levels in cardiovascular control organs elicit targeted immune response, which potentiates systemic HTN and its complications. Thus, oxidative stress induced by pro-inflammatory signals worsens the immunologic response in endothelium, which produces the progressive deterioration of vascular function (Crowley, 2014). Moreover, high levels of oxidative stress in hypertensive states increase Ang II levels, which stimulate the production of ROS through NADPH oxidase (NOX). Isoforms of this enzyme, in particular

NOX1/NOX2, are involved in the development of endothelial dysfunction, HTN and inflammation. Therefore, antioxidant properties of polyphenols can protect from increased ROS levels contributing to vascular homeostasis.

Red grapes are also rich in anthocyanins, which are the responsible of their colour (Khoo et al., 2017). Dietary intake of anthocyanins has been studied for its health-promoting effects, more specifically with respect CVD prevention (Wallace, 2011). Intake of anthocyanin-rich extracts of different sources, such as chokeberry, bilberry or elderberry, led to increased endothelial NO levels via the regulation of endothelial NO synthase (eNOS) expression and activity, promoting vasorelaxation in pig coronary arterial rings (Bell & Gochenaur, 2006). In addition, anthocyanin-rich black soybean has demonstrated antioxidant and antihypertensive properties, preventing oxidative damage, increasing NO bioavailability and reducing NOX activity in lipopolisacharide (LPS)-stimulated RAW 264.7 cells (J. N. Kim et al., 2017). Moreover, three meta-analyses of randomized controlled trials demonstrated the association between consumption of different sources of anthocyanins and the significant reduction of SBP and improved vascular function (Fairlie-Jones et al., 2017; Huang, Chen, Liao, Zhu, & Xue, 2016; Yang et al., 2017). One of these meta-analysis involving 24 clinical studies in healthy and hypertensive subjects showed that acute and chronic anthocyanin consumption improves flow-mediated dilatation and, in the case of acute administration, also the pulse, promoting vascular health (Fairlie-Jones et al., 2017). In addition, a meta-analysis conducted with 22 clinical trials in healthy and hypertensive subjects showed that consumption of berries rich in anthocyanins significantly reduces CVD risk factors including SBP, low density lipoprotein (LDL)-cholesterol and fasting glucose levels (Huang, Chen, Liao, Zhu, & Xue, 2016). Recently, hydroalcoholic extracts of Patagonian Calafate berry (*Berberis microphylla*) containing conjugated anthocyanins, mainly glycosylated anthocyanidins such as delphinidin-3-glucoside, petunidin-3-glucoside and malvidin-3-glucoside, showed strong vasodilatation effects in a rat arterial mesenteric bed bioassay (Calfío & Huidobro-Toro, 2019). Vascular

responses of these glycosylated anthocyanins were endothelium-dependent, mediated by NO production and independent of antioxidant capacity.

Within stilbenes family, resveratrol is the most abundant in grapes, red wine and berries and has been proven to exert numerous beneficial effects, including antihypertensive effects (Bonnefont-Rousselot, 2016). Thus, resveratrol has shown antihypertensive properties in different models of hypertensive rats associated with the improvement of endothelium through vascular relaxation (Akar et al., 2012; Bhatt et al., 2011; Miatello et al., 2005; Mizutani et al., 2000), enhanced eNOS activity (Akar et al., 2012; Bhatt et al., 2011; Miatello et al., 2005) and subsequently increase of NO (Bhatt et al., 2011). This antihypertensive potential of resveratrol is also related to its antioxidant capability. Hence, resveratrol administration to hypertensive rodents led to increased superoxide dismutase (SOD) (Bagul et al., 2012; Bhatt et al., 2011; Franco et al., 2013; Ramar et al., 2012), catalase (Bagul et al., 2012; Franco et al., 2013), glutathione peroxidase (GPx) (Ramar et al., 2012) and reduced glutathione (GSH) (Alturfan et al., 2012; Bagul et al., 2012; Ramar et al., 2012) activity. Furthermore, resveratrol activate sirtuins such as SIRT1 which is implicated in the deacetylation and consequent activation of eNOS (Gertz et al., 2012). However, there is some controversy regarding the effects of resveratrol on BP in humans. Thus, a meta-analysis of randomized controlled trials showed no relationship between resveratrol and pressure drop (Sahebkar et al., 2015). Other meta-analysis in obese or pre-hypertensive subjects showed a reduction in SBP after intake of resveratrol (Asgary et al., 2019; Fogacci et al., 2019; Huang, Chen, Liao, Zhu, Pu, et al., 2016), and, in some cases, this effect was only observed at high doses (Huang, Chen, Liao, Zhu, Pu, et al., 2016).

Regarding the flavonol group, quercetin and kaempferol are the most widely distributed in foods with potential cardiovascular-related benefits (Dabeek & Marra, 2019). Quercetin is found mainly in asparagus, onion and berries and kaempferol in the green leafy vegetables like spinach, but they are also found to a lesser extent in red wine (Dabeek & Marra, 2019). Quercetin stands out for

its capacity of improving endothelial function by the modulation of vasoactive agents, increasing NO levels and reducing the vasoconstrictor factor endothelin-1 (ET-1), in addition to prevent the endothelial cell apoptosis (Marunaka et al., 2017), (Dayoub et al., 2014). Quercetin acts via radical scavenging ability regulating enzymes such as hemeoxygenase-1 (HO-1) and reduces ROS production in macrophages (Ibarra et al., 2003). In hypertensive animal model, quercetin presented the ability to inhibit ACE, reducing BP levels (Ibarra et al., 2003). In subjects with metabolic syndrome, the intake of quercetin decreases SBP (Egert et al., 2009) and their intake in hypertensive subjects reduced the SBP and DBP in hypertensive subjects but not in subjects with pre-hypertension (Marunaka et al., 2017). Endothelial function was also improved in healthy men as shown by a randomized, placebo-controlled, crossover trial where the administration of quercetin increased the levels of NO and reduced ET-1 (Loke et al., 2008). Moreover, a randomised double-blinded controlled cross-over trial in pre-hypertensive subjects showed that intake of a quercetin-rich onion skin extract reduced the arterial BP probably by the regulation of ET-1 production (Brüll et al., 2015). In addition, a meta-analysis of 7 randomized controlled trials showed the reduction of SBP and DBP after supplementation with quercetin (Serban et al., 2016).

4. Antihypertensive effect of grapes, wine and winery by-products

As mentioned above, grapes, wine and winery-derived by-products are rich in phenolic compounds, which have widely shown several beneficial effects including antihypertensive properties. Therefore, they constitute a potential valuable source to obtain new antihypertensive natural therapeutics.

4.1. Grapes. The antihypertensive properties of grapes have been reported by both animals and human studies (**Table 3**). In this regard, the administration of grape powder (600 mg/day) to spontaneously hypertensive rats (SHR) produced an improvement of the endothelial function mediated by an increment of eNOS production, which was accompanied by a BP-lowering effect

(10 mmHg) (Thandapilly et al., 2012). Moreover, administration of 330 mg of grape powder to Sprague-Dawley rats with buthionine sulfoximine (BSO)-induced hypertension reduced BP by 20% via extracellular signal-regulated protein kinase-1/2 (ERK-1/2) (Allam et al., 2013). In addition, an antihypertensive effect has also been evidenced in humans after intake of grape polyphenols. In this sense, a meta-analysis of 10 randomized controlled trials in subjects with HTN or pre-HTN have demonstrated that daily grape polyphenol intake could significantly reduce SBP by 1.48 mmHg when compared to control subjects. Larger reduction was identified when consuming low-dose of grape polyphenols (< 733 mg/day) or in patients with metabolic syndrome. In contrast, diastolic blood pressure was not significantly decreased in the grape polyphenols group as compared to controls (S.-H. Li et al., 2015).

4.2. Wine. Most of wine beneficial effects have been mostly attributed to its content in resveratrol. However, as discussed above, other phenolic compounds found in wine such as anthocyanins and catechins have also shown beneficial properties (Snopek et al., 2018). Thus, different wine extracts rich in phenolic compounds have been shown to exert several beneficial bioactivities including antihypertensive effects (Leikert et al., 2002; Nash et al., 2018; Pechánová et al., 2004; Sarr et al., 2006). Moderate consumption of red wine (Artero et al., 2015; Snopek et al., 2018), dealcoholized red wine (Chiva-Blanch et al., 2012) or red wine extracts (Leikert et al., 2002; Sarr et al., 2006) have also been related to cardioprotective and antioxidant properties, including BP-lowering effects and improvement of endothelial function (Chiva-Blanch et al., 2012; de Figueiredo et al., 2017; Sarr et al., 2006). Production of NO is the main mechanism implicated in the regulation of BP mediated by phenolic compounds of red wine. In this regard, red wine polyphenols prevent HTN by inhibiting *Nox* expression and increasing the release of NO in endothelium (Sarr et al., 2006). Furthermore, red wine reduced plasmatic levels of the vasoconstrictor ET-1 at a single dose of 8.1 dL in hypertensive subjects, being improved in dealcoholized red wine (Kiviniemi et al., 2010). A clinical trial in hypertensive subjects have

shown that the moderate consumption of red wine (272 mL/day for 4 weeks) slightly reduces SBP (2.3 mmHg) and DBP (1 mmHg). Nevertheless, their effect was higher in the same red wine without alcohol with a reduction of 5.8 and 2.3 mmHg in SBP and DBP respectively, and increased the levels of plasmatic NO (Chiva-Blanch et al., 2012). Pechánová et al. also reported that administration of red wine polyphenols at a dose of 40 mg/kg/day during four weeks in hypertensive rats induced by Nw-nitro-L-arginine-methyl-ester (L-NAME), an inhibitor of eNOS, increased *eNos* expression and the release of NO (Pechánová et al., 2004). Similar results were found *in vitro* where red wine polyphenols extract increased *eNos* expression and thus endothelial NO production (Leikert et al., 2002).

4.3. Winery by-products. As mentioned above, winery by-products are rich in phenolic compounds and they have been used to obtain phenolic-enriched extracts. These extracts have been shown to exert a wide range of functionalities, including antihypertensive effects (Rasines-Perea & Teissedre, 2017; Shrikhande, 2000).

Grape pomace. GP is mainly composed by seeds and skins. *In vitro* studies have demonstrated that dry GP extract exerts antioxidant effects in endothelial and muscle cells through the increment of gamma-glutamylcysteine synthetase (GCS) and glutathione S-transferase (GST) enzymes (Goutzourelas, Stagos, Housmekeridou, et al., 2015). GCS is the first enzyme in the biosynthetic pathway of GSH, with a critical role for cell survival (Dalton et al., 2004). GST is induced under conditions of oxidative stress and is involved in the detoxification of organic epoxides, hydroperoxides and unsaturated aldehydes formed after lipid peroxidation. GST detoxifies these products through their conjugation with GSH (Fernández-Iglesias et al., 2014) Moreover, administration of seedless red wine pomace seasoning (RWPS) to SHR at a dose of 300 mg/kg/day during 4 weeks, reduced BP and oxidative damage. Restoration of *eNos*, mitochondrial superoxide dismutase (*Sod2*), *Ho-1* and reduction of *Ace* aortic gene expression

were the leading mechanisms involved in the reduction of BP mediated by RWPS in SHR (Del Pino-García et al., 2017). In addition, the intake of 20 g/day of wine grape pomace flour during 16 weeks improved SBP and DBP with a reduction of 4.3 and 5.3 mmHg respectively in subjects with metabolic syndrome (Urquiaga et al., 2015).

Grape seeds. GS are one of the most studied grape by-products for their antihypertensive effects (Gupta et al., 2020; Rodríguez-Pérez et al., 2019). Different extracts have been elaborated showing different bioactivities, including antihypertensive effects due to their content in phenolic compounds, especially in proanthocyanins (Gupta et al., 2020). In this regard, antihypertensive mechanisms of GSPE have been widely elucidated in many studies *in vitro* and *in vivo* (animal and human). Acute administration of GSPE (375 mg/kg bw) produced a reduction of 50 mmHg over BP in SHR and ameliorated oxidative stress. In addition, it was observed an increase of hepatic GSH levels (M. Quiñones et al., 2013). However, no effects were observed in Wistar Kyoto (WKY) rats after GSPE consumption. Moreover, a single oral dose of GSPE (375 mg/kg bw) to male SHR decreased BP levels by NO and prostacyclin (M. Quiñones et al., 2014). In another study using both cafeteria diet-fed hypertensive rats (CHR) and SHR models, same acute administration of GSPE decreased BP levels through SIRT1, which in turn causes the deacetylation of eNOS increasing the bioavailability of NO and decreased aortic *Nox4* and *Et1* gene expression and plasma ET-1 levels (Pons et al., 2016b). Moreover, same single oral administration of GSPE to CHR, showed a moderate reduction of BP by 17 and 13 mmHg in SBP and DBP respectively, reaching normotensive values of BP. This antihypertensive effect was related to an increase of NO availability and partially to PGI_2 (Pons et al., 2016a). This antihypertensive effect was related to GSPE antioxidant properties since a reduction in MDA liver levels, a marker of lipid peroxidation, was observed (Pons et al., 2014). Regarding GSPE doses, intermediate dose (375 mg/kg bw) has been shown to exert enhanced antihypertensive effect compared to higher dose (500 mg/kg bw) (Pons et al.,

2016a). The same results have been shown in SHR models treated with extracts rich in flavanol-rich compounds such as cocoa or grape seed (Cienfuegos-Jovellanos et al., 2009; M. Quiñones et al., 2013). These findings could be explained by the pro-oxidant properties and the excessive production of ROS caused by high doses of flavanols (Pons et al., 2016a). In addition, chronic administration of GSPE (25 mg/kg bw/day for three weeks) to cafeteria diet-fed rats, reduced SBP and DBP by 15 and 10 mmHg, respectively. This effect was associated with the increment of GSH liver levels observed in these animals which may protect from the oxidative stress associated with HTN (Mas-capdevila et al., 2020). In addition, GSPE (250 mg/kg/day for five weeks) to Sprague-Dawley (SD) rats with HTN induced by administration of Ouabain was shown to regulate NO and ET-1 balance and to suppress the transforming growth factor-β1 (TGF-β1), which is associated with endothelial cells remodelling, decreasing BP (Liu et al., 2012). Administration of GSPE also reduced plasmatic ET-1 levels and in cardiac and renal tissue which restored endothelial dysfunction and oxidative stress in L-NAME-induced HTN pregnant mice (Zhu et al., 2018).

The antihypertensive properties of GSPE have also been demonstrated in hypertensive or pre-hypertensive patients. The results of clinical trials shows that consumption of GSPE (400 mg/kg/day) for 12 weeks could ameliorate vascular stiffness and regulate the BP at a normal stage (Odai et al., 2019). In hypertensive subjects with metabolic syndrome, reduction of oxidative stress by GSPE is one of the main mechanisms involved in BP reduction (Sivaprakasapillai et al., 2009). In addition, different meta-analysis studies have demonstrated the positive correlation among grape seed extract consumption and BP reduction. Thus, a meta-analysis with 16 randomized controlled trials demonstrated the beneficial impact of grape seed extract on BP, which was more evident in younger or obese subjects as well as in people with metabolic syndrome after treatment (Zhang et al., 2016). Other meta-analysis conducted with 9 randomized controlled trials showed that grape seed phenolic extracts

exert their antihypertensive effect over SBP and not over DBP (Feringa et al., 2011). A recent meta-analysis of 6 controlled clinical trials showed that GSPE supplementation reduces SBP and BPD in hypertensive and pre-hypertensive subjects, being interesting in the treatment of HTN or as a preventive method in pre-hypertensive subjects (Feringa et al., 2011).

Grape skin extracts. GSE have also been studied for their antioxidant and antihypertensive activities. Administration of 100 mg/kg bw of GSE during 30 days showed a significant antihypertensive, vasodilator and antioxidant effects in desoxycortisone acetate (DOCA)-salt and L-NAME hypertensive rats probably mediated by restoration of NO levels (R. Soares de Moura et al., 2002). De Costa et at. showed that GSE (200 mg/kg/day for 12 weeks) prevented the development of HTN in SHR due to their antioxidant effect increasing the SOD activity and decreasing MDA levels (De Costa et al., 2020). The increment of NO synthesis and restoration of SOD, catalase, GPx and MDA levels produced by chronic administration of GSE, prevented the development of HTN in rats with metabolic syndrome induced by a high-fat diet (Emiliano et al., 2011; Resende et al., 2013). Additional studies demonstrated that pre-treatment with GSE avoided the HTN stage in rats that were induced HTN by the administration of doxorubicin (Dox). Dox induce cardiomyopathy by the overproduction of ROS (Mokni et al., 2012). Furthermore, studies carry out in hypertensive-induced pregnancy female rats with L-NAME, showed the involvement of NO in the antihypertensive effect of GSE avoiding the HTN stage in rats (Roberto Soares De Moura et al., 2007).

Grape stem. Stems are also a great source of bioactive compounds which may be used to extract phenolic compounds with antioxidant and antihypertensive effects (Nieto et al., 2020). In this sense, antioxidant activity improves the redox system in endothelial cell cultures with an increase of GSH and decrease of MDA levels after treatment with grape stem extract. The antioxidant effect of grape stem extract depend on their qualitative phenolic composition with considerable amounts of trans-resveratrol, gallic acid, catechin, syringic acid or

quercetin (Goutzourelas, Stagos, Housmekeridou, et al., 2015). Grape stem extract presented an ACEi activity of 72.27 % *in vitro* at a concentration of 200 µg/mL and IC_{50} value of 69.5 µg/mL *in vitro* (Lin et al., 2012). Single and chronic dosage of grape stem extract reduced BP in SHR probably due to their phenolic compounds. Specifically, the phenolic compound (+)-visitin A were isolated from grape stem extract by their ACEi activity, showing antihypertensive effects in SHR by increasing NO release from endothelial cells (Lin et al., 2012).

Wine lees. WL are a great source of phenolic compounds and they have been used to obtain extracts with antioxidants effects (Jara-Palacios, 2019; Romero-Díez et al., 2018). It has been evidenced that phenolic compounds identified in WL such as resveratrol, quercetin, gallic acid, (+)-catechin, (−)-epicatechin or malvidin-3-glucoside exhibits antihypertensive effects in hypertensive animal and human models (Fogacci et al., 2019; Jin et al., 2017; Marunaka et al., 2017; Mar Quiñones et al., 2015). Considering these evidence, together with the great antioxidant effect of WL extracts and the relationship between oxidative stress and HTN, WL could be a great alternative to manage HTN. Indeed, we have recently demonstrated WL beneficial effects against HTN. Thus, we evaluated the *in vitro* ACE inhibitory activity of WL from different varieties including Cabernet, Mazuela, and Garnacha grapes, as well as their antihypertensive effects in SHR at a dose of 5 mL/kg bw. Among these, Cabernet WL exhibited a potent antihypertensive activity, similar to that obtained with the drug Captopril. This BP-lowering effect was attributed to the presence of flavanols and anthocyanins (López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021). In other study, an enzyme-assisted extraction was used to obtain WL with increased phenolic content and enhanced bioactivities using Flavourzyme. These enriched-phenolic WL (PWL) contained a 57.20% higher content in phenolic compounds than control WL, being anthocyanins and flavanols the largest families present. In addition, these enriched WL also showed greater antioxidant, ACEi activities and antihypertensive effect. Moreover, this BP-lowering effect was more prolonged than the one produced by Captopril. These

results demonstrated that enzymatic protein hydrolysis is a useful methodology to maximize the extraction of phenolic compounds from WL and to obtain extracts with enhanced functionalities. This BP-lowering effect was related to the high quantities of anthocyanins and flavanols present in these lees (López-Fernández-Sobrino, Margalef, et al., 2021). Three different doses of WL (2.5, 5.0 and 7.5 mL/kg bw) were studied in SHR, where the intermediate dose (5 mL/kg bw) was the most effective to reduce HTN with similar effects than the drug Captopril. In addition, the drying process of WL produced the alcohol elimination showed a significant enhance of the BP-lowering effect. The single dose of 125 mg/kg bw of WL powder (WLPW), equivalent to 5 mL/kg bw, caused a BP-lowering effect more potent than the drug Captopril. In addition, this hypotensive effect was discarded in WKY at the same dose. Furthermore, WLPW have demonstrated to enhance the redox system in HTN stage through the reduction of plasmatic MDA and hepatic ROS, in addition to increase the levels of plasmatic NO and hepatic GSH (López-Fernández-Sobrino, Soliz-Rueda, Ávila-Román, et al., 2021).

5. Extraction of bioactive compounds

As has been mentioned, winery by-products can be considered as a good source of antihypertensive compounds, mainly phenolic compounds. However, many of these phenolic compounds are bound to proteins or to structures of the cell matrix, making their release difficult. Thus, extraction of these bioactive compounds is an important factor when using by-products.

Different extraction procedures allows for the separation of bioactive compounds from inactive compounds present in plants (Pimentel-Moral et al., 2020). Factors such as temperature, pH, pressure, extraction time or solvent are decisive to achieve an effective extraction. In addition to these factors, the structure of the bioactive compounds to be extracted must also be preserved intact, being another factor to take into account when selecting the extraction method (Okolie et al., 2019). Different methods of extraction of phenolic

compounds have been used in order to revalue by-products (Teixeira et al., 2014). They are commonly classified in conventional techniques and green technologies (Pimentel-Moral et al., 2020). Most conventional extraction methods are based on the use of different solvents with the application of heat and/or mixing. Nevertheless, these techniques consume a lot of time, energy, and polluting solvent. Thus, in the last decade, there has been an increasing demand for new extraction techniques with the aim to reduce use of organic solvents, the time of extraction and prevent the pollution between others (Pimentel-Moral et al., 2020). Ultrasound-assisted extraction, microwave-assisted extraction, pressurized-liquid extraction, supercritical fluid extraction or enzyme-assisted extraction are among green technologies (Carciochi et al., 2017). Some of these techniques have been used for the extraction of certain phenolic compounds in winery by-products or for the extraction of phenolic compounds in general. For example, microwave-assisted extraction has been used to extract quercetin, catechin or anthocyanins from grape seeds and skins and supercritical fluid extraction for release total phenolic compounds in grape seeds and pomace (Ahn et al., 2004; Z. Fang & Bhandari, 2010; Louli et al., 2004; Marqués et al., 2013).

Enzyme-assisted extraction is one of the extraction techniques booming by its ability to improve the yield of compound extraction while the use of solvents is reduced in the process (Puri et al., 2012). Enzymatic hydrolysis is commonly used in bioactive compounds extractions for producing softer hydrolysis when the selection of the appropriate hydrolytic enzyme depends of the source of cell walls which mainly consists of lignin, polysaccharides, cellulose, hemicellulose, pectin and proteins (Puri et al., 2012). Winery by-products presented fiber, carbohydrates and proteins, which are susceptible to hydrolysis. Phenolic compounds, one of the main bioactive compounds presents in wine and by-products of winemaking, can be found attached to proteins or other structures of the cell matrix, being of interest the use of extraction methods to release these bioactive compounds (Louli et al., 2004; Teixeira et al., 2014). Enzymatic

hydrolysis allows for the release of phenolic compounds bound to proteins, but proteins can also be hydrolyzed by releasing peptides with different bioactivities, including antihypertensive (Jakubczyk et al., 2020). In this sense, Rodríguez-Morgado et al. have shown enzymatic extraction of GP with protease generate a release of phenolic compounds and peptides which improve its anti-inflammatory properties (Rodríguez-Morgado et al., 2015).

CONCLUSION

In conclusion, this review emphasizes the importance of finding new alternative natural therapeutics against hypertension, which is increasing its prevalence worldwide and which current available pharmacological treatments present several adverse effects. In this regard, grapes, wine and wine industry by-products may be a valuable option as they are rich in phenolic compounds which have shown several beneficial effects including antihypertensive in both animals and human studies. In addition, the use of wine by-products is of particular interest as winery industry generates large amounts of waste that can be reused as they are rich in phenolic compounds, contributing to a more sustainable environment and circular economy. Nevertheless, some of these by-products such as WL, have been little studied regarding their beneficial effects against HTN and further investigations are needed. Moreover, extraction procedures such as enzymatic hydrolysis can be applied to obtain products with enriched phenolic compounds content and enhanced functionality.

Bibliography

Ahn, J., Grün, I. U., & Mustapha, A. (2004). Antimicrobial and Antioxidant Activities of Natural Extracts in Vitro and in Ground Beef. *Journal of Food Protection*, *67*(1), 148–155. https://doi.org/10.4315/0362-028X-67.1.148

Akar, F., Uludağ, O., Aydin, A., Aytekin, Y. A., Elbeg, S., Tuzcu, M., & Sahin, K. (2012). High-fructose corn syrup causes vascular dysfunction associated with metabolic disturbance in rats: Protective effect of resveratrol. *Food and Chemical Toxicology*, *50*(6), 2135–2141. https://doi.org/10.1016/j.fct.2012.03.061

Allam, F., Dao, A. T., Chugh, G., Bohat, R., Jafri, F., Patki, G., Mowrey, C., Asghar, M., Alkadhi, K. A., & Salim, S. (2013). Grape powder supplementation prevents oxidative stress-induced anxiety-like behavior, memory impairment, and high blood pressure in rats. *Journal of Nutrition*, *143*(6), 835–842. https://doi.org/10.3945/jn.113.174649

Alturfan, A. A., Tozan-Beceren, A., Şehirli, A. Ö., Demiralp, E., Şener, G., & Omurtag, G. Z. (2012). Resveratrol ameliorates oxidative DNA damage and protects against acrylamide-induced oxidative stress in rats. *Molecular Biology Reports*, *39*(4), 4589–4596. https://doi.org/10.1007/s11033-011-1249-5

Antonić, B., Jančíková, S., Dordević, D., & Tremlová, B. (2020). Grape Pomace Valorization: A Systematic Review and Meta-Analysis. *Foods*, *9*(11), 1627. https://doi.org/10.3390/foods9111627

Artero, A., Artero, A., Tarín, J. J., & Cano, A. (2015). The impact of moderate wine consumption on health. *Maturitas*, *80*(1), 3–13. https://doi.org/10.1016/j.maturitas.2014.09.007

Asgary, S., Karimi, R., Momtaz, S., Naseri, R., & Farzaei, M. H. (2019). Effect of resveratrol on metabolic syndrome components: A systematic review and meta-analysis. In *Reviews in Endocrine and Metabolic Disorders* (Vol. 20, Issue 2, pp. 173–186). Springer New York LLC. https://doi.org/10.1007/s11154-019-09494-z

Bagul, P. K., Middela, H., Matapally, S., Padiya, R., Bastia, T., Madhusudana, K., Reddy, B. R., Chakravarty, S., & Banerjee, S. K. (2012). Attenuation of insulin resistance, metabolic syndrome and hepatic oxidative stress by resveratrol in fructose-fed rats. *Pharmacological Research*, *66*(3), 260–268. https://doi.org/10.1016/j.phrs.2012.05.003

Banerjee, J., Singh, R., Vijayaraghavan, R., MacFarlane, D., Patti, A. F., & Arora, A. (2017). Bioactives from fruit processing wastes: Green approaches to valuable chemicals. In *Food Chemistry* (Vol. 225, pp. 10–22). Elsevier Ltd. https://doi.org/10.1016/j.foodchem.2016.12.093

Bell, D. R., & Gochenaur, K. (2006). Direct vasoactive and vasoprotective properties of

anthocyanin-rich extracts. *Journal of Applied Physiology*, *100*(4), 1164–1170. https://doi.org/10.1152/japplphysiol.00626.2005

Ben-Othman, S., Jõudu, I., & Bhat, R. (2020). Bioactives from Agri-Food Wastes: Present Insights and Future Challenges. *Molecules*, *25*(3), 510. https://doi.org/10.3390/molecules25030510

Beres, C., Costa, G. N. S., Cabezudo, I., da Silva-James, N. K., Teles, A. S. C., Cruz, A. P. G., Mellinger-Silva, C., Tonon, R. V., Cabral, L. M. C., & Freitas, S. P. (2017). Towards integral utilization of grape pomace from winemaking process: A review. In *Waste Management* (Vol. 68, pp. 581–594). Elsevier Ltd. https://doi.org/10.1016/j.wasman.2017.07.017

Bhatt, S. R., Lokhandwala, M. F., & Banday, A. A. (2011). Resveratrol prevents endothelial nitric oxide synthase uncoupling and attenuates development of hypertension in spontaneously hypertensive rats. *European Journal of Pharmacology*, *667*(1–3), 258–264. https://doi.org/10.1016/j.ejphar.2011.05.026

Bœuf-Gibot, S., Pereira, B., Imbert, J., Kerroum, H., Menini, T., Lafarge, E., De Carvalho, M., Vorilhon, P., Boussageon, R., & Vaillant-Roussel, H. (2020). Benefits and adverse effects of ACE inhibitors in patients with heart failure with reduced ejection fraction: a systematic review and meta-analysis. In *European Journal of Clinical Pharmacology* (Vol. 77, Issue 3, pp. 321–329). Springer Science and Business Media Deutschland GmbH. https://doi.org/10.1007/s00228-020-03018-4

Bonnefont-Rousselot, D. (2016). Resveratrol and cardiovascular diseases. In *Nutrients* (Vol. 8, Issue 5). MDPI AG. https://doi.org/10.3390/nu8050250

Braicu, C., Pilecki, V., Balacescu, O., Irimie, A., & Neagoe, I. B. (2011). The relationships between biological activities and structure of flavan-3-ols. *International Journal of Molecular Sciences*, *12*(12), 9342–9353. https://doi.org/10.3390/ijms12129342

Brüll, V., Burak, C., Stoffel-Wagner, B., Wolffram, S., Nickenig, G., Müller, C., Langguth, P., Alteheld, B., Fimmers, R., Naaf, S., Zimmermann, B. F., Stehle, P., & Egert, S. (2015). Effects of a quercetin-rich onion skin extract on 24 h ambulatory blood pressure and endothelial function in overweight-to-obese patients with (pre-)hypertension: A randomised double-blinded placebo-controlled cross-over trial. *British Journal of Nutrition*, *114*(8), 1263–1277. https://doi.org/10.1017/S0007114515002950

Bustamante, M. A., Moral, R., Paredes, C., Pérez-Espinosa, A., Moreno-Caselles, J., & Pérez-Murcia, M. D. (2008). Agrochemical characterisation of the solid by-products and residues from the winery and distillery industry. *Waste Management*, *28*(2), 372–380. https://doi.org/10.1016/j.wasman.2007.01.013

Cachofeiro, V., Miana, M., de las Heras, N., Martín-Fernández, B., Ballesteros, S., Fernández-Tresguerres, J., & Lahera, V. (2008). Aldosterone and the vascular system. *The Journal of Steroid Biochemistry and Molecular Biology*, *109*(3–5), 331–335. https://doi.org/10.1016/j.jsbmb.2008.03.005

Calfío, C., & Huidobro-Toro, J. P. (2019). Potent vasodilator and cellular antioxidant activity of endemic patagonian calafate berries (berberis microphylla) with nutraceutical potential. *Molecules*, *24*(15), 2700. https://doi.org/10.3390/molecules24152700

Carciochi, R. A., D'Alessandro, L. G., Vauchel, P., Rodriguez, M. M., Nolasco, S. M., & Dimitrov, K. (2017). Valorization of Agrifood By-Products by Extracting Valuable Bioactive Compounds Using Green Processes. In *Ingredients Extraction by Physicochemical Methods in Food* (pp. 191–228). Elsevier. https://doi.org/10.1016/b978-0-12-811521-3.00004-1

Caro, M., Sansone, A., Amezaga, J., Navarro, V., Ferreri, C., & Tueros, I. (2017a). Wine lees modulate lipid metabolism and induce fatty acid remodelling in zebrafish. *Food and Function*, *8*(4), 1652–1659. https://doi.org/10.1039/c6fo01754a

Caro, M., Sansone, A., Amezaga, J., Navarro, V., Ferreri, C., & Tueros, I. (2017b). Wine lees modulate lipid metabolism and induce fatty acid remodelling in zebrafish. *Food and Function*, *8*(4), 1652–1659. https://doi.org/10.1039/c6fo01754a

Cheng, Y. C., Sheen, J. M., Hu, W. L., & Hung, Y. C. (2017). Polyphenols and Oxidative Stress in Atherosclerosis-Related Ischemic Heart Disease and Stroke. In *Oxidative Medicine and Cellular Longevity* (Vol. 2017). Hindawi Limited. https://doi.org/10.1155/2017/8526438

Chiva-Blanch, G., Urpi-Sarda, M., Ros, E., Arranz, S., Valderas-Martínez, P., Casas, R., Sacanella, E., Llorach, R., Lamuela-Raventos, R. M., Andres-Lacueva, C., & Estruch, R. (2012). Dealcoholized red wine decreases systolic and diastolic blood pressure and increases plasma nitric oxide: Short communication. *Circulation Research*, *111*(8), 1065–1068. https://doi.org/10.1161/CIRCRESAHA.112.275636

Cienfuegos-Jovellanos, E., Del Mar Quiñones, 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. https://doi.org/10.1021/jf804045b

Collier, S. R., & Landram, M. J. (2012). Treatment of prehypertension: Lifestyle and/or medication. In *Vascular Health and Risk Management* (Vol. 8, Issue 1, pp. 613–619). Dove Press. https://doi.org/10.2147/VHRM.S29138

Crowley, S. D. (2014). The Cooperative roles of inflammation and oxidative stress in the pathogenesis of hypertension. *Antioxidants and Redox Signaling*, *20*(1), 102–120.

https://doi.org/10.1089/ars.2013.5258

Dabeek, W. M., & Marra, M. V. (2019). Dietary quercetin and kaempferol: Bioavailability and potential cardiovascular-related bioactivity in humans. In *Nutrients* (Vol. 11, Issue 10). MDPI AG. https://doi.org/10.3390/nu11102288

Dalton, T. P., Chen, Y., Schneider, S. N., Nebert, D. W., & Shertzer, H. G. (2004). Genetically altered mice to evaluate glutathione homeostasis in health and disease. In *Free Radical Biology and Medicine* (Vol. 37, Issue 10, pp. 1511–1526). Free Radic Biol Med. https://doi.org/10.1016/j.freeradbiomed.2004.06.040

Dayoub, O., Andriantsitohaina, R., & Clere, N. (2014). Pleiotropic Beneficial Effects of Epigallocatechin Gallate, Quercetin and Delphinidin on Cardiovascular Diseases Associated with Endothelial Dysfunction. *Cardiovascular & Hematological Agents in Medicinal Chemistry*, *11*(4), 249–264. https://doi.org/10.2174/18715257126661403092330 48

De Costa, G. F., Ognibene, D. T., Da Costa, C. A., Teixeira, M. T., Da Silva Cristino Cordeiro, V., De Bem, G. F., Moura, A. S., De Castro Resende, A., & De Moura, R. S. (2020). Vitis vinifera L grape skin extract prevents development of hypretension and altered lipid profile in sponteneously hypertensive tats: Role of oxidative stress. *Preventive Nutrition and Food Science*, *25*(1), 25–31. https://doi.org/10.3746/pnf.2020.25.1.25

de Figueiredo, E. A., Alves, N. F. B., Monteiro, M. M. de O., Cavalcanti, C. de O., da Silva, T. M. S., da Silva, T. M. G., Braga, V. de A., & Oliveira, E. de J. (2017). Antioxidant and antihypertensive effects of a chemically defined fraction of syrah red wine on spontaneously hypertensive rats. *Nutrients*, *9*(6), 10–23. https://doi.org/10.3390/nu9060574

De Iseppi, A., Lomolino, G., Marangon, M., & Curioni, A. (2020). Current and future strategies for wine yeast lees valorization. In *Food Research International* (Vol. 137, p. 109352). Elsevier Ltd. https://doi.org/10.1016/j.foodres.2020.109352

de Moura, R. Soares, Viana, F. S. C., Souza, M. A. V., Kovary, K., Guedes, D. C., Oliveira, E. P. B., Rubenich, L. M. S., Carvalho, L. C. R. M., Oliveira, R. M., Tano, T., & Correia, M. L. G. (2002). Antihypertensive, vasodilator and antioxidant effects of a vinifera grape skin extract. *Journal of Pharmacy and Pharmacology*, *54*(11), 1515–1520. https://doi.org/10.1211/002235702153

De Moura, Roberto Soares, Resende, Â. C., Moura, A. S., & Maradei, M. F. (2007). Protective action of a hydroalcoholic extract of a vinifera grape skin on experimental preeclampsia in rats. *Hypertension in Pregnancy*, *26*(1), 89–100. https://doi.org/10.1080/10641950601147960

Del Borghi, A., Moreschi, L., & Gallo, M. (2020). Circular economy approach to reduce water–energy–food nexus. In *Current Opinion in Environmental Science and*

Health (Vol. 13, pp. 23–28). Elsevier B.V. https://doi.org/10.1016/j.coesh.2019.10.002

Del Pino-García, R., Rivero-Pérez, M. D., González-Sanjosé, M. L., Croft, K. D., & Muñiz, P. (2017). Antihypertensive and antioxidant effects of supplementation with red wine pomace in spontaneously hypertensive rats. *Food and Function*, *8*(7), 2444–2454. https://doi.org/10.1039/c7fo00390k

Del Rio, D., Rodriguez-Mateos, A., Spencer, J. P. E., Tognolini, M., Borges, G., & Crozier, A. (2013). Dietary (poly)phenolics in human health: Structures, bioavailability, and evidence of protective effects against chronic diseases. In *Antioxidants and Redox Signaling* (Vol. 18, Issue 14, pp. 1818–1892). Mary Ann Liebert, Inc. https://doi.org/10.1089/ars.2012.4581

Delgado De La Torre, M. P., Priego-Capote, F., & Luque De Castro, M. D. (2015). Characterization and comparison of wine lees by liquid chromatography-mass spectrometry in high-resolution mode. *Journal of Agricultural and Food Chemistry*, *63*(4), 1116–1125. https://doi.org/10.1021/jf505331f

Di Lorenzo, C., Sousa, L. P. de, Pastor, R. F., Colombo, F., Frigerio, G., & Restani, P. (2015). Beneficial effects of non-alcoholic grape-derived products on human health: A literature review. *BIO Web of Conferences*, *5*(2015), 04002. https://doi.org/10.1051/bioconf/20150504002

Dludla, P. V., Nkambule, B. B., Jack, B., Mkandla, Z., Mutize, T., Silvestri, S., Orlando, P., Tiano, L., Louw, J., & Mazibuko-Mbeje, S. E. (2019). Inflammation and oxidative stress in an obese state and the protective effects of gallic acid. In *Nutrients* (Vol. 11, Issue 1). MDPI AG. https://doi.org/10.3390/nu11010023

Dohadwala, M. M., & Vita, J. A. (2009). Grapes and Cardiovascular Disease. *The Journal of Nutrition*, *139*(9), 1788S-1793S. https://doi.org/10.3945/jn.109.107474

Dujmić, F., Ganić, K. K., Ćurić, D., Karlović, S., Bosiljkov, T., Ježek, D., Vidrih, R., Hribar, J., Zlatić, E., Prusina, T., Khubber, S., Barba, F. J., & Brnčić, M. (2020). Non-thermal ultrasonic extraction of polyphenolic compounds from red wine lees. *Foods*, *9*(4), 472. https://doi.org/10.3390/foods9040472

Durazzo, A., Lucarini, M., Souto, E. B., Cicala, C., Caiazzo, E., Izzo, A. A., Novellino, E., & Santini, A. (2019). Polyphenols: A concise overview on the chemistry, occurrence, and human health. In *Phytotherapy Research* (Vol. 33, Issue 9, pp. 2221–2243). John Wiley and Sons Ltd. https://doi.org/10.1002/ptr.6419

Egert, S., Bosy-Westphal, A., Seiberl, J., Kürbitz, C., Settler, U., Plachta-Danielzik, S., Wagner, A. E., Frank, J., Schrezenmeir, J., Rimbach, G., Wolffram, S., & Müller, M. J. (2009). Quercetin reduces systolic blood pressure and plasma oxidised low-density lipoprotein concentrations in overweight subjects with a high-cardiovascular disease risk phenotype: A double-blinded, placebo-controlled

cross-over study. *British Journal of Nutrition*, *102*(7), 1065–1074. https://doi.org/10.1017/S0007114509359127

Emiliano, A. F., de Cavalho, L. C. R. M., da Silva Cristino Cordeiro, V., da Costa, C. A., de Oliveira, P. B. R., Queiroz, E. F., Col Moreira, D. D., Boaventura, G. T., de Moura, R. S., & Resende, A. C. (2011). Metabolic Disorders and Oxidative Stress Programming in Offspring of Rats Fed a High-fat Diet During Lactation: Effects of a Vinifera Grape Skin (ACH09) Extract. *Journal of Cardiovascular Pharmacology*, *58*(3), 319–328. https://doi.org/10.1097/FJC.0b013e3182244a51

Ettehad, D., Emdin, C. A., Kiran, A., Anderson, S. G., Callender, T., Emberson, J., Chalmers, J., Rodgers, A., & Rahimi, K. (2016). Blood pressure lowering for prevention of cardiovascular disease and death: A systematic review and meta-analysis. *The Lancet*, *387*(10022), 957–967. https://doi.org/10.1016/S0140-6736(15)01225-8

Fairlie-Jones, L., Davison, K., Fromentin, E., & Hill, A. M. (2017). The effect of anthocyanin-rich foods or extracts on vascular function in adults: A systematic review and meta-analysis of randomised controlled trials. In *Nutrients* (Vol. 9, Issue 8, p. 908). https://doi.org/10.3390/nu9080908

Fang, Y., Meng, J., Zhang, A., Liu, J., Xu, T., Yu, W., Chen, S., Li, H., Zhang, Z., & Wang, H. (2011). Influence of shriveling on berry composition and antioxidant activity of Cabernet Sauvignon grapes from Shanxi vineyards. *Journal of the Science of Food and Agriculture*, *91*(4), 749–757. https://doi.org/10.1002/jsfa.4246

Fang, Z., & Bhandari, B. (2010). Encapsulation of polyphenols - A review. In *Trends in Food Science and Technology* (Vol. 21, Issue 10, pp. 510–523). Elsevier. https://doi.org/10.1016/j.tifs.2010.08.003

FAO. (2020). *FAO - News Article: Food wastage: Key facts and figures*. Fao.Org. http://www.fao.org/news/story/en/item/196402/icode/

Feringa, H. H. H., Laskey, D. A., Dickson, J. E., & Coleman, C. I. (2011). The Effect of Grape Seed Extract on Cardiovascular Risk Markers: A Meta-Analysis of Randomized Controlled Trials. *Journal of the American Dietetic Association*, *111*(8), 1173–1181. https://doi.org/10.1016/j.jada.2011.05.015

Fernández-Iglesias, A., Quesada, H., Díaz, S., Pajuelo, D., Bladé, C., Arola, L., Salvadó, M. J., & Mulero, M. (2014). Combination of grape seed proanthocyanidin extract and docosahexaenoic acid-rich oil increases the hepatic detoxification by GST mediated GSH conjugation in a lipidic postprandial state. *Food Chemistry*, *165*, 14–20. https://doi.org/10.1016/j.foodchem.2014.05.057

Figueiredo-Gonzalez, M., Cancho-Grande, B., & Simal-Gandara, J. (2013). Evolution of colour and phenolic compounds during Garnacha Tintorera grape raisining. *Food Chemistry*, *141*(3), 3230–3240. https://doi.org/10.1016/j.foodchem.2013.05.142

Fogacci, F., Tocci, G., Presta, V., Fratter, A., Borghi, C., & Cicero, A. F. G. (2019). Effect of resveratrol on blood pressure: A systematic review and meta-analysis of randomized, controlled, clinical trials. In *Critical Reviews in Food Science and Nutrition* (Vol. 59, Issue 10, pp. 1605–1618). Taylor and Francis Inc. https://doi.org/10.1080/10408398.2017.1422480

Fraga, C. G., Croft, K. D., Kennedy, D. O., & Tomás-Barberán, F. A. (2019). The effects of polyphenols and other bioactives on human health. *Food and Function*, *10*(2), 514–528. https://doi.org/10.1039/c8fo01997e

Franco, J. G., Lisboa, P. C., Lima, N. S., Amaral, T. A. S., Peixoto-Silva, N., Resende, A. C., Oliveira, E., Passos, M. C. F., & Moura, E. G. (2013). Resveratrol attenuates oxidative stress and prevents steatosis and hypertension in obese rats programmed by early weaning. *Journal of Nutritional Biochemistry*, *24*(6), 960–966. https://doi.org/10.1016/j.jnutbio.2012.06.019

Gabaston, J., Valls Fonayet, J., Franc, C., Waffo-Teguo, P., De Revel, G., Hilbert, G., Gomès, E., Richard, T., & Mérillon, J. M. (2020). Characterization of Stilbene Composition in Grape Berries from Wild Vitis Species in Year-To-Year Harvest. *Journal of Agricultural and Food Chemistry*, *68*(47), 13408–13417. https://doi.org/10.1021/acs.jafc.0c04907

García-Lomillo, J., & González-SanJosé, M. L. (2017). Applications of Wine Pomace in the Food Industry: Approaches and Functions. *Comprehensive Reviews in Food Science and Food Safety*, *16*(1), 3–22. https://doi.org/10.1111/1541-4337.12238

Garde-Cerdán, T., Gutiérrez-Gamboa, G., Ayestarán, B., González-Lázaro, M., Rubio-Bretón, P., & Pérez-Álvarez, E. P. (2021). Influence of seaweed foliar application to Tempranillo grapevines on grape and wine phenolic compounds over two vintages. *Food Chemistry*, *345*, 128843. https://doi.org/10.1016/j.foodchem.2020.128843

Garrido, J., & Borges, F. (2013). Wine and grape polyphenols - A chemical perspective. In *Food Research International* (Vol. 54, Issue 2, pp. 1844–1858). Elsevier. https://doi.org/10.1016/j.foodres.2013.08.002

Garza-Reyes, J. A., Kumar, V., Batista, L., Cherrafi, A., & Rocha-Lona, L. (2019). From linear to circular manufacturing business models. In *Journal of Manufacturing Technology Management* (Vol. 30, Issue 3, pp. 554–560). Emerald Group Publishing Ltd. https://doi.org/10.1108/JMTM-04-2019-356

Gertz, M., Nguyen, G. T. T., Fischer, F., Suenkel, B., Schlicker, C., Fränzel, B., Tomaschewski, J., Aladini, F., Becker, C., Wolters, D., & Steegborn, C. (2012). A Molecular Mechanism for Direct Sirtuin Activation by Resveratrol. *PLoS ONE*, *7*(11). https://doi.org/10.1371/journal.pone.0049761

Giacobbo, A., Dias, B. B., Onorevoli, B., Bernardes, A. M., de Pinho, M. N., Caramão, E.

B., Rodrigues, E., & Jacques, R. A. (2019). Wine lees from the 1st and 2nd rackings: valuable by-products. *Journal of Food Science and Technology*, *56*(3), 1559–1566. https://doi.org/10.1007/s13197-019-03665-1

Gómez-Plaza, E., Bautista-Ortín, A. B., Ruiz-García, Y., Fernández-Fernández, J. I., & Gil-Muñoz, R. (2017). Effect of elicitors on the evolution of grape phenolic compounds during the ripening period. *Journal of the Science of Food and Agriculture*, *97*(3), 977–983. https://doi.org/10.1002/jsfa.7823

Goutzourelas, N., Stagos, D., Housmekeridou, A., Karapouliou, C., Kerasioti, E., Aligiannis, N., Skaltsounis, A. L., Spandidos, D. A., Tsatsakis, A. M., & Kouretas, D. (2015). Grape pomace extract exerts antioxidant effects through an increase in GCS levels and GST activity in muscle and endothelial cells. *International Journal of Molecular Medicine*, *36*(2), 433–441. https://doi.org/10.3892/ijmm.2015.2246

Goutzourelas, N., Stagos, D., Spanidis, Y., Liosi, M., Apostolou, A., Priftis, A., Haroutounian, S., Spandidos, D. A., Tsatsakis, A. M., & Kouretas, D. (2015). Polyphenolic composition of grape stem extracts affects antioxidant activity in endothelial and muscle cells. *Molecular Medicine Reports*, *12*(4), 5846–5856. https://doi.org/10.3892/mmr.2015.4216

Guerrero, L., Castillo, J., Quiñones, M., Garcia-Vallvé, S., Arola, L., Pujadas, G., & Muguerza, B. (2012). Inhibition of Angiotensin-Converting Enzyme Activity by Flavonoids: Structure-Activity Relationship Studies. *PLoS ONE*, *7*(11), 49493. https://doi.org/10.1371/journal.pone.0049493

Gupta, M., Dey, S., Marbaniang, D., Pal, P., Ray, S., & Mazumder, B. (2020). Grape seed extract: having a potential health benefits. In *Journal of Food Science and Technology* (Vol. 57, Issue 4, pp. 1205–1215). Springer. https://doi.org/10.1007/s13197-019-04113-w

Gutiérrez-Gamboa, G., Garde-Cerdán, T., Martínez-Lapuente, L., Costa, B. S., Rubio-Bretón, P., & Pérez-Álvarez, E. P. (2020). Phenolic composition of Tempranillo Blanco (*Vitis vinifera* L.) grapes and wines after biostimulation via a foliar seaweed application. *Journal of the Science of Food and Agriculture*, *100*(2), 825–835. https://doi.org/10.1002/jsfa.10094

Gutiérrez-Gamboa, G., Gómez-Plaza, E., Bautista-Ortín, A. B., Garde-Cerdán, T., Moreno-Simunovic, Y., & Martínez-Gil, A. M. (2019). Rootstock effects on grape anthocyanins, skin and seed proanthocyanidins and wine color and phenolic compounds from *Vitis vinifera* L. Merlot grapevines. *Journal of the Science of Food and Agriculture*, *99*(6), 2846–2854. https://doi.org/10.1002/jsfa.9496

Hall, J. E., Guyton, A. C., & Mizelle, H. L. (1990). Role of the renin-angiotensin system in control of sodium excretion and arterial pressure. *Acta Physiologica Scandinavica, Supplement*, *139*(591), 48–62. https://europepmc.org/article/med/2220409

Heiss, C., Jahn, S., Taylor, M., Real, W. M., Angeli, F. S., Wong, M. L., Amabile, N., Prasad, M., Rassaf, T., Ottaviani, J. I., Mihardja, S., Keen, C. L., Springer, M. L., Boyle, A., Grossman, W., Glantz, S. A., Schroeter, H., & Yeghiazarians, Y. (2010). Improvement of endothelial function with dietary flavanols is associated with mobilization of circulating angiogenic cells in patients with coronary artery disease. *Journal of the American College of Cardiology*, *56*(3), 218–224. https://doi.org/10.1016/j.jacc.2010.03.039

Huang, H., Chen, G., Liao, D., Zhu, Y., Pu, R., & Xue, X. (2016). The effects of resveratrol intervention on risk markers of cardiovascular health in overweight and obese subjects: a pooled analysis of randomized controlled trials. *Obesity Reviews*, *17*(12), 1329–1340. https://doi.org/10.1111/obr.12458

Huang, H., Chen, G., Liao, D., Zhu, Y., & Xue, X. (2016). Effects of Berries Consumption on Cardiovascular Risk Factors: A Meta-analysis with Trial Sequential Analysis of Randomized Controlled Trials. *Scientific Reports*, *6*. https://doi.org/10.1038/srep23625

Ibarra, M., Moreno, L., Vera, R., Cogolludo, A., Duarte, J., Tamargo, J., & Perez-Vizcaino, F. (2003). Effects of the Flavonoid Quercetin and its Methylated Metabolite Isorhamnetin in Isolated Arteries from Spontaneously Hypertensive Rats. *Planta Medica*, *69*(11), 995–1000. https://doi.org/10.1055/s-2003-45144

Iglesias-Carres, L., Mas-Capdevila, A., Bravo, F. I., Arola, L., Muguerza, B., & Arola-Arnal, A. (2019). Exposure of Fischer 344 rats to distinct photoperiods influences the bioavailability of red grape polyphenols. *Journal of Photochemistry and Photobiology B: Biology*, *199*, 111623. https://doi.org/10.1016/j.jphotobiol.2019.111623

Iglesias-Carres, L., Mas-Capdevila, A., Sancho-Pardo, L., Bravo, F. I., Mulero, M., Muguerza, B., & Arola-Arnal, A. (2018). Optimized Extraction by Response Surface Methodology Used for the Characterization and Quantification of Phenolic Compounds in Whole Red Grapes (Vitis vinifera). *Nutrients*, *10*(12), 1931. https://doi.org/10.3390/nu10121931

Incalza, M. A., D'Oria, R., Natalicchio, A., Perrini, S., Laviola, L., & Giorgino, F. (2018). Oxidative stress and reactive oxygen species in endothelial dysfunction associated with cardiovascular and metabolic diseases. In *Vascular Pharmacology* (Vol. 100, pp. 1–19). Elsevier Inc. https://doi.org/10.1016/j.vph.2017.05.005

Iriondo-Dehond, M., Miguel, E., & Del Castillo, M. D. (2018). Food byproducts as sustainable ingredients for innovative and healthy dairy foods. In *Nutrients* (Vol. 10, Issue 10). MDPI AG. https://doi.org/10.3390/nu10101358

Izdebska, M., Herbet, M., Gawrońska-Grzywacz, M., Piątkowska-Chmiel, I., Korga, A., Sysa, M., Iwan, M., Natorska-Chomicka, D., Poleszak, E., Wróbel, A., Mandziuk, S., & Dudka, J. (2018). Resveratrol limits lipogenesis and enhance mitochondrial

activity in HepG2 cells. *Journal of Pharmacy and Pharmaceutical Sciences*, *21*(1), 504–515. https://doi.org/10.18433/jpps29994

Jakubczyk, A., Karas, M., Rybczynska-Tkaczyk, K., Zielinska, E., & Zielinski, D. (2020). Current trends of bioactive peptides - New sources and therapeutic effect. In *Foods* (Vol. 9, Issue 7). MDPI Multidisciplinary Digital Publishing Institute. https://doi.org/10.3390/foods9070846

James, P. A., Oparil, S., Carter, B. L., Cushman, W. C., Dennison-Himmelfarb, C., Handler, J., Lackland, D. T., LeFevre, M. L., MacKenzie, T. D., Ogedegbe, O., Smith, S. C., Svetkey, L. P., Taler, S. J., Townsend, R. R., Wright, J. T., Narva, A. S., & Ortiz, E. (2014). 2014 Evidence-based guideline for the management of high blood pressure in adults: Report from the panel members appointed to the Eighth Joint National Committee (JNC 8). In *JAMA - Journal of the American Medical Association* (Vol. 311, Issue 5, pp. 507–520). American Medical Association. https://doi.org/10.1001/jama.2013.284427

Jara-Palacios, M. J. (2019). Wine lees as a source of antioxidant compounds. In *Antioxidants* (Vol. 8, Issue 2). MDPI AG. https://doi.org/10.3390/antiox8020045

Jin, L., Piao, Z. H., Sun, S., Liu, B., Kim, G. R., Seok, Y. M., Lin, M. Q., Ryu, Y., Choi, S. Y., Kee, H. J., & Jeong, M. H. (2017). Gallic Acid Reduces Blood Pressure and Attenuates Oxidative Stress and Cardiac Hypertrophy in Spontaneously Hypertensive Rats. *Scientific Reports*, *7*(1). https://doi.org/10.1038/s41598-017-15925-1

Jones, G. V., & Webb, L. B. (2010). Climate change, viticulture, and wine: Challenges and opportunities. *Journal of Wine Research*, *21*(2), 103–106. https://doi.org/10.1080/09571264.2010.530091

Josep, M. U., Sort, X., Zayas, A., & Rosa, M. P. (2010). Effects of soil and climatic conditions on grape ripening and wine quality of Cabernet Sauvignon. *Journal of Wine Research*, *21*(1), 1–17. https://doi.org/10.1080/09571264.2010.495851

Kadri, S., El Ayed, M., Mabrouk, M., Limam, F., Elkahoui, S., Aouani, E., & Mokni, M. (2019). Characterization, anti-oxidative effect of grape seed powder and in silico affinity profiling of polyphenolic and extra-phenolic compounds for calpain inhibition. *Journal of Pharmaceutical and Biomedical Analysis*, *164*, 365–372. https://doi.org/10.1016/j.jpba.2018.11.003

Kammerer, D., Claus, A., Carle, R., & Schieber, A. (2004). Polyphenol screening of pomace from red and white grape varieties (Vitis vinifera L.) by HPLC-DAD-MS/MS. *Journal of Agricultural and Food Chemistry*, *52*(14), 4360–4367. https://doi.org/10.1021/jf049613b

Katiyar, S. K. (2015). Proanthocyanidins from grape seeds inhibit UV-radiation-induced immune suppression in mice: Detection and analysis of molecular and cellular

targets. In *Photochemistry and Photobiology* (Vol. 91, Issue 1, pp. 156–162). Blackwell Publishing Inc. https://doi.org/10.1111/php.12330

Khoo, H. E., Azlan, A., Tang, S. T., & Lim, S. M. (2017). Anthocyanidins and anthocyanins: Colored pigments as food, pharmaceutical ingredients, and the potential health benefits. In *Food and Nutrition Research* (Vol. 61, Issue 1). Swedish Nutrition Foundation. https://doi.org/10.1080/16546628.2017.1361779

Kim, E. N., Lim, J. H., Kim, M. Y., Ban, T. H., Jang, I.-A., Yoon, H. E., Park, C. W., Chang, Y. S., & Choi, B. S. (2018). Resveratrol, an Nrf2 activator, ameliorates aging-related progressive renal injury. *Aging*, *10*(1), 83–99. https://doi.org/10.18632/aging.101361

Kim, J. N., Han, S. N., Ha, T. J., & Kim, H. K. (2017). Black soybean anthocyanins attenuate inflammatory responses by suppressing reactive oxygen species production and mitogen activated protein kinases signaling in lipopolysaccharide-stimulated macrophages. *Nutrition Research and Practice*, *11*(5), 357–364. https://doi.org/10.4162/nrp.2017.11.5.357

Kiviniemi, T. O., Saraste, A., Lehtimäki, T., Toikka, J. O., Saraste, M., Raitakari, O. T., Hartiala, J. J., Viikari, J., & Koskenvuo, J. W. (2010). Decreased endothelin-1 levels after acute consumption of red wine and de-alcoholized red wine. *Atherosclerosis*, *211*(1), 283–286. https://doi.org/10.1016/j.atherosclerosis.2010.01.017

Landeka Jurčević, I., Dora, M., Guberović, I., Petras, M., Rimac Brnčić, S., & Đikić, D. (2017). Wine Lees Polyphenols as a Novel Functional Bioactive Compound in the Protection against Oxidative Stress and Hyperlipidemia. *Food Technology and Biotechnology*, *55*(1), 109. https://doi.org/10.17113/ftb.55.01.17.4894

Larson, A. J., David Symons, J., & Jalili, T. (2012). Therapeutic potential of quercetin to decrease blood pressure: Review of efficacy and mechanisms. In *Advances in Nutrition* (Vol. 3, Issue 1, pp. 39–46). American Society for Nutrition. https://doi.org/10.3945/an.111.001271

Leikert, J. F., Räthel, T. R., Wohlfart, P., Cheynier, V., Vollmar, A. M., & Dirsch, V. M. (2002). Red wine polyphenols enhance endothelial nitric oxide synthase expression and subsequent nitric oxide release from endothelial cells. *Circulation*, *106*(13), 1614–1617. https://doi.org/10.1161/01.CIR.0000034445.31543.43

Li, S.-H., Zhao, P., Tian, H.-B., Chen, L.-H., & Cui, L.-Q. (2015). Effect of Grape Polyphenols on Blood Pressure: A Meta-Analysis of Randomized Controlled Trials. *PLOS ONE*, *10*(9), e0137665. https://doi.org/10.1371/journal.pone.0137665

Li, Y., Yao, J., Han, C., Yang, J., Chaudhry, M. T., Wang, S., Liu, H., & Yin, Y. (2016). Quercetin, inflammation and immunity. In *Nutrients* (Vol. 8, Issue 3). MDPI AG. https://doi.org/10.3390/nu8030167

Liang, Y., Wang, J., Gao, H., Wang, Q., Zhang, J., & Qiu, J. (2016). Beneficial effects of grape seed proanthocyanidin extract on arterial remodeling in spontaneously hypertensive rats via protecting against oxidative stress. *Molecular Medicine Reports*, *14*(4), 3711–3718. https://doi.org/10.3892/mmr.2016.5699

Lin, Y. S., Lu, Y. L., Wang, G. J., Chen, L. G., Wen, C. L., & Hou, W. C. (2012). Ethanolic extracts and isolated compounds from small-leaf grape (Vitis thunbergii var. taiwaniana) with antihypertensive activities. *Journal of Agricultural and Food Chemistry*, *60*(30), 7435–7441. https://doi.org/10.1021/jf302445x

Lingua, M. S., Fabani, M. P., Wunderlin, D. A., & Baroni, M. V. (2016). From grape to wine: Changes in phenolic composition and its influence on antioxidant activity. *Food Chemistry*, *208*, 228–238. https://doi.org/10.1016/j.foodchem.2016.04.009

Liu, X., Qiu, J., Zhao, S., You, B., Ji, X., Wang, Y., Cui, X., Wang, Q., & Gao, H. (2012). Grape seed proanthocyanidin extract alleviates ouabain-induced vascular remodeling through regulation of endothelial function. *Molecular Medicine Reports*, *6*(5), 949–954. https://doi.org/10.3892/mmr.2012.1026

Loke, W. M., Hodgson, J. M., Proudfoot, J. M., McKinley, A. J., Puddey, I. B., & Croft, K. D. (2008). Pure dietary flavonoids quercetin and (-)-epicatechin augment nitric oxide products and reduce endothelin-1 acutely in healthy men. *American Journal of Clinical Nutrition*, *88*(4), 1018–1025. https://doi.org/10.1093/ajcn/88.4.1018

Lonn, E. M., Bosch, J., López-Jaramillo, P., Zhu, J., Liu, L., Pais, P., Diaz, R., Xavier, D., Sliwa, K., Dans, A., Avezum, A., Piegas, L. S., Keltai, K., Keltai, M., Chazova, I., Peters, R. J. G., Held, C., Yusoff, K., Lewis, B. S., … Yusuf, S. (2016). Blood-Pressure Lowering in Intermediate-Risk Persons without Cardiovascular Disease. *New England Journal of Medicine*, *374*(21), 2009–2020. https://doi.org/10.1056/NEJMoa1600175

López-Fernández-Sobrino, R., Margalef, M., Torres-Fuentes, C., Ávila-Román, J., Aragonès, G., Muguerza, B., & Bravo, F. I. (2021). Enzyme-Assisted Extraction to Obtain Phenolic-Enriched Wine Lees with Enhanced Bioactivity in Hypertensive Rats. *Antioxidants*, *10*(4), 517. https://doi.org/10.3390/antiox10040517

López-Fernández-Sobrino, R., Soliz-Rueda, J. R., Ávila-Román, J., Arola-Arnal, A., Suárez, M., Muguerza, B., & Bravo, F. I. (2021). Blood pressure-lowering effect of wine lees phenolic compounds is mediated by endothelial-derived factors: role of sirtuin-1. *Nutrients*.

López-Fernández-Sobrino, R., Soliz-Rueda, J. R., Margalef, M., Arola-Arnal, A., Suárez, M., Bravo, F. I., & Muguerza, B. (2021). ACE Inhibitory and Antihypertensive Activities of Wine Lees and Relationship among Bioactivity and Phenolic Profile. *Nutrients*, *13*(2), 679. https://doi.org/10.3390/nu13020679

Louli, V., Ragoussis, N., & Magoulas, K. (2004). Recovery of phenolic antioxidants from

wine industry by-products. *Bioresource Technology*, *92*(2), 201–208. https://doi.org/10.1016/j.biortech.2003.06.002

Luo, L., Cui, Y., Zhang, S., Li, L., Li, Y., Zhou, P., & Sun, B. (2016). Preparative separation of grape skin polyphenols by high-speed counter-current chromatography. *Food Chemistry*, *212*, 712–721. https://doi.org/10.1016/j.foodchem.2016.06.009

Maaliki, D., Shaito, A. A., Pintus, G., El-Yazbi, A., & Eid, A. H. (2019). Flavonoids in hypertension: a brief review of the underlying mechanisms. In *Current Opinion in Pharmacology* (Vol. 45, pp. 57–65). Elsevier Ltd. https://doi.org/10.1016/j.coph.2019.04.014

Mahmoudi, M., Charradi, K., Limam, F., & Aouani, E. (2018). Grape seed and skin extract as an adjunct to xenical therapy reduces obesity, brain lipotoxicity and oxidative stress in high fat diet fed rats. *Obesity Research and Clinical Practice*, *12*(1), 115–126. https://doi.org/10.1016/j.orcp.2016.04.006

Mancia, G., & Zanchetti, A. (2008). Choice of antihypertensive drugs in the European Society of Hypertension–European Society of Cardiology guidelines: specific indications rather than ranking for general usage. *Journal of Hypertension*, *26*(2), 164–168. https://doi.org/10.1097/HJH.0b013e3282f52fa8

Manson, J. A. E., Skerrett, P. J., Greenland, P., & VanItallie, T. B. (2004). The Escalating Pandemics of Obesity and Sedentary Lifestyle: A Call to Action for Clinicians. In *Archives of Internal Medicine* (Vol. 164, Issue 3, pp. 249–258). American Medical Association. https://doi.org/10.1001/archinte.164.3.249

Maqsood, S., Adiamo, O., Ahmad, M., & Mudgil, P. (2020). Bioactive compounds from date fruit and seed as potential nutraceutical and functional food ingredients. In *Food Chemistry* (Vol. 308, p. 125522). Elsevier Ltd. https://doi.org/10.1016/j.foodchem.2019.125522

Margalef, M., Bravo, F. I., Arola-Arnal, A., & Muguerza, B. (2017). Natural Angiotensin Converting Enzyme (ACE) Inhibitors with Antihypertensive Properties. In *Natural Products Targeting Clinically Relevant Enzymes* (pp. 45–67). Wiley-VCH Verlag GmbH & Co. KGaA. https://doi.org/10.1002/9783527805921.ch3

Marqués, J. L., Porta, G. Della, Reverchon, E., Renuncio, J. A. R., & Mainar, A. M. (2013). Supercritical antisolvent extraction of antioxidants from grape seeds after vinification. *Journal of Supercritical Fluids*, *82*, 238–243. https://doi.org/10.1016/j.supflu.2013.07.005

Martin, M. Á., & Ramos, S. (2021). Impact of cocoa flavanols on human health. *Food and Chemical Toxicology*, 112121. https://doi.org/10.1016/j.fct.2021.112121

Marunaka, Y., Marunaka, R., Sun, H., Yamamoto, T., Kanamura, N., Inui, T., & Taruno, A. (2017). Actions of quercetin, a polyphenol, on blood pressure. In *Molecules* (Vol. 22, Issue 2). MDPI AG. https://doi.org/10.3390/molecules22020209

Mas-capdevila, A., Iglesias-carres, L., Arola-arnal, A., Suárez, M., & Francisca, I. (2020). Changes in arterial blood pressure caused by long-term administration of grape seed proanthocyanidins in rats with established hypertension. *Food & Function*, *11*(10), 8735–8742. https://doi.org/10.1039/D0FO00981D

Matsumura, N., Takahara, S., Maayah, Z. H., Parajuli, N., Byrne, N. J., Shoieb, S. M., Soltys, C. L. M., Beker, D. L., Masson, G., El-Kadi, A. O. S., & Dyck, J. R. B. (2018). Resveratrol improves cardiac function and exercise performance in MI-induced heart failure through the inhibition of cardiotoxic HETE metabolites. *Journal of Molecular and Cellular Cardiology*, *125*, 162–173. https://doi.org/10.1016/j.yjmcc.2018.10.023

Mattivi, F., Guzzon, R., Vrhovsek, U., Stefanini, M., & Velasco, R. (2006). Metabolite profiling of grape: Flavonols and anthocyanins. *Journal of Agricultural and Food Chemistry*, *54*(20), 7692–7702. https://doi.org/10.1021/jf061538c

Meng, J. F., Yu, Y., Shi, T. C., Fu, Y. S., Zhao, T., & Zhang, Z. W. (2019). Melatonin treatment of pre-veraison grape berries modifies phenolic components and antioxidant activity of grapes and wine. *Food Science and Technology*, *39*(1), 35–42. https://doi.org/10.1590/1678-457x.24517

Messerli, F. H., Williams, B., & Ritz, E. (2007). Essential hypertension. In *Lancet* (Vol. 370, Issue 9587, pp. 591–603). Elsevier. https://doi.org/10.1016/S0140-6736(07)61299-9

Miatello, R., Vázquez, M., Renna, N., Cruzado, M., Zumino, A. P., & Risler, N. (2005). Chronic administration of resveratrol prevents biochemical cardiovascular changes in fructose-fed rats. *American Journal of Hypertension*, *18*(6), 864–870. https://doi.org/10.1016/j.amjhyper.2004.12.012

Mills, K. T., Bundy, J. D., Kelly, T. N., Reed, J. E., Kearney, P. M., Reynolds, K., Chen, J., & He, J. (2019). Global disparities of hypertension prevalence and control. *Circulation*, *134*(6), 441–450. https://doi.org/10.1161/CIRCULATIONAHA.115.018912

Mirvish, S. S., Cardesa, A., Wallcave, L., & Shubik, P. (1975). Induction of mouse lung adenomas by amines or ureas plus nitrite and by N nitroso compounds: effect of ascorbate, gallic acid, thiocyanate, and caffeine. *Journal of the National Cancer Institute*, *55*(3), 633–636. https://doi.org/10.1093/jnci/55.3.633

Mizutani, K., Ikeda, K., Kawai, Y., & Yamori, Y. (2000). Resveratrol attenuates ovariectomy-induced hypertension and bone loss in stroke-prone spontaneously hypertensive rats. *Journal of Nutritional Science and Vitaminology*, *46*(2), 78–83. https://doi.org/10.3177/jnsv.46.78

Mokni, M., Hamlaoui-Guesmi, S., Amri, M., Marzouki, L., Limam, F., & Aouani, E. (2012). Grape seed and skin extract protects against acute chemotherapy toxicity induced

by doxorubicin in rat heart. *Cardiovascular Toxicology*, *12*(2), 158–165. https://doi.org/10.1007/s12012-012-9155-1

Morata, A., Gómez-Cordovés, M. C., Colomo, B., & Suárez, J. A. (2005). Cell wall anthocyanin adsorption by different Saccharomyces strains during the fermentation of Vitis vinifera L. cv Graciano grapes. *European Food Research and Technology*, *220*(3–4), 341–346. https://doi.org/10.1007/s00217-004-1053-8

Muntner, P., & Whelton, P. K. (2017). Using Predicted Cardiovascular Disease Risk in Conjunction With Blood Pressure to Guide Antihypertensive Medication Treatment. In *Journal of the American College of Cardiology* (Vol. 69, Issue 19, pp. 2446–2456). Elsevier USA. https://doi.org/10.1016/j.jacc.2017.02.066

Musee, N., Lorenzen, L., & Aldrich, C. (2007). Cellar waste minimization in the wine industry: a systems approach. *Journal of Cleaner Production*, *15*(5), 417–431. https://doi.org/10.1016/j.jclepro.2005.11.004

Nash, V., Ranadheera, C. S., Georgousopoulou, E. N., Mellor, D. D., Panagiotakos, D. B., McKune, A. J., Kellett, J., & Naumovski, N. (2018). The effects of grape and red wine polyphenols on gut microbiota – A systematic review. *Food Research International*, *113*(April), 277–287. https://doi.org/10.1016/j.foodres.2018.07.019

Nicoletti, I., Bello, C., De Rossi, A., & Corradini, D. (2008). Identification and quantification of phenolic compounds in grapes by HPLC-PDA-ESI-MS on a semimicro separation scale. *Journal of Agricultural and Food Chemistry*, *56*(19), 8801–8808. https://doi.org/10.1021/jf801411m

Nieto, J. A., Santoyo, S., Prodanov, M., Reglero, G., & Jaime, L. (2020). Valorisation of grape stems as a source of phenolic antioxidants by using a sustainable extraction methodology. *Foods*, *9*(5). https://doi.org/10.3390/foods9050604

Obreque-Slier, E., López-Solís, R., Castro-Ulloa, L., Romero-Díaz, C., & Peña-Neira, Á. (2012). Phenolic composition and physicochemical parameters of Carménère, Cabernet Sauvignon, Merlot and Cabernet Franc grape seeds (Vitis vinifera L.) during ripening. *LWT - Food Science and Technology*, *48*(1), 134–141. https://doi.org/10.1016/j.lwt.2012.02.007

Odai, T., Terauchi, M., Kato, K., Hirose, A., & Miyasaka, N. (2019). Effects of grape seed proanthocyanidin extract on vascular endothelial function in participants with prehypertension: A randomized, double-blind, placebo-controlled study. *Nutrients*, *11*(12). https://doi.org/10.3390/nu11122844

OIV, & International Organisation of Vine and Wine. (2019). 2019 Statistical Report on World Vitiviniculture. *2019 Statistical Report on World Vitiviniculture*, 23. https://doi.org/64/19/6835 [pii]\n10.1158/0008-5472.CAN-04-1678

Okolie, C. L., Akanbi, T. O., Mason, B., Udenigwe, C. C., & Aryee, A. N. A. (2019). Influence

of conventional and recent extraction technologies on physicochemical properties of bioactive macromolecules from natural sources: A review. In *Food Research International* (Vol. 116, pp. 827–839). Elsevier Ltd. https://doi.org/10.1016/j.foodres.2018.09.018

Pantelić, M., Dabić Zagorac, D., Natić, M., Gašić, U., Jović, S., Vujović, D., & Popović Djordjević, J. (2016). Impact of clonal variability on phenolics and radical scavenging activity of grapes and wines: A study on the recently developed Merlot and Cabernet Franc clones (Vitis vinifera L.). *PLoS ONE*, *11*(10), e0163823. https://doi.org/10.1371/journal.pone.0163823

Pantelić, M. M., Dabić Zagorac, D., Davidović, S. M., Todić, S. R., Bešlić, Z. S., Gašić, U. M., Tešić, Ž. L., & Natić, M. M. (2016). Identification and quantification of phenolic compounds in berry skin, pulp, and seeds in 13 grapevine varieties grown in Serbia. *Food Chemistry*, *211*, 243–252. https://doi.org/10.1016/j.foodchem.2016.05.051

Park, E., Edirisinghe, I., Choy, Y. Y., Waterhouse, A., & Burton-Freeman, B. (2016). Effects of grape seed extract beverage on blood pressure and metabolic indices in individuals with pre-hypertension: A randomised, double-blinded, two-arm, parallel, placebo-controlled trial. *British Journal of Nutrition*, *115*(2), 226–238. https://doi.org/10.1017/S0007114515004328

Pasini, F., Chinnici, F., Caboni, M. F., & Verardo, V. (2019). Recovery of oligomeric proanthocyanidins and other phenolic compounds with established bioactivity from grape seed by-products. *Molecules*, *24*(4), 677. https://doi.org/10.3390/molecules24040677

Pea-Neira, A., Cceres, A., & Pastenes, C. (2007). Low molecular weight phenolic and anthocyanin composition of grape skins from cv. Syrah (Vitis vinifera L.) in the Maipo Valley (Chile): Effect of clusters thinning and vineyard yield. *Food Science and Technology International*, *13*(2), 153–158. https://doi.org/10.1177/1082013207077920

Pechánová, O., Bernátová, I., Babál, P., Martínez, M. C., Kyselá, S., Stvrtina, S., & Andriantsitohaina, R. (2004). Red wine polyphenols prevent cardiovascular alterations in L-NAME-induced hypertension. *Journal of Hypertension*, *22*(8), 1551–1559. https://doi.org/10.1097/01.hjh.0000133734.32125.c7

Peng, R.-M., Lin, G.-R., Ting, Y., & Hu, J.-Y. (2018). Oral delivery system enhanced the bioavailability of stilbenes: Resveratrol and pterostilbene. *BioFactors*, *44*(1), 5–15. https://doi.org/10.1002/biof.1405

Pereira, G. E., Padhi, E. M. T., Girardello, R. C., Medina-Plaza, C., Tseng, D., Bruce, R. C., Erdmann, J. N., Kurtural, S. K., Slupsky, C. M., & Oberholster, A. (2020). Trunk Girdling Increased Stomatal Conductance in Cabernet Sauvignon Grapevines, Reduced Glutamine, and Increased Malvidin-3-Glucoside and Quercetin-3-

Glucoside Concentrations in Skins and Pulp at Harvest. *Frontiers in Plant Science*, *11*. https://doi.org/10.3389/fpls.2020.00707

Pereira, G. E., Padhi, E. M. T., Sudarshana, M. R., Fialho, F. B., Medina-Plaza, C., Girardello, R. C., Tseng, D., Bruce, R. C., Erdmann, J. N., Slupsky, C. M., & Oberholster, A. (2020). Impact of grapevine red blotch disease on primary and secondary metabolites in 'Cabernet Sauvignon' grape tissues. *Food Chemistry*, *342*, 128312. https://doi.org/10.1016/j.foodchem.2020.128312

Pérez-Álvarez, E. P., Martínez-Vidaurre, J. M., & Garde-Cerdán, T. (2019). Anthocyanin composition of grapes from three different soil types in cv. Tempranillo A.O.C. Rioja vineyards. *Journal of the Science of Food and Agriculture*, *99*(10), 4833–4841. https://doi.org/10.1002/jsfa.9741

Pérez-Serradilla, J. A., & de Castro, M. D. L. (2008). Role of lees in wine production: A review. In *Food Chemistry* (Vol. 111, Issue 2, pp. 447–456). Elsevier. https://doi.org/10.1016/j.foodchem.2008.04.019

Pérez-Serradilla, J. A., & Luque de Castro, M. D. (2011). Microwave-assisted extraction of phenolic compounds from wine lees and spray-drying of the extract. *Food Chemistry*, *124*(4), 1652–1659. https://doi.org/10.1016/j.foodchem.2010.07.046

Pimentel-Moral, S., Cádiz-Gurrea, M. de la L., Rodríguez-Pérez, C., & Segura-Carretero, A. (2020). Recent advances in extraction technologies of phytochemicals applied for the revaluation of agri-food by-products. In *Functional and Preservative Properties of Phytochemicals* (pp. 209–239). Elsevier. https://doi.org/10.1016/b978-0-12-818593-3.00007-5

Pons, Z., Guerrero, L., Margalef, M., Arola, L., Arola-Arnal, A., & Muguerza, B. (2014). Effect of low molecular grape seed proanthocyanidins on blood pressure and lipid homeostasis in cafeteria diet-fed rats. *Journal of Physiology and Biochemistry*, *70*(2), 629–637. https://doi.org/10.1007/s13105-014-0329-0

Pons, Z., Margalef, M., Bravo, F. I., Arola-Arnal, A., & Muguerza, B. (2016a). Acute administration of single oral dose of grape seed polyphenols restores blood pressure in a rat model of metabolic syndrome: role of nitric oxide and prostacyclin. *European Journal of Nutrition*, *55*(2), 749–758. https://doi.org/10.1007/s00394-015-0895-0

Pons, Z., Margalef, M., Bravo, F. I., Arola-Arnal, A., & Muguerza, B. (2016b). Grape seed flavanols decrease blood pressure via Sirt-1 and confer a vasoprotective pattern in rats. *Journal of Functional Foods*, *24*, 164–172. https://doi.org/10.1016/j.jff.2016.03.030

Pons, Z., Margalef, M., Bravo, F. I., Arola-Arnal, A., & Muguerza, B. (2017). Chronic administration of grape-seed polyphenols attenuates the development of hypertension and improves other cardiometabolic risk factors associated with the

metabolic syndrome in cafeteria diet-fed rats. *British Journal of Nutrition*, *117*(2), 200–208. https://doi.org/10.1017/S0007114516004426

Portu, J., López, R., Baroja, E., Santamaría, P., & Garde-Cerdán, T. (2016). Improvement of grape and wine phenolic content by foliar application to grapevine of three different elicitors: Methyl jasmonate, chitosan, and yeast extract. *Food Chemistry*, *201*, 213–221. https://doi.org/10.1016/j.foodchem.2016.01.086

Portu, J., López, R., Santamaría, P., & Garde-Cerdán, T. (2017). Elicitation with methyl jasmonate supported by precursor feeding with phenylalanine: Effect on Garnacha grape phenolic content. *Food Chemistry*, *237*, 416–422. https://doi.org/10.1016/j.foodchem.2017.05.126

Portu, J., Santamaría, P., López-Alfaro, I., López, R., & Garde-Cerdán, T. (2015). Methyl jasmonate foliar application to tempranillo vineyard improved grape and wine phenolic content. *Journal of Agricultural and Food Chemistry*, *63*(8), 2328–2337. https://doi.org/10.1021/jf5060672

Puri, M., Sharma, D., & Barrow, C. J. (2012). Enzyme-assisted extraction of bioactives from plants. In *Trends in Biotechnology* (Vol. 30, Issue 1, pp. 37–44). Elsevier Current Trends. https://doi.org/10.1016/j.tibtech.2011.06.014

Quiñones, M., Guerrero, L., Fernández-Vallinas, S., Pons, Z., Arola, L., Aleixandre, A., & Muguerza, B. (2014). Involvement of nitric oxide and prostacyclin in the antihypertensive effect of low-molecular-weight procyanidin rich grape seed extract in male spontaneously hypertensive rats. *Journal of Functional Foods*, *6*(1), 419–427. https://doi.org/10.1016/j.jff.2013.11.008

Quiñones, M., Guerrero, L., Suarez, M., Pons, Z., Aleixandre, A., Arola, L., & Muguerza, B. (2013). Low-molecular procyanidin rich grape seed extract exerts antihypertensive effect in males spontaneously hypertensive rats. *Food Research International*, *51*(2), 587–595. https://doi.org/10.1016/j.foodres.2013.01.023

Quiñones, Mar, Margalef, M., Arola-Arnal, A., Muguerza, B., Miguel, M., & Aleixandre, A. (2015). The blood pressure effect and related plasma levels of flavan-3-ols in spontaneously hypertensive rats. *Food and Function*, *6*(11), 3479–3489. https://doi.org/10.1039/c5fo00547g

Rafe, T., Shawon, P. A., Salem, L., Chowdhury, N. I., Kabir, F., Bin Zahur, S. M., Akhter, R., Noor, H. B., Mohib, M. M., & Sagor, M. A. T. (2019). Preventive Role of Resveratrol Against Inflammatory Cytokines and Related Diseases. *Current Pharmaceutical Design*, *25*(12), 1345–1371. https://doi.org/10.2174/1381612825666190410153307

Ramar, M., Manikandan, B., Raman, T., Priyadarsini, A., Palanisamy, S., Velayudam, M., Munusamy, A., Marimuthu Prabhu, N., & Vaseeharan, B. (2012). Protective effect of ferulic acid and resveratrol against alloxan-induced diabetes in mice. *European*

Journal of Pharmacology, *690*(1–3), 226–235. https://doi.org/10.1016/j.ejphar.2012.05.019

Rasines-Perea, Z., Ky, I., Cros, G., Crozier, A., & Teissedre, P.-L. (2018). Grape Pomace: Antioxidant Activity, Potential Effect Against Hypertension and Metabolites Characterization after Intake. *Diseases*, *6*(3), 60. https://doi.org/10.3390/diseases6030060

Rasines-Perea, Z., & Teissedre, P. L. (2017). Grape Polyphenols' effects in human cardiovascular diseases and diabetes. In *Molecules* (Vol. 22, Issue 1). MDPI AG. https://doi.org/10.3390/molecules22010068

Reaux, A., Fournie-Zaluski, M. C., David, C., Zini, S., Roques, B. P., Corvol, P., & Llorens-Cortes, C. (1999). Aminopeptidase A inhibitors as potential central antihypertensive agents. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(23), 13415–13420. https://doi.org/10.1073/pnas.96.23.13415

Ren, J., An, J., Chen, M., Yang, H., & Ma, Y. (2021). Effect of proanthocyanidins on blood pressure: A systematic review and meta-analysis of randomized controlled trials. In *Pharmacological Research* (Vol. 165, p. 105329). Academic Press. https://doi.org/10.1016/j.phrs.2020.105329

Resende, A. C., Emiliano, A. F., Cordeiro, V. S. C., de Bem, G. F., de Cavalho, L. C. R. M., de Oliveira, P. R. B., Neto, M. L., Costa, C. A., Boaventura, G. T., & de Moura, R. S. (2013). Grape skin extract protects against programmed changes in the adult rat offspring caused by maternal high-fat diet during lactation. *Journal of Nutritional Biochemistry*, *24*(12), 2119–2126. https://doi.org/10.1016/j.jnutbio.2013.08.003

Rio, S., Atsushi, K., Ryusuke, H., Takashi, H., & Yoshiyuki, H. (2018). Resveratrol Decreases Oxidative Stress by Restoring Mitophagy and Improves the Pathophysiology of Dystrophin-Deficient mdx Mice. *Oxidative Medicine and Cellular Longevity*, *2018*, 9179270. https://doi.org/10.1155/2018/9179270

Rockenbach, I. I., Jungfer, E., Ritter, C., Santiago-Schübel, B., Thiele, B., Fett, R., & Galensa, R. (2012). Characterization of flavan-3-ols in seeds of grape pomace by CE, HPLC-DAD-MS n and LC-ESI-FTICR-MS. *Food Research International*, *48*(2), 848–855. https://doi.org/10.1016/j.foodres.2012.07.001

Rodrigo, R., Brito, R., Castillo, G., González, J., & Valls, N. (2015). Oxidative Stress in Hypertension: Mechanisms and Therapeutic Opportunities. *Oxidative Stress in Hypertension … Exp Clin Endocrinol Diabetes*, *123*, 325–335. https://doi.org/10.1055/s-0035-1548765

Rodríguez-Morgado, B., Candiracci, M., Santa-María, C., Revilla, E., Gordillo, B., Parrado, J., & Castaño, A. (2015). Obtaining from Grape Pomace an Enzymatic Extract with Anti-inflammatory Properties. *Plant Foods for Human Nutrition*, *70*(1), 42–49.

https://doi.org/10.1007/s11130-014-0459-0

Rodríguez-Pérez, C., García-Villanova, B., Guerra-Hernández, E., & Verardo, V. (2019). Grape seeds proanthocyanidins: An overview of in vivo bioactivity in animal models. In *Nutrients* (Vol. 11, Issue 10). MDPI AG. https://doi.org/10.3390/nu11102435

Rodríguez Montealegre, R., Romero Peces, R., Chacón Vozmediano, J. L., Martínez Gascueña, J., & García Romero, E. (2006). Phenolic compounds in skins and seeds of ten grape Vitis vinifera varieties grown in a warm climate. *Journal of Food Composition and Analysis*, *19*(6–7), 687–693. https://doi.org/10.1016/j.jfca.2005.05.003

Romero-Díez, R., Matos, M., Rodrigues, L., Bronze, M. R., Rodríguez-Rojo, S., Cocero, M. J., & Matias, A. A. (2019). Microwave and ultrasound pre-treatments to enhance anthocyanins extraction from different wine lees. *Food Chemistry*, *272*, 258–266. https://doi.org/10.1016/j.foodchem.2018.08.016

Romero-Díez, R., Rodríguez-Rojo, S., Cocero, M. J., Duarte, C. M. M., Matias, A. A., & Bronze, M. R. (2018). Phenolic characterization of aging wine lees: Correlation with antioxidant activities. *Food Chemistry*, *259*, 188–195. https://doi.org/10.1016/j.foodchem.2018.03.119

Roth, G. A., Abate, D., Abate, K. H., Abay, S. M., Abbafati, C., Abbasi, N., Abbastabar, H., Abd-Allah, F., Abdela, J., Abdelalim, A., Abdollahpour, I., Abdulkader, R. S., Abebe, H. T., Abebe, M., Abebe, Z., Abejie, A. N., Abera, S. F., Abil, O. Z., Abraha, H. N., … Murray, C. J. L. (2018). Global, regional, and national age-sex-specific mortality for 282 causes of death in 195 countries and territories, 1980–2017: a systematic analysis for the Global Burden of Disease Study 2017. *The Lancet*, *392*(10159), 1736–1788. https://doi.org/10.1016/S0140-6736(18)32203-7

Sahebkar, A., Serban, C., Ursoniu, S., Wong, N. D., Muntner, P., Graham, I. M., Mikhailidis, D. P., Rizzo, M., Rysz, J., Sperling, L. S., Lip, G. Y. H., & Banach, M. (2015). Lack of efficacy of resveratrol on C-reactive protein and selected cardiovascular risk factors - Results from a systematic review and meta-analysis of randomized controlled trials. *International Journal of Cardiology*, *189*(1), 47–55. https://doi.org/10.1016/j.ijcard.2015.04.008

Samadian, F., Dalili, N., & Jamalian, A. (2016). Lifestyle modifications to prevent and control hypertension. *Iranian Journal of Kidney Diseases*, *10*(5), 237–263.

Sarr, M., Chataigneau, M., Martins, S., Schott, C., El Bedoui, J., Oak, M. H., Muller, B., Chataigneau, T., & Schini-Kerth, V. B. (2006). Red wine polyphenols prevent angiotensin II-induced hypertension and endothelial dysfunction in rats: Role of NADPH oxidase. *Cardiovascular Research*, *71*(4), 794–802. https://doi.org/10.1016/j.cardiores.2006.05.022

Schini-Kerth, V. B., Auger, C., Kim, J. H., Étienne-Selloum, N., & Chataigneau, T. (2010). Nutritional improvement of the endothelial control of vascular tone by polyphenols: Role of NO and EDHF. In *Pflugers Archiv European Journal of Physiology* (Vol. 459, Issue 6, pp. 853–862). Pflugers Arch. https://doi.org/10.1007/s00424-010-0806-4

Serban, M. C., Sahebkar, A., Zanchetti, A., Mikhailidis, D. P., Howard, G., Antal, D., Andrica, F., Ahmed, A., Aronow, W. S., Muntner, P., Lip, G. Y. H., Graham, I., Wong, N., Rysz, J., & Banach, M. (2016). Effects of Quercetin on Blood Pressure: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. *Journal of the American Heart Association*, *5*(7). https://doi.org/10.1161/JAHA.115.002713

Shrikhande, A. J. (2000). Wine by-products with health benefits. In *Food Research International* (Vol. 33, Issue 6, pp. 469–474). Elsevier Ltd. https://doi.org/10.1016/S0963-9969(00)00071-5

Sivaprakasapillai, B., Edirisinghe, I., Randolph, J., Steinberg, F., & Kappagoda, T. (2009). Effect of grape seed extract on blood pressure in subjects with the metabolic syndrome. *Metabolism: Clinical and Experimental*, *58*(12), 1743–1746. https://doi.org/10.1016/j.metabol.2009.05.030

Snopek, L., Mlcek, J., Sochorova, L., Baron, M., Hlavacova, I., Jurikova, T., Kizek, R., Sedlackova, E., & Sochor, J. (2018). Contribution of red wine consumption to human health protection. *Molecules*, *23*(7), 1–16. https://doi.org/10.3390/molecules23071684

Sochorova, L., Prusova, B., Cebova, M., Jurikova, T., Mlcek, J., Adamkova, A., Nedomova, S., Baron, M., & Sochor, J. (2020). Health Effects of Grape Seed and Skin Extracts and Their Influence on Biochemical Markers. In *Molecules (Basel, Switzerland)* (Vol. 25, Issue 22). NLM (Medline). https://doi.org/10.3390/molecules25225311

Sovak, M. (2001). Grape extract, resveratrol, and its analogs: A review. *Journal of Medicinal Food*, *4*(2), 93–105. https://doi.org/10.1089/109662001300341752

Sturrock, E. D., Lubbe, L., Cozier, G. E., Schwager, S. L. U., Arowolo, A. T., Arendse, L. B., Belcher, E., & Acharya, K. R. (2019). Structural basis for the C-domain-selective angiotensin-converting enzyme inhibition by bradykinin-potentiating peptide b (BPPb). *Biochemical Journal*, *476*(10), 1553–1570. https://doi.org/10.1042/BCJ20190290

Subramanian, K. R. (2018). *The Crisis of Consumption of Natural Resources SUSTAINING GLOBALIZATION IN AN ENVIRONMENT OF RISING NATIONALISM View project*. www.ijriar.com

Teixeira, A., Baenas, N., Dominguez-Perles, R., Barros, A., Rosa, E., Moreno, D. A., & Garcia-Viguera, C. (2014). Natural bioactive compounds from winery by-products as health promoters: A review. In *International Journal of Molecular Sciences* (Vol.

15, Issue 9, pp. 15638–15678). MDPI AG. https://doi.org/10.3390/ijms150915638

Thandapilly, S. J., LeMaistre, J. L., Louis, X. L., Anderson, C. M., Netticadan, T., & Anderson, H. D. (2012). Vascular and Cardiac Effects of Grape Powder in the Spontaneously Hypertensive Rat. *American Journal of Hypertension*, *25*(10), 1070–1076. https://doi.org/10.1038/ajh.2012.98

Thomopoulos, C., Parati, G., & Zanchetti, A. (2014). Effects of blood pressure lowering on outcome incidence in hypertension.1. Overview, meta-analyses, and meta-regression analyses of randomized trials. In *Journal of Hypertension* (Vol. 32, Issue 12, pp. 2285–2295). Lippincott Williams and Wilkins. https://doi.org/10.1097/HJH.0000000000000378

Tomiyama, J. M., Takagi, D., & Kantar, M. B. (2020). The effect of acute and chronic food shortage on human population equilibrium in a subsistence setting. *Agriculture and Food Security*, *9*(1), 6. https://doi.org/10.1186/s40066-020-00261-x

Trigo, J. P., Alexandre, E. M. C., Saraiva, J. A., & Pintado, M. E. (2020). High value-added compounds from fruit and vegetable by-products–Characterization, bioactivities, and application in the development of novel food products. In *Critical Reviews in Food Science and Nutrition* (Vol. 60, Issue 8, pp. 1388–1416). Taylor and Francis Inc. https://doi.org/10.1080/10408398.2019.1572588

Unger, T., Borghi, C., Charchar, F., Khan, N. A., Poulter, N. R., Prabhakaran, D., Ramirez, A., Schlaich, M., Stergiou, G. S., Tomaszewski, M., Wainford, R. D., Williams, B., & Schutte, A. E. (2020). 2020 International Society of Hypertension Global Hypertension Practice Guidelines. *Hypertension*, *75*(6), 1334–1357. https://doi.org/10.1161/HYPERTENSIONAHA.120.15026

Urquiaga, I., D'Acuña, S., Pérez, D., Dicenta, S., Echeverría, G., Rigotti, A., & Leighton, F. (2015). Wine grape pomace flour improves blood pressure, fasting glucose and protein damage in humans: A randomized controlled trial. *Biological Research*, *48*(1), 49. https://doi.org/10.1186/s40659-015-0040-9

Visioli, F., Panaite, S. A., & Tomé-Carneiro, J. (2020). Wine's phenolic compounds and health: A pythagorean view. In *Molecules* (Vol. 25, Issue 18). MDPI AG. https://doi.org/10.3390/molecules25184105

Wallace, T. C. (2011). Anthocyanins in cardiovascular disease. In *Advances in Nutrition* (Vol. 2, Issue 1, pp. 1–7). Adv Nutr. https://doi.org/10.3945/an.110.000042

Wang, X., Ouyang, Y. Y., Liu, J., & Zhao, G. (2014). Flavonoid intake and risk of CVD: A systematic review and meta-analysis of prospective cohort studies. In *British Journal of Nutrition* (Vol. 111, Issue 1, pp. 1–11). Cambridge University Press. https://doi.org/10.1017/S000711451300278X

Wang, Y., He, Y. N., Chen, W. K., He, F., Chen, W., Cai, X. D., Duan, C. Q., & Wang, J. (2018). Effects of cluster thinning on vine photosynthesis, berry ripeness and

flavonoid composition of Cabernet Sauvignon. *Food Chemistry*, *248*, 101–110. https://doi.org/10.1016/j.foodchem.2017.12.021

Weir, M. (1999). The renin-angiotensin-aldosterone system: a specific target for hypertension management. *American Journal of Hypertension*, *12*(4), 205–213. https://doi.org/10.1016/s0895-7061(99)00103-x

WHO. (2019). *Hypertension*. https://www.who.int/news-room/fact-sheets/detail/hypertension

Wightman, J. D., & Heuberger, R. A. (2015). Effect of grape and other berries on cardiovascular health. *Journal of the Science of Food and Agriculture*, *95*(8), 1584–1597. https://doi.org/10.1002/jsfa.6890

Yang, L. P., Ling, W. H., Du, Z. C., Chen, Y. M., Li, D., Deng, S. Z., Liu, Z. M., & Yang, L. L. (2017). Effects of anthocyanins on cardiometabolic health: A systematic review and meta-analysis of randomized controlled trials. In *Advances in Nutrition* (Vol. 8, Issue 5, pp. 684–693). American Society for Nutrition. https://doi.org/10.3945/an.116.014852

Ye, J., Chen, X., Bao, L., & Tarantino, G. (2019). Effects of wine on blood pressure, glucose parameters, and lipid profile in type 2 diabetes mellitus: A meta-analysis of randomized interventional trials (PRISMA Compliant). *Medicine (United States)*, *98*(23). https://doi.org/10.1097/MD.0000000000015771

Yilmaz, Y., & Toledo, R. T. (2004). Major Flavonoids in Grape Seeds and Skins: Antioxidant Capacity of Catechin, Epicatechin, and Gallic Acid. *Journal of Agricultural and Food Chemistry*, *52*(2), 255–260. https://doi.org/10.1021/jf030117h

Zanchetti, A. (2016). Hypertension: Lower or higher blood-pressure targets for high-risk patients? In *Nature Reviews Cardiology* (Vol. 13, Issue 11, pp. 637–638). Nature Publishing Group. https://doi.org/10.1038/nrcardio.2016.165

Zhang, H., Liu, S., Li, L., Liu, S., Liu, S., Mi, J., & Tian, G. (2016). The impact of grape seed extract treatment on blood pressure changes: A meta-analysis of 16 randomized controlled trials. In *Medicine (United States)* (Vol. 95, Issue 33). Lippincott Williams and Wilkins. https://doi.org/10.1097/MD.0000000000004247

Zhao, C. N., Meng, X., Li, Y., Li, S., Liu, Q., Tang, G. Y., & Li, H. Bin. (2017). Fruits for prevention and treatment of cardiovascular diseases. In *Nutrients* (Vol. 9, Issue 6). MDPI AG. https://doi.org/10.3390/nu9060598

Zhu, F., Hu, J., Lv, F., Cheng, P., & Gao, S. (2018). Effects of oligomeric grape seed proanthocyanidins on *L*- NAME - induced hypertension in pregnant mice: Role of oxidative stress and endothelial dysfunction. *Phytotherapy Research*, *32*(9), 1836–1847. https://doi.org/10.1002/ptr.6119

TABLES

Table 1. Phenolic composition of different grape varieties.

	Compound	Grape variety					References
		Garnacha (mg/kg)	Cabernet (mg/kg)	Tempranillo (mg/kg)	Merlot (mg/kg)	Syrah (mg/kg)	
Flavanols	Catechin	13.1-1365.7	7.4-164.7	27.1-240.2	17-601	13.7-29.3	Y. Fang et al., 2011; Figueiredo-Gonzalez et al., 2013; Gabaston et al., 2020; Garde-Cerdán et al., 2021; Gómez-Plaza, et al., 2017; Gutiérrez-Gamboa et al., 2020, 2019; Iglesias-Carres et al., 2019, 2018; Kammerer, et al., 2004; Lingua, et al., 2016; Mattivi, et al., 2019; Nicoletti, et al., 2008; Obreque-Slier, et al., 2012; M. Pantelić, et al., 2016; M. Pantelić, et al., 2016; Pea-Neira, et al., 2007; Pereira, Padhi, Girardello, et al., 2020; Pereira, Padhi, Sudarshana, et al., 2020;
	Epicatechin	7.2-430.6	25.0-1067.4	17.1-353.6	1.56-980	10.3-74.8	
	Catechin gallate	44.6-85.4	2.1-38.9	-	4.62-29	21.2	
	Epicatechin gallate	0.1-34.5	2.4-75.6	9.6-30.5	0.4-2.6	3.9-25.4	
	Procyanidin B2	27.3-319.4	15.8-285.1	5.1-18.3	280-366.7	-	
	Procyanidin dimer	240.2-340.5	73.2-432.4	-	147.0-331.8	13.1	
Flavonols	Quercetin	1.4-1.5	23.4-45.1	7.1-77.0	12.2	0.2	
	Quercetin-3-O-glucoside	4.4-471.6	5.4-19.2	6.2-24.3	2.3-45.8	3.9-10.9	
	Kaempferol-3-O-glucoside	1.2-57.2	0.5-76.8	0.8-8.5	0.3-97.7	0.1-0.8	
	Isorhamnetin-3-O-glucoside	3.5-76.7	5.7-34.5	0.4-2.6	0.3-1.1	0.9-3.1	
	Rutin	20.0-25.2	19.3-87.5	-	5.1-89.6	0.1-1.3	
	Kaempferol	-	2.1-6.5	3.1-4.5	3.4	0.1	
Phenolic acids	Gallic acid	2.5-19.3	0.1-216.8	0.4-9.2	3.4-153.8	0.6-89.6	
	Protocatechuid acid	11.1-15.8	-	-	0.1-329.7	0.1-1.0	
	p-Coumaric acid	0.4-0.9	5.1-18.3	2.7-0.9	0.2-6.1	-	
	Caffeic acid	0.6	9.6-28.4	0.2-0.8	0.2-5.3	0.4-1.2	
	Ferrulic acid	4.4-5.6	16.9-30.7	-	4.7-10.7	0.4-3.4	
	Hydroxybenzoic acid		2.3-18.9	-	2.1-13.7	0.2-9.0	

	Benzoic acid	7.5-9.6	2.2-4	-	2.2	-	Pérez-Álvarez, et al., 2019; Portu, et al., 2016; Portu, et al., 2017; Portu, et al., 2015; Rodríguez Montealegre, et al., 2006; Sochorova et al., 2020; Wang et al., 2018
Stilbenes	Resveratrol	4.5-7.8	0.2-0.4	-	-	-	
	Resveratrol-O-glucoside	17.1-151.7	-	-	-	-	
	trans-resveratrol	-	0.2-1.0	0.1	7.3-9.2	0.1	
	Picceatanol	-	0.7-5.3	0.32-1.60	26.3	-	
Anthocyanins	Malvidin-3-O-glucoside	384.1-1025.2	152.7-809.7	298.5-729.4	204.4-1065.0	324.2-963.7	
	Cyanidin-3-O-glucoside	6.6-48.3	4.2-38.5	23.7-84.3	14.7-74.8	9.1-21.5	
	Delphinidin-3-O-glucoside	24.9-199.2	34.0-267.4	126.2-354.0	47.7-177.9	33.0-148.5	
	Petunidin-3-O-glucoside	30.4-312.5	25.2-154.4	93.1-242.2	40.4-172.8	64.8-158.3	
	Peonidin-3-O-glucoside	43.4-733.3	23.6-126.8	44.0-115.7	81.3-245.1	56.3-192.4	
	Malvidin-O-coumaroylglucoside	5.3-831.2	2.2-20.6	7.1-209.8	57.5-409.8	128.8-637.3	
	Peonidin-O-acetyl glucoside	3.0-57.2	6.9-36.6	0.2-6.3	17.1-82.9	31.6-68.2	
	Malvidin-O-acetyl glucoside	11.9-49.4	60.5-338.2	5.8-35.4	80.1-602.8	146.9-457.2	

Table 2. Phenolic profile, identification and quantification in wine lees.

	Compound	Identification method	Quantification	Ref.
Flavanols	Catechin	HPLC-MS/MS	121 ± 6 µg/mL	(Caro et al., 2017b)
		UHPLC-Q-TOF-MS	39.27-97.63 mg/mL	(López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021)
	Catechin gallate	UHPCL-Q-TOF-MS	0.79-1.53 mg/mL	(López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021)
	Epicatechin	LC–QqTOF-MS/MS	n.q.	(Delgado De La Torre et al., 2015)
		UHPLC-Q-TOF-MS	12.94-43.48 mg/mL	(López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021)
	(Epi)catechin O-glucoside iso1	UHPLC-Q-TOF-MS	0.50-0.90 mg/mL	(López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021)
	(Epi)catechin O-glucoside iso1	UHPLC-Q-TOF-MS	0.29-0.43 mg/mL	
	(Epi)catechin O-glucoside iso1	UHPLC-Q-TOF-MS	0.77-1.64 mg/mL	
	Procyanidin B2	LC–QqTOF-MS/MS	n.q.	(Delgado De La Torre et al., 2015)
	Procyanidin dimer B2	UHPLC-Q-TOF-MS	9.61-34.60 mg/mL	(López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021)
	Procyanidin dimer iso1	UHPLC-Q-TOF-MS	32.11-64.21 mg/mL	
	Procyanidin dimer iso2	UHPLC-Q-TOF-MS	4.19-14.26 mg/mL	
	Procyanidin dimer iso3	UHPLC-Q-TOF-MS	0.84-2.96 mg/mL	
	Procyanidin dimer iso4	UHPLC-Q-TOF-MS	2.71-12.80 mg/mL	
	Procyanidin dimer iso5	UHPLC-Q-TOF-MS	1.54-4.32 mg/mL	
	Procyanidin trimer iso1	UHPLC-Q-TOF-MS	5.14-16.28 mg/mL	
	Procyanidin trimer iso2	UHPLC-Q-TOF-MS	4.62-14.53 mg/mL	
	Procyanidin trimer iso3	UHPLC-Q-TOF-MS	2.37-6.11 mg/mL	
	Procyanidin trimer iso4	UHPLC-Q-TOF-MS	1.19-3.22 mg/mL	
	Procyanidin trimer iso5	UHPLC-Q-TOF-MS	2.70-13.79 mg/mL	

Flavonols	Quercetin	HPLC	620 ± 42 mg/kg	(Landeka Jurčević et al., 2017)
		HPLC-DAD-MS	n.q.	(Giacobbo et al., 2019)
		HPLC-MS/MS	0.85-1.42 mg/g	(Dujmić et al., 2020)
		UHPLC-Q-TOF-MS	25.35-36.78 mg/mL	(López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021)
		HPLC-MS/MS	1216 ± 61 µg/mL	(Caro et al., 2017b)
	Quercetin-3-O-glucuronide	HPLC-DAD-MS	n.q.	(Giacobbo et al., 2019)
		UHPLC-Q-TOF-MS	2.42-4.86 mg/mL	(López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021)
	Quercetin-3-O-glucoside	UHPLC-Q-TOF-MS	1.63-7.68 mg/mL	
	Kaempferol	HPLC	59 ± 6 mg/kg	(Landeka Jurčević et al., 2017)
		HPLC-MS/MS	0.04-0.17 mg/g	(Dujmić et al., 2020)
		UHPLC-Q-TOF-MS	1.63-9.35 mg/mL	(López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021)
	Kaempferol-3-O-galactoside	HPLC-DAD-MS	n.q.	(Giacobbo et al., 2019)
	Kaempferol-3-O-glucuronide	UHPLC-Q-TOF-MS	2.42-5.62 mg/mL	(López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021)
	Myricetin	HPLC-DAD-MS	n.q.	(Giacobbo et al., 2019)
	Myricetin-3-O-glucoside	HPLC-DAD-MS	n.q.	
	Rutin	HPLC-MS/MS	2640 ± 132 µg/mL	(Caro et al., 2017b)
	Isorhamnetin	UHPLC-Q-TOF-MS	4.92-11.16 mg/mL	(López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021)
Phenolic acids	Gallic acid	HPLC-DAD-MS	n.q.	(Giacobbo et al., 2019)
		HPLC	620 ± 42 mg/kg	(Landeka Jurčević et al., 2017)
		UHPLC-Q-TOF-MS	96.11-121.19 mg/mL	(López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021)
	Caffeic acid	HPLC	128 ± 14 mg/kg	(Landeka Jurčević et al., 2017)
		UHPLC-Q-TOF-MS	1.09-3.27 mg/mL	(López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021)
	Caffeic acid O-glucoside iso1	UHPLC-Q-TOF-MS	0.13-1.21 mg/mL	

	Caffeic acid O-glucoside iso2	UHPLC-Q-TOF-MS	0.20-1.13 mg/mL	
	p-Coumaric acid	HPLC	158 ± 17 mg/kg	(Landeka Jurčević et al., 2017)
		UHPLC-Q-TOF-MS	1.15-3.73 mg/mL	(López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021)
	Egallic acid	HPLC	219 ± 23 mg/kg	(Landeka Jurčević et al., 2017)
	Chlorogenic acid	HPLC	82 ± 9 mg/kg	
	4-Hydroxybenzoic acid	UHPLC-Q-TOF-MS	0.89-1.67 mg/mL	(López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021)
	Ferulic acid	UHPLC-Q-TOF-MS	0.27-0.75 mg/mL	
	Vanillic acid	UHPLC-Q-TOF-MS	2.15-2.67 mg/mL	
Stilbenes	trans-resveratrol	HPLC-MS/MS	42 ± 2 µg/mL	(Caro et al., 2017b)
		HPLC-MS/MS	0.03-0.13 mg/g	(Dujmić et al., 2020)
		UHPLC-Q-TOF-MS	3.12-4.60 mg/mL	(López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021)
	Resveratrol iso1	UHPLC-Q-TOF-MS	0.97-2.95 mg/mL	(López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021)
	Resveratrol O-glucoside iso1	UHPLC-Q-TOF-MS	0.27-1.22 mg/mL	
	Resveratrol O-glucoside iso2	UHPLC-Q-TOF-MS	1.35-6.01 mg/mL	
	trans-resveratrol-3-O-glucoside	HPLC-MS/MS	0.01-0.78 mg/g	(Dujmić et al., 2020)
	Piceatannol	UHPLC-Q-TOF-MS	4.04-4.96 mg/mL	(López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021)
	Piceatannol 3-O-glucoside iso1	UHPLC-Q-TOF-MS	0.04-0.22 mg/mL	
	Piceatannol 3-O-glucoside iso2	UHPLC-Q-TOF-MS	1.35-6.01 mg/mL	
	Viniferin-iso1	UHPLC-Q-TOF-MS	0.15-0.33 mg/mL	
	Viniferin-iso2	UHPLC-Q-TOF-MS	0.81-1.05 mg/mL	
	Delphinidin-3-O-glucoside	LC-MS/MS	n.q.	(Romero-Díez et al., 2019)
		UHPLC-Q-TOF-MS	1.39-3.69 mg/mL	(López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021)

Anthocyanins	Delphinidin-3-O-(6''-p-acetyl-glucoside)	LC-MS/MS	n.q.	(Romero-Díez et al., 2019)
	Delphinidin-(6-acetyl)-3-glucoside	UHPLC-Q-TOF-MS	0.02-0.91 mg/mL	(López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021)
	Delphinidin-3-O-(6''-p-coumaroyl-glucoside)	LC-MS/MS	n.q.	(Romero-Díez et al., 2019)
	Delphinidin-3-(6-coumaroyl)-hexoside	HPLC-DAD-MS	n.q.	(Giacobbo et al., 2019)
	Delphinidin-(6-coumaroyl)-3-glucoside	UHPLC-Q-TOF-MS	0.09-0.55 mg/mL	(López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021)
	Delphinidin-3-hexoside	HPLC-DAD-MS	n.q.	(Giacobbo et al., 2019)
	Petunidin-3-O-glucoside	LC-MS/MS	n.q.	(Romero-Díez et al., 2019)
	Petunidin-3-glucoside	HPLC-MS/MS	0.97-3.41 mg/g	(Dujmić et al., 2020)
		UHPLC-Q-TOF-MS	2.18-5.03 mg/mL	(López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021)
	Petunidin-3-(6-coumaroyl)-hexoside	HPLC-DAD-MS	n.q.	(Giacobbo et al., 2019)
	Petunidin-3-O-(6''-p-coumaroyl-glucoside)	LC-MS/MS	n.q.	(Romero-Díez et al., 2019)
		UHPLC-Q-TOF-MS	0.16-0.78 mg/mL	(López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021)
	Petunidin-3-hexoside	HPLC-DAD-MS	n.q.	(Giacobbo et al., 2019)
	Petunidin-3-(6-acetyl)-hexoside	HPLC-DAD-MS	n.q.	
	Petunidin-(6-acetyl)-glucoside	UHPLC-Q-TOF-MS	0.04-1.29 mg/mL	(López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021)
	Petunidin-3-glucoside-pyruvic acid	UHPLC-Q-TOF-MS	0.03-0.09 mg/mL	
	Malvidin-3-hexoside	HPLC-DAD-MS	n.q.	(Giacobbo et al., 2019)
	Malvidin-3-glucoside	HPLC-MS/MS	2.36-4.24 mg/g	(Dujmić et al., 2020)

		UHPLC-Q-TOF-MS	26.78-60.67 mg/mL	
	Malvidin-3-glucoside-(epi)catechin	UHPLC-Q-TOF-MS	0.50-1.11 mg/mL	(López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021)
	Malvidin-3-glucoside-vinylguaiacol	UHPLC-Q-TOF-MS	0.17-0.59 mg/mL	
	Malvidin-3-glucoside-ethyl-(epi)catechin	UHPLC-Q-TOF-MS	0.09-2.63 mg/mL	
	Malvidin-3-O-glucoside	LC-MS/MS	n.q.	(Romero-Díez et al., 2019)
	Malvidin-glucoside-vinyl-catechin	UHPLC-Q-TOF-MS	0.08-0.16 mg/mL	(López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021)
	Malvidin-3-(6-O-acetyl)-glucoside	HPLC-MS/MS	1.06-1.74 mg/g	(Dujmić et al., 2020)
	Malvidin-3-(6-acetyl)-hexoside	HPLC-DAD-MS	n.q.	(Giacobbo et al., 2019)
	Malvidin-3-(6-coumaroyl)-hexoside	HPLC-DAD-MS	n.q.	
	Malvidin-(6-coumaroyl)-3-glucoside	UHPLC-Q-TOF-MS	2.31-10.77 mg/mL	(López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021)
	Malvidin-(6-acetyl)-3-glucoside	UHPLC-Q-TOF-MS	0.79-28.39 mg/mL	
	Malvidin-3-O-6''-acetyl-glucoside	LC-MS/MS	n.q.	(Romero-Díez et al., 2019)
	Malvidin-3-(6-O-p-coumaroyl)-glucoside	HPLC-MS/MS	0.79-1.80 mg/g	(Dujmić et al., 2020)
	Malvidin-3-O-(6''-p-coumaroyl-glucoside)	LC-MS/MS	n.q.	(Romero-Díez et al., 2019)
	Malvidin-3-hexoside-pyruvate	HPLC-DAD-MS	n.q.	(Giacobbo et al., 2019)
	Malvidin-(6-caffeoyl)-3-glucoside	UHPLC-Q-TOF-MS	0.04-0.36 mg/mL	(López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021)
	Malvidin 3-O-glucoside 4-vinylphenol (Pigment A)	UHPLC-Q-TOF-MS	0.44-0.64 mg/mL	

	Malvidin acetyl 3-O-glucoside 4-vinylphenol (Acetyl-pigment A)	UHPLC-Q-TOF-MS	0.02-0.38 mg/mL	
	Gallocatechin-Malvidin-3-glucoside dimer	UHPLC-Q-TOF-MS	0.10-0.25 mg/mL	
	Catechin-ethyl-Malvidin-3-acetylglucoside dimer	UHPLC-Q-TOF-MS	0.03-0.88 mg/mL	
	Catechin-ethyl-malvidin-3-coumaroylglucoside dimer	UHPLC-Q-TOF-MS	0.21-0.68 mg/mL	
	Visitin A (malvidin-3-glucoside-pyruvic acid)	UHPLC-Q-TOF-MS	0.35-1.23 mg/mL	
	Visitin B (malvidin-3-glucoside-acetaldehyde)	UHPLC-Q-TOF-MS	3.06-5.11 mg/mL	
	Pinotin A (malvidin-3-glucoside-vinylcatechol)	UHPLC-Q-TOF-MS	0.18-0.88 mg/mL	
	Cyanidin-3-hexoside	HPLC-DAD-MS	n.q.	(Giacobbo et al., 2019)
	Cyanidin-3-glucoside	UHPLC-Q-TOF-MS	0.16-0.23 mg/mL	(López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021)
	Cyanidin-3-(6-coumaroyl)-hexoside	HPLC-DAD-MS	n.q.	(Giacobbo et al., 2019)
	Cyanidin-(6-coumaroyl)-3-glucoside	UHPLC-Q-TOF-MS	0.03-0.11 mg/mL	(López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021)
	Cyanidin-3-O-(6''-p-acetylglucoside)	LC-MS/MS	n.q.	(Romero-Díez et al., 2019)
		UHPLC-Q-TOF-MS	0.01-0.20 mg/mL	(López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021)
	Peonidin-3-hexoside	HPLC-DAD-MS	n.q.	(Giacobbo et al., 2019)
	Peonidin-3-glucoside	UHPLC-Q-TOF-MS	1.83-2.72 mg/mL	(López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021)
	Peonidin-3-(6-coumaroyl)-hexoside	HPLC-DAD-MS	n.q.	(Giacobbo et al., 2019)

	Peonidin-(6coumaroyl)-3-glucoside	UHPLC-Q-TOF-MS	0.42-0.94 mg/mL	(López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021)
	Peonidin-3-glucoside-pyruvic acid	UHPLC-Q-TOF-MS	0.02-0.04 mg/mL	
	Peonidin-(6-acetyl)-3-glucoside	UHPLC-Q-TOF-MS	0.08-1.32 mg/mL	
	Pelargonidin-3-hexoside	HPLC-DAD-MS	n.q.	(Giacobbo et al., 2019)
	10-carboxypyranomalvidin-3-6''-p-coumaroyl-glucoside	LC-MS/MS	n.q.	(Romero-Díez et al., 2019)
	Acetylvisitin A	UHPLC-Q-TOF-MS	0.10-0.79 mg/mL	(López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021)
	Acetylvisitin B	UHPLC-Q-TOF-MS	0.27-1.66 mg/mL	
	Coumaroylvisitin A	UHPLC-Q-TOF-MS	0.04-0.20 mg/mL	
	Coumaroylvisitin B	UHPLC-Q-TOF-MS	0.91-1.12 mg/mL	
	Acetyl-pinotin A	UHPLC-Q-TOF-MS	0.01 mg/mL	

n.q. (non-quantified)

Table 3. Grapes, wine and winemaking by-products consumption antihypertensive effects in both animals and human studies.

Model used		Product	Dose (mg/kg bw)	Administration	SBP decrement	DBP decrement	Action mechanism	Ref.
Animal	SHR	Grape powder	600	10 weeks	8	11 mmHg	↑ eNOS	(Thandapilly et al., 2012)
	Sprague-Dawley rats with BSO	Grape powder	300	3 weeks	20%	20%	↓ ERK-1/2	(Allam et al., 2013)
	Wistar rats with Ang II	Red wine	150	20 days	~20 mmHg	-	↑ Renin ↓ NADPH oxidase ↓ ROS	(Sarr et al., 2006)
	Wistar rats with L-NAME	Red wine polyphenols	40	4 weeks	18%	-	↑ eNOS, NO	(Pechánová et al., 2004)
	SHR	Red wine pomace	300	4 weeks	30 mmHg	-	↑ NO, *Ho-1*, *Sod2* ↓ *Ace*	(Del Pino-García et al., 2017)
	SHR	Grape pomace	25	4 weeks	~25 mmHg	-	-	(Rasines-Perea et al., 2018)
	Wistar rats with L-NAME or DOCA-salt	Grape skin extract	100	30 days	~30 mmHg	~35 mmHg	↑ NO	(R. Soares de Moura et al., 2002)

SHR	Grape skin extract	200	12 weeks	~60 mmHg	-		↑ SOD	(De Costa et al., 2020)
Wistar rats with high fat diet (HF)	Grape skin extract	200	180 days	~30 mmHg	-	↑	NO, SOD, CAT, GPx ↓ MDA	(Emiliano et al., 2011; Resende et al., 2013)
Pregnancy female rats	Grape skin extract	200	Chronic	-	-		NO	(Roberto Soares De Moura et al., 2007)
SHR	Grape seed procyanidin extract	375	Acute	48 mmHg	57.4 mmHg		↑ GSH	(M. Quiñones et al., 2013)
SHR	Grape seed procyanidin extract	375	Acute	35.7 mmHg	34.5 mmHg	↑	PGFα1, NO, PGI_2	(M. Quiñones et al., 2014)
Wistar rats with cafeteria diet	Grape seed proanthocyanidins extract	375	Acute	~18 mmHg	~13 mmHg		↓ MDA	(Pons et al., 2014)
Wistar rats with cafeteria diet	Polyphenol grape seed extract	375	Acute	21 mmHg	22 mmHg		↑ NO, PGI_2	(Pons et al., 2016a)

Wistar rats with cafeteria diet and SHR	Grape seed polyphenol extract	375	Acute	21 mmHg	22 mmHg	↑ *eNos, Sirt1* ↓ *Nox4, Et1*	(Pons et al., 2016b)
Wistar rats with cafeteria diet	Grape seed proanthocyanidin extract	25	3 weeks	18 mmHg	17 mmHg	↑ GSH, *Sirt1* ↓ *Et1*	(Mas-capdevila et al., 2020)
Wistar rats with cafeteria diet	Grape seed proanthocyanidin extract	25, 100, 200	12	-	-	-	(Pons et al., 2017)
Sprague-Dawley rats with nitric sodium or ouabain	Grape seed proanthocyanidin extract	250	5 weeks	~20 mmHg	-	↑ NO ↓ ET-1, TGF-β1	(Liu et al., 2012)
Kunming mice with L-NAME	Grape seed proanthocyanidins	-	3 weeks	~20 mmHg	~10 mmHg	↑ eNOS, NO, SOD ↓ MDA, ET-1	(Zhu et al. 2018)
SHR	Wine lees	5 mL/kg	Acute	36.4 mmHg	38.8 mmHg	-	(López-Fernández-Sobrino, Soliz-Rueda, Margalef, et al., 2021)
SHR	Wine lees	5 mL/kg	Acute	32.5 mmHg	32.2 mmHg	-	(López-Fernández-Sobrino, Margalef, et al., 2021)
SHR	Phenolic-enriched wine lees	5 mL/kg	Acute	35.6 mmHg	35.2 mmHg	-	

	SHR	Wine lees powder	125	Acute	48.0 mmHg	42.6 mmHg	↑ NO, GSH ↓ MDA, ROS	(López-Fernández-Sobrino, Soliz-Rueda, Ávila-Román, et al., 2021)
Humans	Pre-hypertensive men and women	Grape seed extract	300 mg/day	6 weeks	9 mmHg	4 mmHg	-	(Park et al., 2016)
	Adults with metabolic syndrome	Grape seed extract	150 or 300 mg/day	4 weeks	11 mmHg	6-7 mmHg	↑ eNOS	(Sivaprakasapillai et al., 2009)
	Pre-hypertensive middle-aged Japanese men and women	Grape seed proanthocyanidin extract	400 mg/day	12 weeks	13.1 mmHg	6.5 mmHg	-	(Odai et al., 2019)
	Men with high cardiovascular risk	Dealcoholized red wine	272 mL/day	4 weeks	5.8 mmHg	2.3 mmHg	NO	(Chiva-Blanch et al., 2012)
	Healthy men	Red wine and dealcoholized red wine	8.1± 0.9 dL	Acute	-	-	↓ ET-1	(Kiviniemi et al., 2010)

Men with metabolic syndrome	Wine grape pomace	20 g/day	16 weeks	4.3 mmHg	5.3 mmHg	-	(Urquiaga et al., 2015)
Hypertensive patients	Grape polyphenols	150-1400 mg/day	2-16 weeks	1.48 mmHg	-	↑ NO	(S.-H. Li et al., 2015)

FIGURES

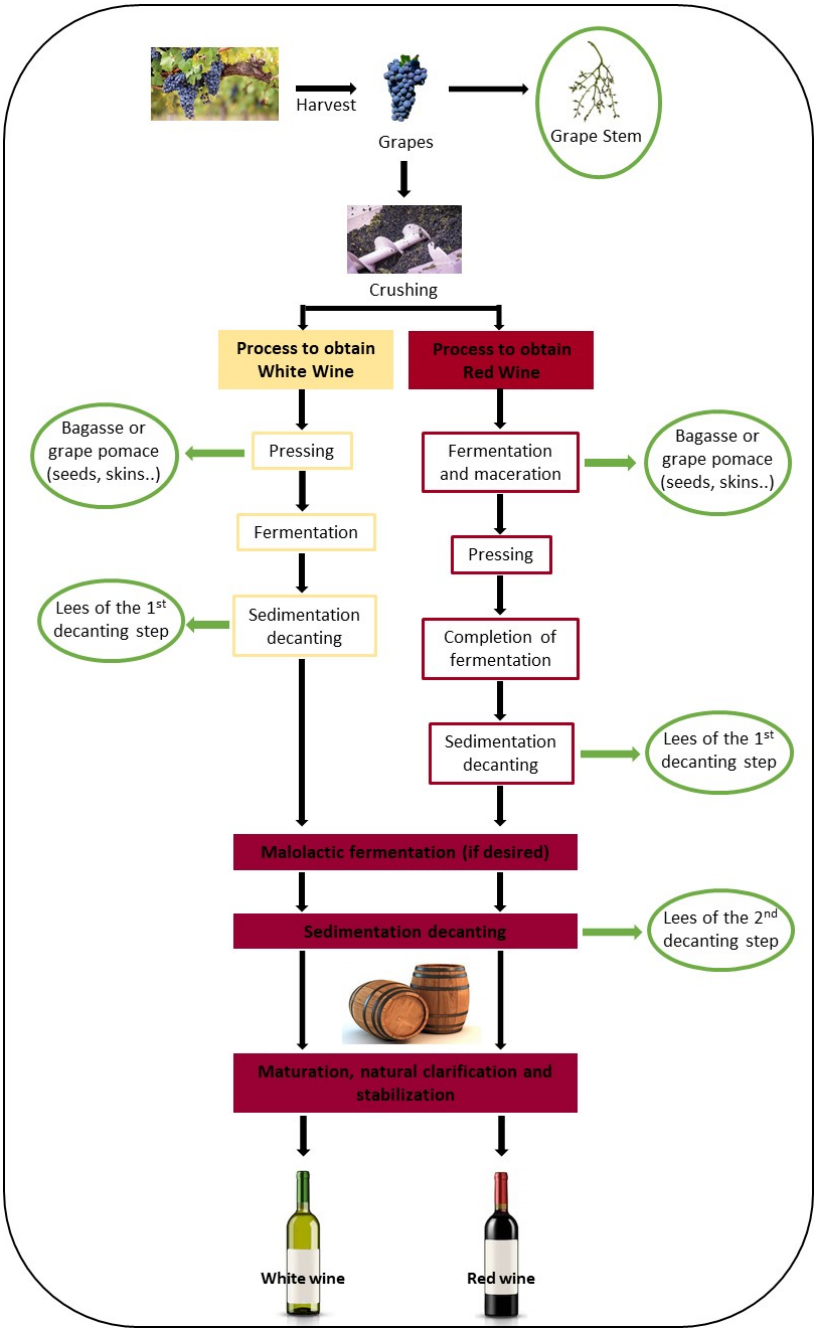

Figure 1. General procedure of winemaking process. Grapes are crushing and in the case of red wine, fermentation and maceration is carried out with the juice and pomace. Generated grape juice is pressed to remove grape bagasse (also called grape pomace) grape skin and grape seed. Then the fermentation

can continue in the tanks or end. After the fermentation process, wine is racking to be separated of solid residues called lees (lees of first decanting step). The obtained wine continues with the malolactic fermentation in the tanks and obtain lees of the second decanting step after sedimentation decanting. Finally, wine suffer a process of maturation in barrels and natural clarification and stabilization before bottling. In white winemaking process, the first fermentation process with pomace is not carried out, the rest of the process is the same that in red wine.

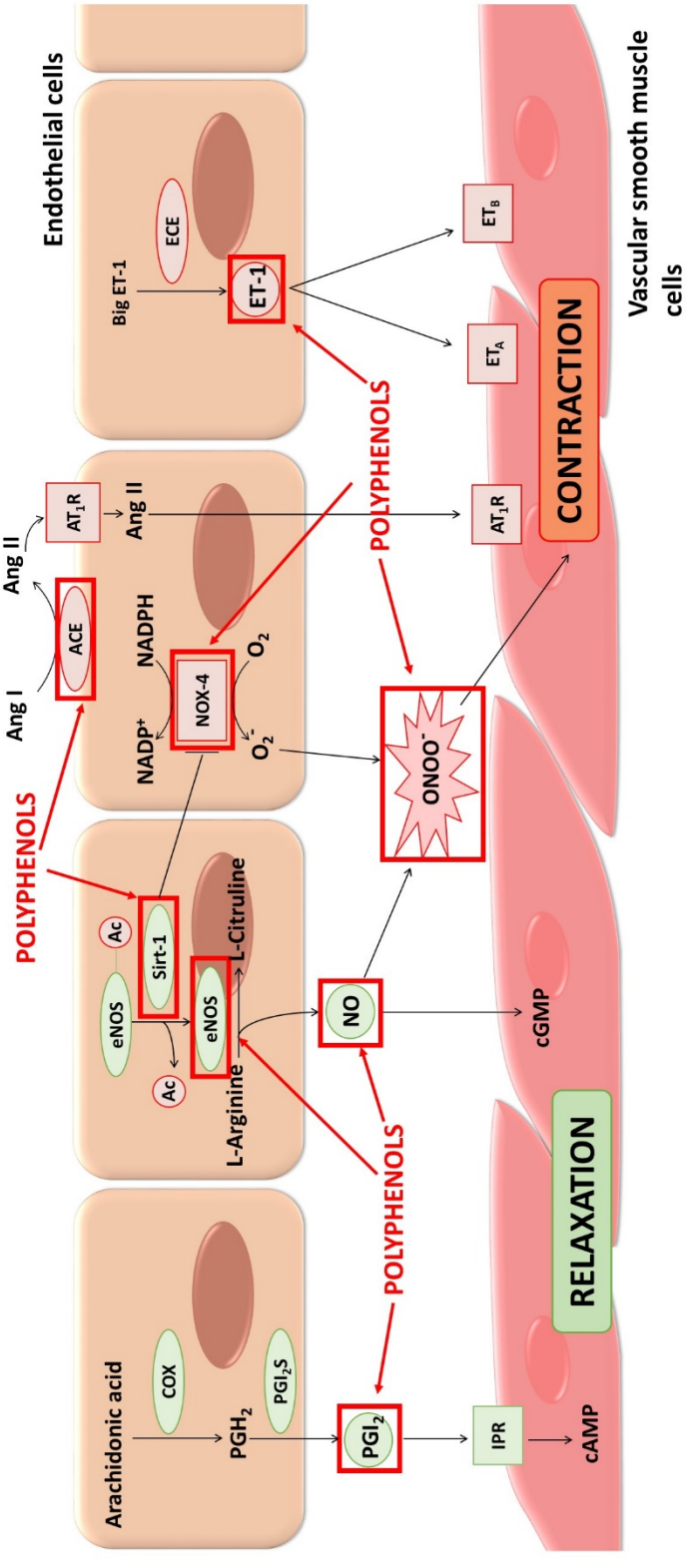

Figure 2. Endothelial-derived vasoactive molecules and their hypertension manage by polyphenols. Vasorelaxant enzymes and molecules are represented in green and vasoconstrictor enzymes and molecules in red. The boxes in red indicate the molecules or enzymes through which polyphenols exert their antihypertensive effect. ACE: angiotensin converting enzyme; Ang: angiotensin; AT_1R: Angiotensin type 1 receptor; cAMP: cyclic adenosine monophosphate; cGMP: cyclic guanosine monophosphate; COX: cyclooxygenase; ECE: endothelin

converting enzyme; ET: endothelin; ET_A: endothelin receptor A; ET_B: endothelin receptor B; eNOS: endothelial nitric oxide synthase; L-Arg: L-arginine; NADPH: nicotinamide adenine dinucleotide phosphate; NOX-4: nicotinamide adenine dinucleotide phosphate-oxidase 4; NO: nitric oxide; PGI_2: prostaglandin I2 or prostacyclin; PGI_2S: Prostaglandin I2 synthase; ROS: reactive oxygen species; Sirt-1: Sirtuin 1; IPR: prostaglandin receptor.

Patent:

Objective:

To study the antihypertensive effect of wine lees and their uses

Wine lees, derivatives thereof and their uses

Francisca Isabel Bravo Vázquez, Raúl López Fernández, Juan María Alcaide-Hidalgo, María Margalef Jornet, Anna Mas Capdevila, Jose María del Bas Prior, María Eugenia Hernández De Pablo, María Begoña Muguerza Marquínez.

European Patent.

Applicant: GRANDES VINOS Y VIÑEDOS S.A.

State: Presented

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DE INDUSTRIA, ENERGÍA
Y TURISMO

Oficina Española
de Patentes y Marcas

Acknowledgement of receipt

We hereby acknowledge receipt of your request for grant of a European patent as follows:

Submission number	300363060	
Application number	EP20382358.8	
File No. to be used for priority declarations	EP20382358	
Date of receipt	30 April 2020	
Your reference	20200399	
Applicant	GRANDES VINOS Y VIÑEDOS SA	
Country	ES	
Title		
Documents submitted	package-data.xml	ep-request.xml
	application-body.xml	ep-request.pdf (5 p.)
	SPECNONEPO.pdf\description.pdf (31 p.)	DRAWNONEPO.pdf\drawings-es.pdf (4 p.)
	SEQLTXT.txt\20200399_Seqlist_ST25.txt	f1002-1.pdf (2 p.)
Submitted by	CN=Silvia Moreno Gordo 63659	
Method of submission	Online	
Date and time receipt generated	30 April 2020, 15:02:09 (CEST)	
Official Digest of Submission	5C:EE:54:A3:79:66:43:4F:40:BB:03:2D:4C:B9:6C:8D:26:2E:5D:AD	

/Madrid, Oficina Receptora/

Form 1002 - 1: Public inventor(s)

Designation of inventor

User reference: 20200399
Application No:

Public

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Signature(s)

Place:	**Madrid**
Date:	**30 April 2020**
Signed by:	**/Gustavo Adolfo Gonzalez Peces/**
Association:	**HERRERO & ASOCIADOS, S.L.**
Representative name:	**Gustavo Adolfo Gonzalez Peces**
Capacity:	**(Representative)**

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Acknowledgement of receipt

We hereby acknowledge receipt of your request for the processing of an international application according to the Patent Cooperation Treaty as follows:

Submission number	9483946
PCT application number	PCT/EP2021/053051
Date of receipt	09 February 2021
Receiving Office	European Patent Office, The Hague
Your reference	20210133
Applicant	GRANDES VINOS Y VIÑEDOS SA
Number of applicants	1
Country	ES
Title	WINE LEES, DERIVATIVES THEREOF AND THEIR USES

Documents submitted	eolf-pkda.xml	eolf-requ.xml
	eolf-appb.xml	eolf-seql.txt
	eolf-fees.xml	eolf-vlog.xml
	eolf-othd-000001.pdf (33 p.)	eolf-appb-P000001.pdf (5 p.)

Submitted by	CN=Silvia Moreno Gordo 63659
Method of submission	Online
Date and time receipt generated	09 February 2021, 13:28 (CET)
Message Digest	56:DD:1D:13:05:2C:64:C0:9F:14:1D:C7:2D:73:9A:3D:3D:31:3C:79

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DAS access code
The access code generated for this application and used to retrieve the priority documents from WIPO's Digital Access Service (DAS) is indicated in the document appended to this acknowledgement of receipt. Please note that the appended document is non-public and will not be published.

/European Patent Office/

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DAS access code

To access and retrieve the priority document from WIPO's Digital Access Service (DAS) in respect of

Application number	PCT/EP2021/053051
Applicant	GRANDES VINOS Y VIÑEDOS SA

the European Patent Office has generated the following code:

DAS access code	F762

For further information, see OJ EPO 03/2019.

Date and time receipt generated	09 February 2021, 13:28 (CET)

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PCT REQUEST

(Original in Electronic Form)

0	**For receiving Office use only**	
0-1	International Application No.	
0-2	International Filing Date	
0-3	Name of receiving Office and "PCT International Application"	

0-4	**Form PCT/RO/101 PCT Request**	
0-4-1	Prepared Using	PCT Online Filing Version 3.51.000.269e MT/FOP 20141031/0.20.5.24
0-5	**Petition**	
	The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty	
0-6	**Receiving Office (specified by the applicant)**	European Patent Office (EPO) (RO/EP)
0-7	**Applicant's or agent's file reference**	20210133
I	**Title of Invention**	WINE LEES, DERIVATIVES THEREOF AND THEIR USES
II	**Applicant**	
II-1	This person is	Applicant only
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II-6	State of nationality	ES
II-7	State of residence	ES
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III-3	**Applicant and/or inventor**	
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III-8-3	Inventor for	**All designated States**
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III-8-5	Address	**C/ Marina alta, 6, Esc. 14, pta. 5 46015 VALENCIA Spain**

PCT REQUEST

(Original in Electronic Form)

IV-1	**Agent or common representative; or address for correspondence** The person identified below is hereby/ has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:	**Agent**
IV-1-1	Name (LAST, First)	**GONZALEZ PECES, Gustavo, Adolfo**
IV-1-2	Address	**Herrero & Asociados, S.L.** **Cedaceros, 1** **28014 MADRID** **Spain**
IV-1-5	e-mail	**notificaciones@herrero.es**
IV-1-5(a)	E-mail authorization The receiving Office, the International Searching Authority, the International Bureau and the International Preliminary Examining Authority are authorized to use this e-mail address, if the Office or Authority so wishes, to send notifications issued in respect of this international application:	**as advance copies followed by paper notifications**
V	**DESIGNATIONS**	
V-1	**The filing of this request constitutes under Rule 4.9(a), the designation of all Contracting States bound by the PCT on the international filing date, for the grant of every kind of protection available and, where applicable, for the grant of both regional and national patents.**	
VI-1	**Priority claim of earlier regional application**	
VI-1-1	Filing date	**30 April 2020 (30.04.2020)**
VI-1-2	Number	**20382358.8**
VI-1-3	Regional Office	**EP**
VI-2	**Priority document request** The International Bureau is requested to obtain from a digital library a certified copy of the earlier application(s) identified above as item(s), using, where applicable, the access code(s) indicated:	**VI-1 Access code: B370**
VI-3	**Incorporation by reference :** where an element of the international application referred to in Article 11(1)(iii)(d) or (e) or a part of the description, claims or drawings referred to in Rule 20.5(a), or an element or part of the description, claims or drawings referred to in Rule 20.5bis(a) is not otherwise contained in this international application but is completely contained in an earlier application whose priority is claimed on the date on which one or more elements referred to in Article 11(1)(iii) were first received by the receiving Office, that element or part is, subject to confirmation under Rule 20.6, incorporated by reference in this international application for the purposes of Rule 20.6.	
VII-1	**International Searching Authority Chosen**	**European Patent Office (EPO) (ISA/EP)**

PCT REQUEST

(Original in Electronic Form)

VII-2	**Request to use results of earlier search; reference to that search**		
VII-2-1	Filing date	**30 April 2020 (30.04.2020)**	
VII-2-2	Application Number	**20382358.8**	
VII-2-3	Country (or regional Office)	**EP**	
VII-2-4	Statement (Rule 4.12(ii)):	**This international application is the same, or substantially the same, as the application in respect of which the earlier search was carried out except, where applicable, that it is filed in a different language.**	
VII-2-5	Documents are available to the ISA in a form and a manner acceptable to it, and therefore do not need to be submitted by the applicant to the receiving Office, or to the ISA (Rules 12bis1.(c) and (d) and 12bis.2(b)):	**A copy of the results of the earlier search** **A copy of the earlier application** **A copy of any document cited in the earlier search results**	
VIII	**Declarations**	Number of declarations	
VIII-1	Declaration as to the identity of the inventor	-	
VIII-2	Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent	-	
VIII-3	Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application	-	
VIII-4	Declaration of inventorship (only for the purposes of the designation of the United States of America)	-	
VIII-5	Declaration as to non-prejudicial disclosures or exceptions to lack of novelty	-	
IX	**Check list**	Number of sheets	Electronic file(s) attached
IX-1	Request (including declaration sheets)	5	✓
IX-2	Description	29	✓
IX-3	Claims	3	✓
IX-4	Abstract	1	✓
IX-5	Drawings	5	✓
IX-6a	Sequence listing part of the description (also to be used for the purposes of international search)	-	✓
IX-7	TOTAL	43	
	Accompanying Items	Paper document(s) attached	Electronic file(s) attached
IX-8	Fee calculation sheet	-	✓
IX-20	**Figure of the drawings which should accompany the abstract**		
IX-21	**Language of filing of the international application**	**English**	

PCT REQUEST

(Original in Electronic Form)

X-1	**Signature of applicant, agent or common representative**	/Gustavo Adolfo Gonzalez Peces/
X-1-1	Name (LAST, First)	GONZALEZ PECES, Gustavo, Adolfo
X-1-3	Capacity (if such capacity is not obvious from reading the request)	(Representative)

FOR RECEIVING OFFICE USE ONLY

10-1	**Date of actual receipt of the purported international application**	
10-2	**Drawings:**	
10-2-1	Received	
10-2-2	Not received	
10-3	**Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application**	
10-4	**Date of timely receipt of the required corrections under PCT Article 11(2)**	
10-5	**International Searching Authority**	ISA/EP
10-6	**Transmittal of search copy delayed until search fee is paid**	

FOR INTERNATIONAL BUREAU USE ONLY

11-1	**Date of receipt of the record copy by the International Bureau**	