Universidade de Trás-os-Montes e Alto Douro

Evaluation of biological activities of grape (*Vitis vinifera* L.) stems for potential application in the cosmetic and pharmaceutical industries

Dissertação de Mestrado em Enologia e Viticultura

Carla Alexandra Carneiro Leal

Orientadores: Professora Doutora Ana Isabel Ramos Novo Amorim de Barros
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Vila Real, 2020

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Declara-se sob compromisso de honra que este trabalho foi expressamente elaborado pelo autor como dissertação original para o efeito da obtenção do grau de Mestre em Enologia e Viticultura, na Universidade de Trás-os-Montes e Alto Douro. Todas as contribuições não originais foram devidamente referenciadas com indicação da fonte.

Carla Alexandra Carneiro Leal

(Carla Alexandra Carneiro Leal)

Vila Real, abril 2020

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Results in this master's dissertation published/submitted or presented in meetings

Poster communications:

[1] Carla Leal, Irene Gouvinhas, Carlos Matos, Ana Barros. Quantification of essencial minerals and toxic metals in grape (*Vitis vinifera* L.) stems, Semana da Ciência e Tecnologia, 25 fevereiro – 1 março de **2019**, UTAD.

[2] Carla Leal, Rosa Pinto, Marcelo Queiroz, Rafaela Santos, Maria José Saavedra, Irene Gouvinhas, Ana Barros. Antimicrobial activity of white Portuguese grape (*Vitis vinifera* L.) stems against gastrointestinal pathogens. Infowine - 6th edition. 23-24th May **2018**, Theater of Vila Real (Portugal).

Articles published/submitted in international journals referenced in SCI

[1] Carla Leal, Rafaela A. Santos, Rosa Pinto, Marcelo Queiroz, Miguel Rodrigues, Maria José Saavedra, Ana Barros, Irene Gouvinhas. Recovery of bioactive compounds from white grape (*Vitis vinifera* L.) stems as potential antimicrobial agents. *Saudi Journal of Biological Sciences* (**2020**).

[2] Carla Leal, Irene Gouvinhas, Rafaela A. Santos, Maria José Saavedra, Eduardo Rosa, Amélia M. Silva, Ana I.R.N.A Barros. Potential application of grape (*Vitis vinifera* L.) stem extracts in the cosmetic and pharmaceutical industries: valorization of a by-product. Submitted to: *Industrial Crops and Products*.

[3] Carla Leal, Carlos M. Costa, Ana I. R. N. A. Barros, Irene Gouvinhas. Assessing the relationship between the phenolic content and elemental composition of Grape (*Vitis vinifera* L.) stems. Submitted to: *Waste and Biomass Valorization.*

Resumo

A produção de uva gera grandes quantidades de subprodutos, como o engaço de uva. Este resíduo é maioritariamente descartado em áreas abertas, onde a sua difícil deterioração origina problemas ambientais e compromete a sustentabilidade das empresas. Visto que, este subproduto possui compostos bioativos, com benefícios para a saúde, a sua aplicação como fonte de compostos fenólicos em novos produtos pode ser promissora, contudo, é essencial garantir que este subproduto é seguro para os consumidores. Assim, o objetivo deste trabalho foi analisar espectrometricamente e fluorimetricamente o conteúdo mineral de seis variedades de engaço de uva, procedendo-se depois à determinação da composição fenólica por métodos colorimétricos (fenóis totais, *orto*-difenóis e flavonóides), e à identificação e quantificação dos compostos maioritários por HPLC. As atividades biológicas dos extratos de engaço foram também avaliadas, nomeadamente: a atividade antioxidante através dos métodos ABTS, DPPH e FRAP; a atividade antimicrobiana pelos métodos de difusão em disco e Concentração Mínima Inibitória contra isolados bacterianos de pacientes hospitalares; a atividade anti-inflamatória na linha celular RAW 264.7; e a atividade anti-envelhecimento através da capacidade de inibição das atividades das enzimas tirosinase e elastase.

Os resultados demonstraram que o engaço de uva possui um alto teor de minerais essenciais, sendo os mais abundantes o Ca e K, com concentrações médias de 1.41 e 24.45 g/Kg peso seco, respetivamente. Relativamente aos metais tóxicos, o Al apresentou maior abundância com uma concentração média de 200.80 mg/Kg peso seco. Também para a composição fenólica foram observadas diferenças significativas, com teores variando de 30,91 ± 0,73 a 96,12 ± 8,14 mg ácido gálico/g peso seco, 32,17 ± 1,04 a 77,26 ± 5,31 mg ácido gálico/g peso seco, e 25,76 ± 1,14 a 65,14 ± 0,65 mg catequina/g peso seco para os fenóis totais, *orto*-difenóis e flavonóides, correspondentemente. Ademais, onze compostos fenólicos foram identificados por HPLC, sendo a catequina o composto mais abundante (0,44 ± 0,02 a 2,03 ± 0,08 mg/g peso seco). Em relação à atividade antioxidante, atingiram-se valores de 0,84 ± 0,06, 0,64 ± 0,05 e 1,03 ± 0,06 mmol Trolox/g peso seco para os métodos ABTS, DPPH e FRAP, respetivamente, enquanto que, para a atividade antimicrobiana, os extratos apresentaram alta eficácia contra bactérias Gram-positivas. A capacidade anti-inflamatória dos extratos mostrou inibições de 16,52% a 35,25% na produção de NO, e pela primeira vez, os extratos de engaço apresentaram atividades anti-tirosinase e anti-elastase, com inibições máximas de 53,82% e 98,02%, respetivamente.

O engaço demonstrou ser rico em minerais essenciais e compostos fenólicos, que aliados com as suas propriedades biológicas, indicam que este subproduto pode ser utilizado no desenvolvimento de novos antibióticos contra a resistência bacteriana aos antibióticos, em fármacos que diminuam/eliminem a inflamação, em produtos cosméticos, e em suplementos alimentares e vitaminas para humanos. Porém, estudos adicionais sobre a sua toxicidade são necessários, assim como, descobrir os compostos fenólicos responsáveis pelas propriedades biológicas. No entanto, numa primeira fase, é vital um maior cuidado na utilização de fertilizantes/pesticidas, para que este subproduto se torne totalmente seguro para ser aplicado nas indústrias cosmética, farmacêutica e alimentar.

Palavras-chave: Engaço de uva; Minerais essenciais; Compostos fenólicos; Atividades biológicas; Indústrias cosmética/farmacêutica.

Abstract

Grape production generates large amounts of by-products, such as grape stem. This residue is mostly discarded in open areas, where its difficult deterioration causes environmental problems and compromises the sustainability of the companies. Since this by-product has bioactive compounds with health benefits, its application as a source of phenolic compounds in new products may be promising, however, it is essential to ensure that this by-product is safe for consumers. Thus, the objective of this work was to analyze spectrometrically and fluorimetrically the mineral content of six grape stem varieties, followed by the determination of phenolic composition by colorimetric methods (total phenols, *ortho*-diphenols, and flavonoids), and the identification and quantification of major compounds by HPLC. The biological activities of grape stem extracts were also evaluated: the antioxidant activity through the ABTS, DPPH, and FRAP assays; the antimicrobial activity by the disk diffusion and Minimum Inhibitory Concentration methods against bacterial isolates from hospital patients; the anti-inflammatory activity by RAW 264.7 cell line; and the anti-aging activity by the capacity to inhibit the activity of tyrosinase and elastase enzymes.

The results demonstrated that grape stem has a high content of essential minerals, being the most abundant Ca and K, with average concentrations of 1.41 and 24.45 g/Kg dry weight, respectively. Regarding the toxic metals, Al presented higher abundance with an average concentration of 200.80 mg/Kg dry weight. Significant differences were also observed for phenolic composition, with contents ranging from 30.91 ± 0.73 to 96.12 ± 8.14 mg gallic acid/g dry weight, 32.17 ± 1.04 to 77.26 ± 5.31 mg gallic acid/g dry weight, and 25.76 ± 1.14 to 65.14 ± 0.65 mg catechin/g dry weight for total phenols, *ortho*-diphenols and flavonoids, respectively. In addition, eleven compounds were identified by HPLC, with catechin being the most abundant compound (0.44 ± 0.02 to 2.03 ± 0.08 mg/g dry weight). Concerning to antioxidant activity, values of 0.84 ± 0.06, 0.64 ± 0.05 and 1.03 ± 0.06 mmol Trolox/g dry weight were obtained for the ABTS, DPPH and FRAP methods, respectively, while for antimicrobial activity, the extracts presented high efficacy against Gram-positive bacteria. The anti-inflammatory capacity of the extracts showed inhibitions of 16.52% to 35.25% in NO production, and for the first time, grape stem extracts exhibited anti-tyrosinase and anti-elastase activities, with maximum inhibitions of 53.82% and 98.02%, respectively.

Grape stem has been shown to be rich in essential minerals and phenolic compounds, which, combined with their biological properties, indicate that this by-product can be used in the development of new antibiotics against bacterial resistance to antibiotics, in drugs that

decrease/eliminate inflammation, in cosmetic products, and in dietary supplements and vitamins for humans. Nevertheless, further studies on their toxicity are needed, as well as discovering the phenolic compounds responsible for biological activities. But first, greater care in the use of fertilizer/pesticides is vital for this by-product to become totally safe to be applied in the cosmetic, pharmaceutical, and food industries.

Keywords: Grape stem; Essential minerals; Phenolic compounds; Biological activities; Cosmetic/pharmaceutical industries.

Abbreviations

AB	Alamar Blue®
ABTS	2,2-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt
Al	Aluminum
AR	Arinto
As	Arsenic
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BHI	Brain Heart Infusion
Ca	Calcium
CAT	Catechin
Cd	Cadmium
CECT	Spanish Type Culture Collection
CHTMAD	Hospital Centre of Trás-os-Montes and Alto Douro
CIP10	Ciprofloxacin (10 µg)
CN10	Gentamicin (10 µg)
CN30	Gentamicin (30 µg)
Co	Cobalt
CO_2	Carbon Dioxide
COX	Cyclooxygenase
Cr	Chromium
CT	Castelão
Cu	Cupper
DAD	Diode Array Detector
DAN	2,3-diaminonaphthalene
DMEM	Dulbeco's modified Eagle's medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPPH	2,2-Diphenyl-1-picrylhidracyl radical
dw	dry weight
***E. aerogenes* MJMC534A**	*Enterobacter aerogenes* MJMC534A

***E. coli* MJS260**	*Eschericia coli* MJS260
EDTA	2,2',2",2"'-(Ethane-1,2-diyldinitrilo)tetraacetic acid
***E. faecalis* MJS257**	*Enterococcus faecalis* MJS257
EFSA	European Food Safety Authority
FAAS	Flame Atomic Absorption Spectrometry
FAES	Flame Atomic Emission Spectrometry
FBS	Fetal bovine serum
Fe	Iron
FP	Fernão Pires
FRAP	Ferric Reducing Antioxidant Power
GA	Gallic acid
Glc	Glucoside
Glt	Galactoside
GFAAS	Graphite Furnace Atomic Absorption Spectrometry
HPLC	High Performance Liquid Chromatograph
IL	Interleukin
iNOS	Inducible nitric oxide synthase
K	Potassium
***K. pneumoniae* MJH602**	*Klebsiella pneumoniae* MJH602
***K. pneumoniae* MJS281**	*Klebsiella pneumoniae* MJS281
***L. monocytogenes* ATCC 15313**	*Listeria monocytogenes* ATCC 15313
LPS	Lipopolysaccharide
Mg	Magnesium
MIC	Minimum Inhibitory Concentration
MJH	Maria José Hospital
MJMC	Maria José and Maria da Conceição
MJS	Maria José Saavedra
Mn	Manganese
Mv	Malvidin
Na	Sodium
ND	Not detected
NF-kB	Nuclear factor kappa B
Ni	Nickel

NO	Nitric Oxide
OIV	International Organisation of Vine and Wine
***P. aeruginosa* ATCC 10145**	*Pseudomonas aeruginosa* ATCC 10145
Pb	Lead
Q	Quercetin
RIZD	Relative Inhibition Zone Diameter
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
Rut	Rutinoside
***S. aureus* CECT 976**	*Staphylococcus aureus* CECT 976
***S. aureus* MJMC109**	*Staphylococcus aureus* MJMC109
***S. aureus* MJMC534B**	*Staphylococcus aureus* MJMC534B
***S. aureus* MJS241**	*Staphylococcus aureus* MJS241
Se	Selenium
SH	Syrah
SPSS	Statistical package for the social science
T	Trolox
TN	Touriga Nacional
TNF-α	Tumor necrosis factor-α
TPTZ	2,4,6-Tris(2-pyridyl)-*s*-triazine
TR	Tinta Roriz
Tris/HCl	Tris(hydroxymethyl) aminomethane hydrochloride
Trolox	6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
UTAD	Universidade de Trás-os-Montes e Alto Douro
UV	Ultraviolet
Zn	Zinc

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Chapter I:

Introduction

1. Introduction

1.1. State of the art

Grape production is one of the main economic activities in the agri-food sector, around the world (Barros *et al*., 2015), reaching 73.3 million tons in 2017. It is estimated that 52% of this production goes to the wine industry (OIV, 2018), where large quantities of by-products are generated, namely, organic residues (grape pomace, seeds, pulp, skins, grape leaves, grape stems and wine lees), greenhouse gas wastes (CO_2, organic volatile compounds, etc) and inorganic residues (diatomaceous earth, bentonite clay, and perlite) (Gouvinhas *et al*., 2019; Mateo *&* Maicas, 2015).

Currently in Europe, it is calculated that about 14.5 million tons of grape by-products are produced (Karvela *et al*., 2011), where Portugal contributes about 1.5 million tons (Domínguez-Perles *et al.*, 2014), causing environmental problems, and consequently, compromising the sustainability and competitiveness of the wine industries (Barba *et al*., 2016; Teixeira *et al*., 2014).

Thus, in search of an environmental-friendly production, the adoption of new approaches for waste prevention started, providing the application of these by-products in the development of innovative and value-added products (Barros *et al*., 2015) in the cosmetic, pharmaceutical and food industries, since they are rich in bioactive compounds (Makris *et al*., 2007) with beneficial biological properties for the human health (Vaquero *et al*., 2007).

1.2. Grape stem

Grape stem, which correspond to 25% of the total of by-products, is the less characterized and valued of all by-products generated in the wine industry (Barros *et al*., 2014), representing up to 7% of the raw material processed (Mateo *&* Maicas, 2015). Primarily, the grapes are harvested, and before the vinification process the stems are removed (Spatafora *et al*., 2013), since their presence increases the astringency, due to its richness in proanthocyanidins, affecting the quality of the wines (Anastasiadi *et al*., 2012).

This by-product is a rich source of phenolic compounds, as well as of celluloses, hemicelluloses and lignins, enhancing its use in diverse industries (Gouvinhas *et al*., 2019). However, it is usually destined to the production of spirits, dietary fiber, vegetable protein concentrates, fertilizers (Domínguez-Perles *et al.*, 2014), and animal feed (Sahpazidou *et al*., 2014).

1.2.1. Chemical composition of grape stem

Regarding the physical-chemical characteristics of grape stems, it was determined that the moisture content of this by-product can range from 55% to 80%, depending on grape variety (Teixeira *et al*., 2014). Grape stems contain macromolecules, being the most abundant the cellulose (30.3%), followed by hemicellulose (21%) and lignin (17.4%), presenting also a high tannin content (15.9%) (Gouvinhas *et al*., 2019).

In addition, this matrix is rich in dietary fibre, ranging from 60 to 90% of the dry matter, proteins (up to 7%), soluble sugars (up to 2%) and bioactive compounds (Nunes *et al*., 2017).

1.2.2. Phenolic composition of grape stem

In the last years, bioactive compounds present in grapes and their by-products have attracted attention due to their health benefits, leading to a growing interest of the scientific community in the exploration of these by-products as sources of active ingredients (Gouvinhas *et al*., 2018). In grape stem, the content of these compounds is about 5.8% of dry weight (Karvela *et al*., 2011), being particularly rich in flavonoids (anthocyanins, flavonols and flavanols), stilbenes, such as Ɛ-viniferin, and phenolic acids (Anastasiadi *et al*., 2012; Che *et al*., 2017). However, the concentration of these bioactive compounds may vary depending on the variety, cultural practices, and environmental conditions (Flamini *et al*., 2013).

These bioactive or phenolic compounds are secondary metabolites synthesized in the normal development of plants through two main biosynthetic pathways, the shikimate pathway and/or phenylpropanoid metabolism (Gouvinhas *et al*., 2019), however they are also produced by plants as a mechanism of defense in stress conditions, against ultraviolet radiations, aggression of pathogens, parasites or predators, among others (Dai & Mumper, 2010; Machado & Domínguez-Perles, 2017).

Structurally, the phenolic compounds are formed by an aromatic ring which contain one or more hydroxyl groups, ranging from single molecules to highly polymerized compounds (Balasundram *et al*., 2006; Crozier *et al*., 2009), since they can be supplemented with glycosides, esters, among others, originating functional compounds with different biological properties (Machado & Domínguez-Perles, 2017). They also play an important role in sensory characteristics of plant foods (such as fruits, vegetables, cereals and chocolate) and beverages (like coffee, wine, tea and beer), where they are responsible for the color, aroma, flavour, astringency or bitterness (Dai & Mumper, 2010; Teixeira *et al*., 2014).

Phenolic compounds can be divided in two main classes: flavonoids, which present subclasses, namely flavonols, flavan-3-ols, anthocyanins, flavones, and flavanones, and non-flavonoids, which are divided into phenolic acids (hydroxycinnamic acids and hydroxybenzoic acids) and stilbenes (Teixeira *et al*., 2013; Tsao, 2010). The tannins are also a group of phenolic compounds, divided into condensed tannins and hydrolysable tannins, which belong to the classes of flavonoids and non-flavonoids, respectively (Crozier *et al*., 2009).

1.2.2.1. Flavonoids

Flavonoids are bioactive compounds present in various food and beverages of vegetable origin (Ross & Kasum, 2002). They are potentially beneficial compounds in the fight against diseases, such as cardiovascular or degenerative diseases, principally due to its antioxidant power (Pascual-Teresa *et al*., 2010).

In grape, it was described that the peak of its concentration was reached at 3 to 4 weeks after the summer, and together with the moment of harvest, can influence the qualitative and quantitative content of these bioactive compounds in the vinification residues (Teixeira *et al*., 2014). Flavonoids are formed by 15 carbon atoms with two aromatic rings attached by a three carbon bridge (C_6-C_3-C_6) (Figure 1.1) (Del Rio *et al*., 2013), and as previously mentioned, their main classes are flavonols, flavan-3-ols, anthocyanins, flavones and flavanones, which differ by the degree of oxidation of the central pyran-ring (Teixeira *et al*., 2014).

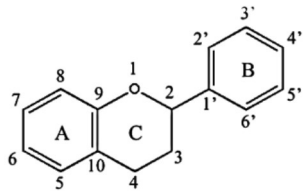


Figure 1.1. Basic flavonoid structure.

Flavonols are the most abundant flavonoids in food (El Gharras, 2009). They are yellow compounds that are present in grapes and wines, being responsible for the color of white wines, while in red wines they are camouflaged by anthocyanins (red pigments). Despite this, flavonols are important in the process of copigmentation of red wines, where this mechanism causes an increase in the extraction of anthocyanins during the vinification and

promotes color stabilization, since reactions are originated, which lead to the preservation of color (Favre *et al*., 2018). In addition, these phenolic compounds have a high antioxidant activity principally in white wines, where they are in higher concentration (Castillo-Muñoz *et al*., 2007). In the chemical structure they have a double bond between the atoms C_2 and C_3, a hydroxyl group in C_3 and about 90% of these compounds are hydroxylated at C_3, C_5 and C_7 positions (Figure 1.2A) (Garrido & Borges, 2013). Flavonols have six aglycones, namely, kaempferol, isorhamnetin, myricetin, quercetin, syringetin, and laricitrin, which can be linked at the C_3 position to different sugars originating glycosides, glucuronides, galactosides and diglycosides (for example, glycosylarabinoside or glycosylgalactose) (Castillo-Muñoz *et al*., 2007; Jeffery *et al*., 2008).

Concerning the existence of these compounds in grape stems, several studies have identified the presence of flavonols such as quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucuronide and kaempferol-3-*O*-rutinoside, among others, being the most abundant quercetin-3-*O*-glucuronide (Barros *et al*., 2014; Dias *et al*., 2015; Domínguez-Perles *et al*., 2016; Gouvinhas *et al*., 2018; Teixeira *et al*., 2018).

Flavan-3-ols or flavanols are present in fruits, teas, cereals and cocoa, yet they are almost non-existent in vegetables and legumes. Flavan-3-ols is a very complex group, ranging from monomeric flavan-3-ols to polymeric procyanidins (condensed tannins) (Pascual-Teresa *et al*., 2010). In its structure a hydroxyl group is present at the C_3 position, but unlike other flavonoids they do not have a double bond between C_2 and C_3, or a carbonyl group at the C_4 position (Figure 1.2B) (Garrido & Borges, 2013; Tsao, 2010).

There are five flavan-3-ols in grape, namely, (+)-catechin, (-)-epicatechin, (+)-gallocatechin, (-)-epigallocatechin and catechin-3-*O*-gallate. In wine, catechins are responsible for bitterness and are partially related with their astringency (Teixeira *et al.*, 2013). The flavan-3-ols can be found in grape, apple and blueberry skins, while catechins and epicatechins, and their derivates, for example gallocatechins, are found in tea leaves and cocoa beans (Tsao, 2010). Several studies have detected the presence of catechin and epicatechin in grape stems of red and white varieties (Anastasiadi *et al*., 2012; Apostolou *et al*., 2013; Sahpazidou *et al*., 2014; Spatafora *et al*., 2013) and comparatively with other by-products (skins, leaves and seeds), grape stems are the most abundant in flavan-3-ols (namely catechin) in both red and white varieties (Teixeira *et al*., 2014).

Anthocyanins are pigments responsible for the colors of many fruits and vegetables, ranging from blue to purple, red or orange. These compounds are very important at the food

level, since they have an impact on the sensorial characteristics of foods but are also important for health, because they present different biological activities that can prevent diseases. In nature they are found in glycosides form, however the aglycone form of anthocyanins are anthocyanidins (Pascual-Teresa *et al*., 2010), being the most common pelargonidin, cyanidin, delphinidin, malvidin, peonidin and petunidin (Crozier *et al*., 2009). Anthocyanidins have an aromatic ring (A) attached to a heterocyclic ring (C) that contains oxygen, which is also attached by a carbon-carbon bond to a third aromatic ring (B) (Figure 1.2C) (Castañeda-Ovando *et al*., 2009; Machado *&* Domínguez-Perles, 2017), however they form conjugates with sugars and organic acids originating anthocyanins of different colors (Del Rio *et al*., 2013).

In grape (*Vitis vinifera*), the anthocyanins most frequently found are delphinidin, cyanidin, petunidin, peonidin and malvidin 3-glucosides, 3-(6-acetyl)-glucosides and 3-(6-*p*-coumaryol)-glucosides. Furthermore, the majority anthocyanin is malvidin-3-*O*-glucoside along with their acylated forms (Teixeira *et al*., 2013). Anthocyanins are responsible for the color in red grapes and red wines, being the skin, the part of the grape with the highest concentration in these phenolic compounds (Teixeira *et al*., 2014). In relation to the grape stem, it was identified the presence of anthocyanins malvidin-3-*O*-glucoside, malvidin-3-*O*-(6-*O*-caffeyol)-glucoside and malvidin-3-*O*-rutinoside (Barros *et al*., 2015; Queiroz *et al*., 2017).

Flavones are present in fruit and vegetables being daily consumed in our diet. These compounds are crystalline substances that can vary between colorless and yellow (Singh *et al*., 2014) and have in their structure a double bond between C_2 and C_3 carbons, but differ from other flavonoids due to the absence of the hydroxyl group at the C_3 position (Figure 1.2D) (Garrido *&* Borges, 2013).

These flavonoids are present in leaves, fruits and flowers as glycosides, being celery, red pepper, parsley and chamomile the main sources of flavones. There are several flavones, namely luteolin, apigenin, tangeritin, nobiletin, and sinensetin, among others (Panche *et al*., 2017). Concerning the presence of flavones in grape, they are not in significant quantities, except for luteolin (Garrido *&* Borges, 2013). In winery by-products these phytochemicals were identified, although in very low concentrations, with no differences detected between the white and red varieties on grape stem (Teixeira *et al*., 2014).

Flavanones can be found in citrus fruits, where they are in high concentrations, but also in tomatoes and herbs, such as mint (El Gharras, 2009). Structurally they are

characterized by the presence of a saturated three-carbon chain and an oxygen atom in the C_4 position (Figure 1.2E) (Ignat *et al*., 2011). Flavanones are generally found in the glycosylated form with a disaccharide at the C_7 position, although it may also be found with OH and O-methylated derivatives.

The main flavanones are hesperidin and naringenin, being the first the most common in the form of a glycoside (hesperidin-7-*O*-rutinoside) (Del Rio *et al*., 2013). Concerning the presence of these compounds in grape, the most abundant flavanone is eriodicitol (Garrido & Borges, 2013).

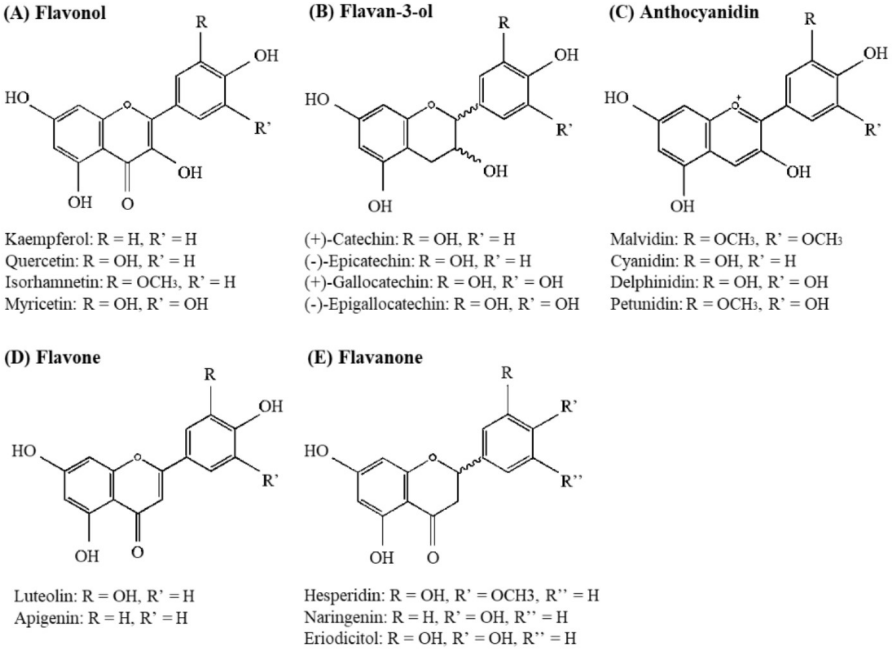


Figure 1.2. Structures of the main classes of flavonoids: (A) Flavonol, (B) Flavan-3-ol, (C) Anthocyanidin, (D) Flavone, and (E) Flavanone.

1.2.2.2. Phenolic acids

Phenolic acids represent up to one-third of the polyphenols and may be present in plants in two forms: free or bound. In the bound form, they are linked to other plant components through ester, ether or acetal bonds (Ignat *et al*., 2011). These phenolic

compounds can be divided into two groups, hydroxycinnamic acids and hydroxybenzoic acids, with a C_6-C_3 and C_6-C_1 base structure, respectively (Figure 1.3) (Balasundram *et al*., 2006; Crozier *et al*., 2009).

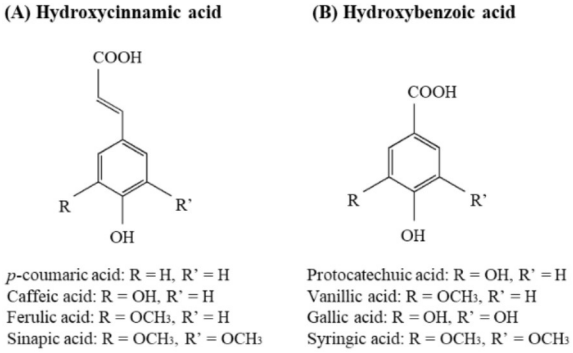


Figure 1.3. Chemical structure of hydroxycinnamic (A) and hydroxybenzoic (B) acids.

Hydroxycinnamic acids are a group of aromatic carboxylic acids, which can be found in various food, such as cinnamon, coffee beans, grape, tea, cacao, citrus, vegetables, among others (Guzman, 2014). Concerning the presence of these compounds in grape, they are generally accumulated in the skin and pulp of white and red varieties, however, despite being found in red wines, they are more concentrated in white wines, where they contribute to the oxidation (Teixeira *et al*., 2013). Ferulic, synaptic, caffeic and *p*-coumaric acids are the most abundant in the class of hydroxycinnamic acids (Figure 1.3A) (Balasundram *et al*., 2006; Crozier *et al*., 2009), and are also the most abundant in grape, except for synaptic acid. Moreover, these three acids are found in grape esterified with tartaric acid, being designated coutaric acid (*trans*-*p*-coumaroyl-tartaric acid), caftaric acid (*trans*-caffeoyl-tartaric acid), and fertaric acid (*trans*-feruloyl-tartaric acid) (Teixeira *et al*., 2013).

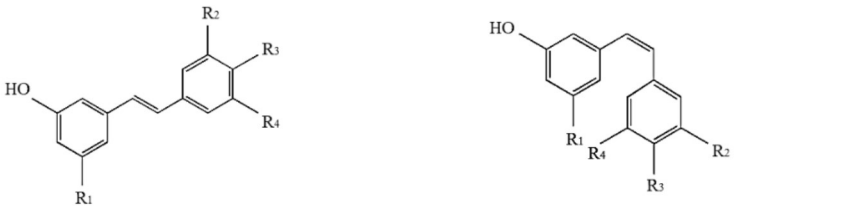
In grape stems, hydroxycinnamic acids are also detected, being identified as the main, caftaric, caffeic, ferulic, coumaric, and coutaric acids. These acids show to be in higher concentrations in the red varieties (Gouvinhas *et al*., 2019).

Relatively to hydroxybenzoic acids, the most common in grape and wine are gallic, vanillic, protocatechuic, *p*-hydroxybenzoic, and syringic acids (Figure 1.3B) (Garrido & Borges, 2013), which are found mainly in their free form. Gallic acid is the most

abundant (Teixeira *et al*., 2013) and the most important because it is the precursor of hydrolysable tannins. These acids are also present in grape by-products, but with regard to the grape stem, gallic acid is the major, followed by syringic acid (Gouvinhas *et al*., 2019; Teixeira *et al*., 2014).

1.2.2.3. Stilbenes

Stilbenes can be found in several plants, but only a few of them are present in our diet. They are produced by plants in response to stress situations, ultraviolet radiation, injury and fungal infection (*Botrytis cinearea*) (Kasiotis *et al*., 2013). These phenolic compounds have in their structure two benzene rings linked through an ethylene unit (C_6-C_2-C_6), which form a compact ring structure, separated by a double bond (Figure 1.4) (Machado & Domínguez-Perles, 2017). Compounds of this class are promising due to their biological activities, and within this group, resveratrol (3,5,40-trihydroxystilbene) is the main one, since studies show that it is a compound with anticancer, antioxidant and anti-inflammatory activities (Flamini *et al*., 2013).



trans-resveratrol: R_1 = OH, R_2 = H, R_3 = OH, R_4 = H
trans-piceid: R_1 = Gluc, R_2 = H, R_3 = OH, R_4 = H
trans-piceatannol: R_1 = OH, R_2 = OH, R_3 = H, R_4 = OH

cis-resveratrol: R_1 = OH, R_2 = H, R_3 = OH, R_4 = H
cis-piceid: R_1 = Gluc, R_2 = H, R_3 = OH, R_4 = H
cis-piceatannol: R_1 = OH, R_2 = OH, R_3 = H, R_4 = OH

Figure 1.4. Stilbenes structure.

In grape, some stilbenes can be found, namely *cis*- and *trans*-resveratrol, piceid, piceatannol (Figure 1.4), and viniferins (Flamini *et al*., 2013). Viniferins are an important group of resveratrol oligomers and the most important are α-, β-, γ-, δ-, and ε-viniferins (Teixeira *et al*., 2013). In the case of wines, red wines generally have higher concentrations of stilbenes than roses and white wines, this happens due to the contact of the musts with the skins during the fermentation, and to the high content of phenols in these red varieties (Ali & Maltese, 2010). Also, in by-products the presence of stilbenes was detected, however

differences are shown between red and white varieties. These differences translate into the presence of these phenolic compounds in stems, seeds, pomace and leaves of red varieties, whereas in the white varieties, they are only present in stems and skins (Teixeira *et al*., 2014). In relation to the stilbenes identified in grape stems, studies show the presence of *trans*-resveratrol, ε-viniferin, *trans*-piceid, *cis*-piceid, piceatannol, and vitisin-B (Gouvinhas *et al*., 2019).

1.2.2.4. Tannins

Tannins are a group of phenolic compounds that can be divided into two classes, condensed tannins and hydrolysable tannins. They are bioactive compounds with diverse biological effects, among them, metals ion chelators, precipitating agents and antioxidants (Ignat *et al*., 2011).

Condensed tannins or proanthocyanidins can be found in tea, coffee, grapes, wine, mint, and basil (Laddha & Kulkarni, 2018). These compounds are derived from the oligomerization of flavan-3-ol units, such as epicatechin and epigallocatechin, originating procyanidins (Figure 1.5A) and prodelfinidins, respectively. They can be found in skin and grape seeds (Versari *et al*., 2013), where procyanidins are the most abundant in seeds, being chemically constituted by flavan-3-ols (-)-epicatechin and/or (+)-catechin units linked by C_4-C_8 or C_4-C_6 bonds (B-type structures) or doubly linked by an additional C_2-C_7 ether bond (A-type structures). Dimeric compounds belong to the B-series, trimers to C and tetrameric to D, in addition the dimers and trimers are composed by catechins and epicatechins (Machado & Domínguez-Perles, 2017).

Proanthocyanidins are extremely important in red wines. These compounds contain astringent and bitter properties, influencing sensory quality, and also play an important role in the color stability of red wines over time, through chemical reactions of copigmentation and/or condensation with anthocyanins (Ky *et al*., 2015).

As previously mentioned, grape seeds and skins contain condensed tannins and the same happens in grape stem, where some of these compounds have been identified, namely procyanidins B1, B2, B3, B4 (Figure 1.5A), B2-gallate, C1, C2, D1, and D2 (Machado & Domínguez-Perles, 2017).

Hydrolysable tannins are synthetized by plants and trees, and can be found in wood, bark, leaves and galls. They are designated hydrolysable, since they can be hydrolysed, releasing gallic acid and/or ellagic acid (Figure 1.5B) (Ky *et al*., 2015). These compounds can

be separated into gallotannins and ellagitannins, being the first formed by the esterification of hydroxyl groups of D-glucose and gallic acid in polymer chains, while the second are esters of hexahydroxydiphenic acid and polyols, such as glucose or quinic acid (Amarowicz & Janiak, 2018).

The main source of hydrolysable tannins, more specifically ellagitannins, such as vescalagin and its epimer castalagin C1, is the wood of barrels where the wines age. Once present in wine, these compounds are slowly transformed by condensation, hydrolysis, and oxidation reactions. Moreover, they can influence the color stability and sensory profiles of red wines, as well as, protect it against oxidation (Ky *et al*., 2015).

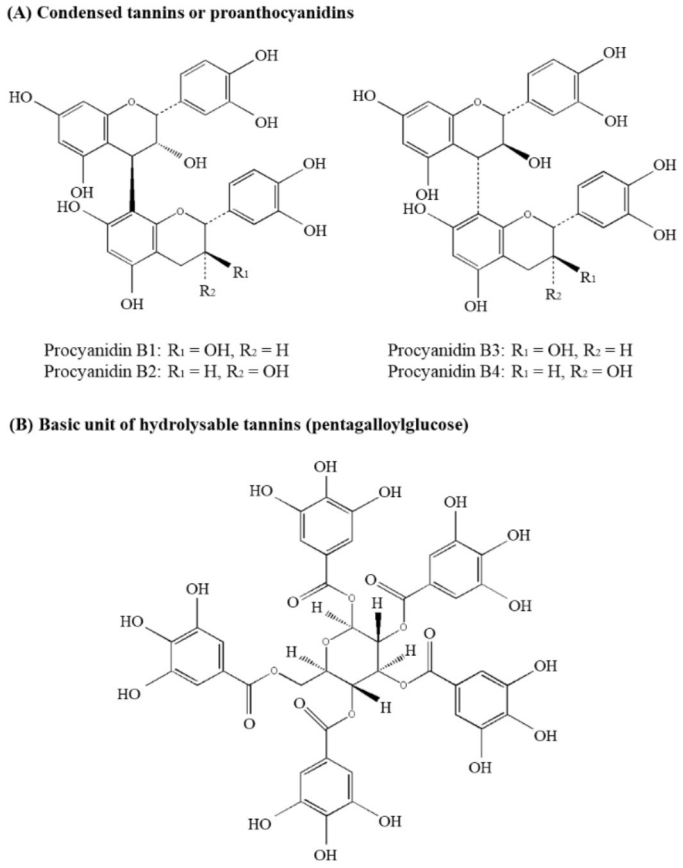


Figure 1.5. Structure of some procyanidins (A) and basic structure of hydrolysable tannins (B).

1.3. Biological activities

Grapes and red wines contain bioactive compounds that have deserve the attention of the scientific community, originating studies that show the positive effects of grape and their by-products for human health (Xia *et al*., 2013). These beneficial effects are due to the fact that phenolic compounds are potent antioxidants, which include other properties, such as anti-inflammatory, antidiabetic, cardioprotective, neuroprotective, antitumor, and anti-aging (Vuolo *et al*., 2019).

Relatively to grape stem, there are already studies in the literature that prove that this by-product has several biological activities, among them, antioxidant, antimicrobial, anti-inflammatory, and anti-carcinogenic activities (Figure 1.6) (Apostolou *et al*., 2013; Dias *et al*., 2015; Gouvinhas *et al*., 2018; Queiroz *et al*., 2017; Sahpazidou *et al*., 2014).

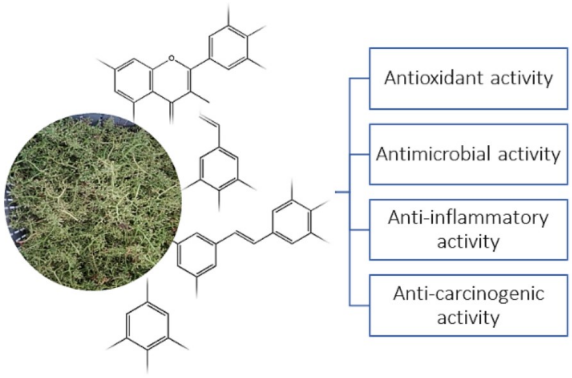


Figure 1.6. Grape stems biological activities.

1.3.1. Antioxidant activity

Antioxidants are compounds that can slow, inhibit or prevent oxidation, eliminating free radicals and reducing oxidative stress. Oxidative stress causes the development of chronic degenerative diseases, cancer and aging, due to the alteration of biological molecules such as lipids, proteins, nucleic acids and enzymes (Dai & Mumper, 2010). It is caused by the production in large concentrations of reactive oxygen species (ROS)/reactive nitrogen species (RNS) during the auto-oxidation of polyunsaturated fatty acids. These species produced include the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH^{\bullet}$), the hypochlorite ion (ClO^-), nitrogen dioxide (NO_2), peroxynitrite ($ONOO^-$), and the lipid

oxyl and peroxyl radicals (RO˙, ROO˙) (Khan & Dangles, 2013). Auto-oxidation encompasses several reactions, which are the initiation, where it occurs the formation of free radicals, the propagation, and the termination, where the production of non-radical products occurs.

The initiation and propagation reactions can be delayed, inhibited or interrupted by important antioxidant compounds designated phenolic compounds (Shahidi & Ambigaipalan, 2015). Phenolic compounds generally act as radical scavengers of lipid peroxidation chain reactions (chains breakers), since they can neutralize free radicals by donating an electron or hydrogen atom, thus making the radicals stable and interrupting chain reactions. In addition, these antioxidant compounds are chelators of transition metals, being able to avoid the oxidation caused by highly reactive hydroxyl radicals (Tsao, 2010).

Grape and wine are of the main sources of antioxidants due to their polyphenolic content. Also, grape stem is a matrix rich in phenolic compounds, and therefore, has significant antioxidant activities (Goutzourelas *et al*., 2015), which are demonstrated in several studies (Anastasiadi *et al*., 2012; Apostolou *et al*., 2013; Barros *et al*., 2014; Domínguez-Perles *et al*., 2014; Domínguez-Perles *et al*., 2016; Goutzourelas *et al*., 2015; Ruiz-Moreno *et al*., 2015; Spatafora *et al*., 2013).

Moreover, Gouvinhas *et al*. (2018), prove in their study that grape stems can be stored during several months, not losing their antioxidant activities. Additionally, Queiroz *et al*. (2017), showed in their work that the use of individual phenolic compounds (quercetin-3-*O*-glucuronide, malvidin-3-*O*-glucoside and malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside) presented more antioxidant activity than the whole grape stem extract. Besides, synergies were also tested between individual compounds and vitamins C and E, which exhibited a high antioxidant capacity.

1.3.2. Antimicrobial activity

Infectious diseases have a major impact on public health, being one of the main causes of morbidity and mortality worldwide. To combat these diseases, antibiotics are used, which currently cannot be effective due to the growing antimicrobial resistance of organisms. This resistance is a major concern worldwide and arises due to the lack of knowledge in the use of antibiotics, and to excessive and inappropriate use of these. Nowadays, in order to overcome the lack of efficacy of antibiotics, scientists are looking for alternative compounds with

different modes of action, with natural antibiotics being one of the most promising solutions (Bostanghadiri *et al*., 2017; Lima *et al*., 2019).

Natural antibiotics can be found from various sources, namely, plants, animals, bacteria, *algae* and *fungi*. Phenolic compounds, derived from plants (Gyawali & Ibrahim, 2014), are those that have a greater interest due to the high antimicrobial activity, mainly of the flavan-3-ols, flavonols and tannins, and also by exhibiting a great synergism with antibiotics. The synergy between phenolic compounds and antibiotics enhances efficacy, provide the use of a smaller amount of antibiotics, and decreases the adverse reactions to them (Daglia, 2012).

Regarding the antimicrobial compounds' mechanism of action, phenolic compounds can cause changes in the cell membranes of microbial cells, making them permeable, and consequently, causing the loss of biomolecules inside the cells. The structure of phenolic compounds influences their antimicrobial potential, since the OH groups are the major responsible for the inhibitory activity and the disruption of the cell membrane, leading to cell death (Mattos *et al*., 2017; Pisoschi *et al*., 2017). Thus, microbial growth may be activated or inhibited depending on the concentration and constitution of the phenolic compounds (Vaquero *et al*., 2007), being that, at low concentrations there is an inhibition of the activity of the microbial enzyme, while at high concentrations the denaturation of the proteins is caused (Pisoschi *et al*., 2017).

Grape contains polyphenols that have been used as natural antimicrobial agents (Xia *et al*., 2013). The same happens in their by-products, where there are already some studies showing their antimicrobial activity, such as, skins (Katalinić *et al*., 2010) and grape pomace (Oliveira *et al.*, 2013; Peixoto *et al*., 2018). Also, grape stems can be a source of antimicrobial agents. Dias *et al*. (2015), prove in their study that the bioactive compounds present in grape stems have antimicrobial activity, being more effective than antibiotics in inhibiting the growth of *Listeria monocytogenes* and *Pseudomonas aeruginosa*, and in general, show good efficacy in inhibiting the growth of Gram-positive bacteria. As well, Gouvinhas *et al*. (2018), demonstrate in their work, once again, that grape stems have antimicrobial activity, being the extracts more effective than antibiotics in inhibiting the growth of Gram-positive bacteria, particularly, *Staphylococcus aureus* and *Enterococcus faecalis*, being this characteristic maintained during the storage of this by-product, not losing this property.

1.3.3. Anti-inflammatory activity

The skin is a biological barrier between our body and the external environment. This organ has several functions, from antimicrobial defense to restriction of inflammation, acting as a defensive barrier (Rodrigues *et al*., 2018) against harmful ultraviolet radiation and exogenous chemicals, which cause a redox unbalance in the organism inducing aging, malignant process and inflammation (Queiroz *et al*., 2017).

Inflammation appears in the affected tissue through swelling, pain, heat, and redness, being a manifestation of immune defense, and therefore, helping to supress those responsible for the damage, such as virus and bacteria, and initiating healing (Khan & Dangles, 2013). Immune cells initially recognize tissue damage, and move to the site of injury releasing inflammatory mediators, like cytokines, nitric oxide, prostaglandins, among others (Dvorakova & Landa, 2017). During inflammation, large amounts of ROS and RNS are produced, constituted mainly by nitric oxide (NO). This increase of free radicals causes the activation of enzymes and transcriptional factors (transcription factor AP-1 and nuclear factor kappa B (NF-kB), for example), which will regulate the secretion of signalling molecules, which in turn, lead to tissue inflammation and release of immune cells (Gouvinhas *et al*., 2019). However, if the inflammation is prolonged it becomes chronic, causing chronic diseases, such as, for example, cancer, Alzheimer's, and cardiovascular disease (Soto *et al*., 2015).

Inflammation is usually treated with nonsteroidal anti-inflammatory drugs and corticosteroids, but these have limited efficacy and serious side effects, so there is a growing demand for new compounds with anti-inflammatory activity (Dvorakova & Landa, 2017). These new compounds, like grape polyphenols, can reduce inflammation, achieving greater effectiveness than some synthetic drugs (Soto *et al*., 2015). These bioactive compounds acquire the functions of development regulators and signalling molecules, which together with the antioxidant activity, that they possess, can interact with proteins responsible for the intracellular signalling cascades activated during inflammation, and thus, resolve the inflammatory process (Ribeiro *et al*., 2014).

Some studies have proven that grape, wine and seeds have compounds with anti-inflammatory activity (Xia *et al*., 2013). Regarding the presence of these in grape stems, Queiroz *et al*. (2017) show in their study that this by-product has anti-inflammatory activity. In this work, this biological activity was tested in a macrophage cell line RAW 264.7, where several compounds isolated from grape stems were applied, as well as the whole extract,

along with the vitamins C and E, experiencing the possible synergies between them. The results obtained showed that the isolated compounds are more effective in inhibiting NO production than the whole extract. Nevertheless, it was the synergy between malvidin-3-*O*-glucoside and vitamin E that achieved a greater inhibition of NO production (71%).

1.3.4. Anti-aging activity

Skin aging is a biological process that can be caused by intrinsic factors, such as genetic changes, vitamin deficiencies, and hormonal disorders, and extrinsic factors, like environmental toxins, UV radiation, and inadequate care (Rodrigues *et al*., 2018). Skin has an endogenous antioxidant system whose function is to avoid oxidative damage, maintaining the balance between pro-oxidants and antioxidants. However, this system can be compromised if the amount of natural antioxidants present is not enough to fight against these factors, thus causing oxidative stress and originating skin wrinkling, photoaging, lack of elasticity, appearance of fine lines and/or hyperpigmentation.

Excessive free radicals cause the loss of cellular integrity, since modifications occur in DNA and abnormal expression of cellular genes, leading to an increase in matrix metalloproteinases, which will cause protein degradation of the extracellular matrix (Soto *et al*., 2015). The matrix metalloproteinases are endopeptidases, which form the main proteolytic enzyme group responsible for dermal connective tissue degradation, and therefore, inhibition of the activity of these enzymes, such as elastases, may be essential to prevent premature aging of the skin, as well as skin changes. For this inhibition to happen, it is necessary the elimination of free radicals (ROS), since these are responsible for the activation of these enzymes. Phenolic compounds are powerful antioxidants that can eliminate free radicals, furthermore, these bioactive compounds can also inhibit the activity of proteolytic enzymes by transforming themselves into complexing or precipitation agents (Wittenauer *et al*., 2015). Due to these characteristics, the use of polyphenols in dermal cosmetics products is promising (Działo *et al*., 2016), having as main enzymatic targets, elastase and tyrosinase (Chiocchio *et al*., 2018).

Elastase is a proteolytic enzyme responsible for the breakdown of proteins, especially elastin. Elastin can be found inside the extracellular matrix, having as the main function to give elasticity to the skin, lungs, arteries and ligaments. Tyrosinase is an enzyme responsible

for the production of melanin in the hair and skin, and its inhibition leads to skin lightening properties (Taghouti *et al*., 2018).

Grape is a matrix rich in polyphenols beneficial to the skin, and, as expected, their by-products, especially the skins and seeds (Rodrigues *et al.*, 2018). Although there are no studies in the literature that prove that grape stem has anti-aging activity, there are already some about grape pomace. Effectively, Wittenauer *et al*. (2015) and Ferri *et al*. (2017) have proven in their studies that grape pomace extracts can inhibit the activity of the enzymes collagenase and elastase, and tyrosinase, respectively, demonstrating their potential for cosmetic applications.

1.4. Essential minerals /toxic metals present in grapes and by-products

1.4.1. Essential Minerals

Essential minerals are a group formed by various elements, for example, Na, K, Mg, Ca, Mn, Zn, Cu, Fe, and Se. If these elements are not present in the human diet, or are ingested in insufficient quantities, several deficiencies are caused in the organism (Mir-Marqués *et al*., 2016; Rodríguez-Solana *et al*., 2014), such as, hypertension, stopping of growth, depression, weakness, among others. Contrary, excessive consumption leads to symptoms of toxicity, for example nausea, hair and nail loss, and hallucinations. Thus, the consumption of essential minerals can range from a few micrograms to 1 g per day, depending on the mineral (Nosratpour *et al*., 2018).

Regarding the presence of essential minerals in grape, Acuña-Avila *et al*. (2016) detected in their study the presence of several elements, being K the most abundant followed by Ca, Mg and Na, whereas, Vystavna *et al*. (2014) only quantified the elements Cu, Pb and Zn in grape, where Cu presented the highest content. Regarding the presence of essentials minerals in raisins, in the work of Fabani *et al*. (2017), numerous elements were quantified, being K the most abundant, followed by Ca and Mg. With respect to by-products, Canizo *et al*. (2019) quantified various elements in grape skins, presenting Fe element the highest concentration. Pantelić *et al*. (2017) detected in their study several essentials minerals in leaves, being K the majority, and having a remarkable content in B, Ca, Na, Mg, and S. Again, Vystavna *et al*. (2014) quantified Cu, Pb, and Zn in leaves, where Zn showed the highest content.

1.4.2. Toxic metals

The elements belonging to this group, such as, Al, As, Cd, and Pb, may cause undesirable effects on human health (Mir-Marqués *et al*., 2016), since their accumulation in the body causes serious injuries, mainly in the respiratory, nervous, reproductive, and digestive systems (Kim *et al*., 2019). Due to these damages, toxic metals represent a public health problem, and therefore legislation has been created to limit their quantities in food (Gouvinhas *et al*., 2015).

Some studies demonstrated the presence of toxic metals in grape and by-products. Their presence may be due to environmental factors, such as geography, climate, type and composition of soil, and grape variety, and also to anthropogenic factors, like vineyard pollution (soil contamination and water quality), viticulture management practices (use of chemicals products, fertilizers, etc) and vinification technology (Vystavna *et al*., 2014).

Once again, Acuña-Avila *et al.* (2016) presented in their work the existence of toxic metals in grapes, such as Pb, Sr, Tl and Al, however they are present in small quantities and they not represent a danger to health. Also, Fabani *et al*. (2017) decided to analyse the presence of toxic metals in raisins, and although they evaluated the contents of eleven metals (Ag, Al, As, Bi, Cd, Co, Ga, Ni, Pb, Te and Tl), only Al was above the limit of quantification, but still below the established maximum limits, being safe its consumption. Relatively to the by-products, Canizo *et al*. (2019) and Pantelić *et al*. (2017), analysed the content of toxic metals, such as Pb, As and Cd, in grape skins and leaves, respectively, being these metals detected in small concentrations, and therefore their use was considered safe.

1.5. Objectives

The production of large quantities of by-products by the wine industry, such as, grape stems, put at risk the sustainability of the environment, since most of these by-products are unexplored and discarded. Thus, in order to ensure sustainable production to the environment it is necessary to reuse these by-products. In this sense, it is estimated that there may be the ability to develop innovative products using the grape stem, due to the bioactive compounds potentially beneficial to human health it contains, encouraging further research with this by-product.

Therefore, the main objective of this work is the enhancement of the grape stem through the extraction of phenolic compounds from this matrix, which will be later applied in

new products. In this way, it is required to evaluate the phytochemical composition and *in vitro* biological activities of this by-product.

Thus, to the main objective to be performed, it is essential to accomplish the following purposes:

- To evaluate the presence of essential minerals and toxic metals in grape stem;
- To quantify the phenolic compounds and determine which are the most abundant in grape stem extracts;
- To determine the antioxidant activity of grape stem extracts;
- To evaluate the antimicrobial activity of grape stem extracts against gastrointestinal and diabetic foot wound bacteria of hospital patients;
- To determine the potential toxicity of grape stem extracts in cell cultures (mouse macrophages), and subsequently evaluate their anti-inflammatory activity;
- To evaluate the anti-aging ability of grape stem extracts by inhibiting the elastase and tyrosinase enzymes.

1.6. References

Acuña-Avila, P. E., Vásquez-Murrieta, M. S., Olivia, M., Hernández, F., & López-Cortéz, M. S. (2016). Relationship between the elemental composition of grapeyards and bioactive compounds in the Cabernet Sauvignon grapes Vitis vinifera harvested in Mexico. *Food Chemistry*, 203, 79–85. https://doi.org/10.1016/j.foodchem.2016.02.031

Ali, K., & Maltese, F. (2010). Metabolic constituents of grapevine and grape-derived products. *Phytochemistry Reviews*, 9, 357–378. https://doi.org/10.1007/s11101-009-9158-0

Amarowicz, R., & Janiak, M. (2018). Hydrolysable Tannins. *Reference Module in Food Science*. Elsevier. https://doi.org/10.1016/B978-0-08-100596-5.21771-X

Anastasiadi, M., Pratsinis, H., Kletsas, D., Skaltsounis, A. L., & Haroutounian, S. A. (2012). Grape stem extracts: Polyphenolic content and assessment of their in vitro antioxidant properties. *LWT - Food Science and Technology*, 48(2), 316–322. https://doi.org/10.1016/j.lwt.2012.04.006

Apostolou, A., Stagos, D., Galitsiou, E., Spyrou, A., Haroutounian, S., Portesis, N., … Kouretas, D. (2013). Assessment of polyphenolic content, antioxidant activity, protection against ROS-induced DNA damage and anticancer activity of Vitis vinifera stem extracts. *Food and Chemical Toxicology*, 61, 60–68. https://doi.org/10.1016/j.fct.2013.01.029

Balasundram, N., Sundram, K., & Samman, S. (2006). Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chemistry*, 99(1), 191–203. https://doi.org/10.1016/j.foodchem.2005.07.042

Barba, F. J., Zhu, Z., Koubaa, M., Sant'Ana, A. S., & Orlien, V. (2016). Green alternative methods for the extraction of antioxidant bioactive compounds from winery wastes and by-products: A review. *Trends in Food Science and Technology*, 49, 96–109. https://doi.org/10.1016/j.tifs.2016.01.006

Barros, A., Gironés-Vilaplana, A., Teixeira, A., Collado-González, J., Moreno, D. A., Gil-Izquierdo, A., … Domínguez-Perles, R. (2014). Evaluation of grape (Vitis vinifera L.) stems from Portuguese varieties as a resource of (poly)phenolic compounds: A comparative study. *Food Research International*, 65(PC), 375–384. https://doi.org/10.1016/j.foodres.2014.07.021

Barros, A., Gironés-Vilaplana, A., Texeira, A., Baenas, N., & Domínguez-Perles, R. (2015). Grape stems as a source of bioactive compounds: application towards added-value commodities and significance for human health. *Phytochemistry Reviews*, 14(6), 921–931. https://doi.org/10.1007/s11101-015-9421-5

Bostanghadiri, N., Pormohammad, A., Salimi, A., & Pouriran, R. (2017). Comprehensive review on the antimicrobial potency of the plant polyphenol Resveratrol. *Biomedicine & Pharmacotherapy*, 95(September), 1588–1595. https://doi.org/10.1016/j.biopha.2017.09.084

Canizo, B. V, Escudero, L. B., Pellerano, R. G., & Wuilloud, R. G. (2019). Data mining approach based on chemical composition of grape skin for quality evaluation and traceability prediction of grapes. *Computers and Electronics in Agriculture*, 162(September 2018), 514–522. https://doi.org/10.1016/j.compag.2019.04.043

Castañeda-Ovando, A., Pacheco-Hernández, M. D. L., Páez-Hernández, M. E., Rodríguez, J. A., & Galán-Vidal, C. A. (2009). Chemical studies of anthocyanins: A review. *Food Chemistry*, 113(4), 859–871. https://doi.org/10.1016/j.foodchem.2008.09.001

Castillo-Muñoz, N., Gómez-Alonso, S., García-Romero, E., & Hermosín-Gutiérrez, I. (2007). Flavonol profiles of Vitis vinifera red grapes and their single-cultivar wines. *Journal of Agricultural and Food Chemistry*, 55(3), 992–1002. https://doi.org/10.1021/jf062800k

Che, D. N., Xie, G. H., Cho, B. O., Shin, J. Y., Kang, H. J., & Jang, S. Il. (2017). Protective effects of grape stem extract against UVB-induced damage in C57BL mice skin. *Journal of Photochemistry and Photobiology B: Biology*, 173(May), 551–559. https://doi.org/10.1016/j.jphotobiol.2017.06.042

Chiocchio, I., Mandrone, M., Sanna, C., Maxia, A., Tacchini, M., & Poli, F. (2018). Screening of a hundred plant extracts as tyrosinase and elastase inhibitors, two enzymatic targets of cosmetic interest. *Industrial Crops & Products*, 122(April), 498–505. https://doi.org/10.1016/j.indcrop.2018.06.029

Crozier, A., Jaganath, I. B., & Clifford, M. N. (2009). Dietary phenolics: chemistry, bioavailability and effects on health. *Natural Product Reports*, 26(8), 965–1096. https://doi.org/10.1039/b802662a

Daglia, M. (2012). Polyphenols as antimicrobial agents. *Current Opinion in Biotechnology*, 23(2), 174–181. https://doi.org/10.1016/j.copbio.2011.08.007

Dai, J., & Mumper, R. J. (2010). Plant phenolics: Extraction, analysis and their antioxidant and anticancer properties. *Molecules*, 15(10), 7313–7352. https://doi.org/10.3390/molecules15107313

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. *Antioxidants & Redox Signaling*, 18(14), 1818–1892. https://doi.org/10.1089/ars.2012.4581

Dias, C., Domínguez-Perles, R., Aires, A., Teixeira, A., Rosa, E., Barros, A., & Saavedra, M. J. (2015). Phytochemistry and activity against digestive pathogens of grape (Vitis vinifera L.) stem's (poly)phenolic extracts. *LWT - Food Science and Technology*, 61(1), 25–32. https://doi.org/10.1016/j.lwt.2014.11.033

Domínguez-Perles, R., Teixeira, A. I., Rosa, E., & Barros, A. I. (2014). Assessment of (poly)phenols in grape (Vitis vinifera L.) stems by using food/pharma industry compatible solvents and Response Surface Methodology. *Food Chemistry*, 164, 339–346. https://doi.org/10.1016/j.foodchem.2014.05.020

Domínguez-Perles, Raúl, Guedes, A., Queiroz, M., Silva, A. M., & Barros, A. I. R. N. A. (2016). Oxidative stress prevention and anti-apoptosis activity of grape (Vitis vinifera L.) stems in human keratinocytes. *Food Research International*, 87, 92–102. https://doi.org/10.1016/j.foodres.2016.06.030

Dvorakova, M., & Landa, P. (2017). Anti-inflammatory activity of natural stilbenoids: A review. *Pharmacological Research*, 124(October), 126–145. https://doi.org/10.1016/j.phrs.2017.08.002

Działo, M., Mierziak, J., Korzun, U., Preisner, M., Szopa, J., & Kulma, A. (2016). The Potential of Plant Phenolics in Prevention and Therapy of Skin Disorders. *International Journal of Molecular Sciences*, 17(160), 1–41. https://doi.org/10.3390/ijms17020160

El Gharras, H. (2009). Polyphenols: Food sources, properties and applications - A review. *International Journal of Food Science and Technology*, 44(12), 2512–2518. https://doi.org/10.1111/j.1365-2621.2009.02077.x

Fabani, M. P., Baroni, M. V, Luna, L., Lingua, M. S., Monferran, M. V, Paños, H., … Egly, G. (2017). Journal of Food Composition and Analysis Changes in the phenolic pro fi le of Argentinean fresh grapes during production of sun-dried raisins. *Journal of Food Composition and Analysis*, 58, 23–32. https://doi.org/10.1016/j.jfca.2017.01.006

Favre, G., González-Neves, G., Piccardo, D., Gómez-Alonso, S., Pérez-Navarro, J., & Hermosín-Gutiérrez, I. (2018). New acylated flavonols identified in Vitis vinifera grapes

and wines. *Food Research International*, 112(October), 98–107. https://doi.org/10.1016/j.foodres.2018.06.019

Ferri, M., Rondini, G., Maddalena, M., Michelini, E., Vallini, V., Fava, F., … Tassoni, A. (2017). White grape pomace extracts, obtained by a sequential enzymatic plus ethanol-based extraction, exert antioxidant, anti-tyrosinase and anti-inflammatory activities. *New Biotechnology*, 39, Part A(October), 51–58. https://doi.org/10.1016/j.nbt.2017.07.002

Flamini, R., Mattivi, F., De Rosso, M., Arapitsas, P., & Bavaresco, L. (2013). Advanced knowledge of three important classes of grape phenolics: Anthocyanins, stilbenes and flavonols. *International Journal of Molecular Sciences*, 14(10), 19651–19669. https://doi.org/10.3390/ijms141019651

Garrido, J., & Borges, F. (2013). Wine and grape polyphenols - A chemical perspective. *Food Research International*, 54(2), 1844–1858. https://doi.org/10.1016/j.foodres.2013.08.002

Goutzourelas, N., Stagos, D., Spanidis, Y., Liosi, M., Apostolou, A., Priftis, A., … Kouretas, D. (2015). Polyphenolic composition of grape stem extracts affects antioxidant activity in endothelial and muscle cells. *Molecular Medicine Reports*, 12, 5846–5856. https://doi.org/10.3892/mmr.2015.4216

Gouvinhas, I., Machado, N., Cunha, M., Pereira, M., Matos, C., Gomes, S., … Barros, A. I. R. N. A. (2015). Trace element content of monovarietal and commercial Portuguese olive oils. *Journal of Oleo Science*, 64(10). https://doi.org/10.5650/jos.ess15101

Gouvinhas, I., Santos, R. A., Queiroz, M., Leal, C., Saavedra, M. J., Domínguez-Perles, R., … Barros, A. I. R. N. A. (2018). Monitoring the antioxidant and antimicrobial power of grape (*Vitis vinifera* L.) stems phenolics over long-term storage. *Industrial Crops and Products*, 126. https://doi.org/10.1016/j.indcrop.2018.10.006

Gouvinhas, Irene, Queiroz, M., Rodrigues, M., & Barros, A. I. R. N. A. (2019). Evaluation of the Phytochemistry and Biological Activity of Grape (*Vitis vinifera* L.) Stems: Toward a Sustainable Winery Industry. *Polyphenols in Plants* (2nd ed.). Elsevier Inc. https://doi.org/10.1016/B978-0-12-813768-0.00023-2

Guzman, J. D. (2014). Natural Cinnamic Acids, Synthetic Derivatives and Hybrids with Antimicrobial Activity. *Molecules*, 19, 19292–19349. https://doi.org/10.3390/molecules191219292

Gyawali, R., & Ibrahim, S. A. (2014). Natural products as antimicrobial agents. *Food Control*. https://doi.org/10.1016/j.foodcont.2014.05.047

Ignat, I., Volf, I., & Popa, V. I. (2011). A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables. *Food Chemistry*, 126(4), 1821–1835. https://doi.org/10.1016/j.foodchem.2010.12.026

Jeffery, D. W., Parker, M., & Smith, P. A. (2008). Flavonol composition of Australian red and white wines determined by high-performance liquid chromatography. *Australian Journal of Grape and Wine Research*, 14(3), 153–161. https://doi.org/10.1111/j.1755-0238.2008.00017.x

Karvela, E., Makris, D. P., Kalogeropoulos, N., & Karathanos, V. T. (2011). Deployment of response surface methodology to optimise recovery of grape (*Vitis vinifera*) stem polyphenols. *Talanta*, 79(5), 1311–1321. https://doi.org/10.1016/j.talanta.2009.05.042

Kasiotis, K. M., Pratsinis, H., Kletsas, D., & Haroutounian, S. A. (2013). Resveratrol and related stilbenes: Their anti-aging and anti-angiogenic properties. *Food and Chemical Toxicology*, 61, 112–120. https://doi.org/10.1016/j.fct.2013.03.038

Katalinić, V., Možina, S. S., Skroza, D., Generalić, I., Abramovič, H., Miloš, M., … Boban, M. (2010). Polyphenolic profile, antioxidant properties and antimicrobial activity of grape skin extracts of 14 Vitis vinifera varieties grown in Dalmatia (Croatia). *Food Chemistry*, 119(2), 715–723. https://doi.org/10.1016/j.foodchem.2009.07.019

Khan, M. K., & Dangles, O. (2013). A comprehensive review on flavanones, the major citrus polyphenols. *Journal of Food Composition and Analysis*. https://doi.org/10.1016/j.jfca.2013.11.004

Kim, J., Kim, Y., & Kumar, V. (2019). Heavy metal toxicity: an update of chelating therapeutic strategies. *Journal of Trace Elements in Medicine and Biology*. https://doi.org/10.1016/j.jtemb.2019.05.003

Ky, I., Le Floch, A., Zeng, L., Pechamat, L., Jourdes, M., & Teissedre, P. L. (2015). Tannins. *Encyclopedia of Food and Health*, 247–255. https://doi.org/10.1016/B978-0-12-384947-2.00683-8

Laddha, A. P., & Kulkarni, Y. A. (2018). Tannins and Vascular Complications of Diabetes: An update. *Phytomedicine*, 56(March), 229–245. https://doi.org/10.1016/j.phymed.2018.10.026

Lima, M. C., Sousa, C. P. De, Harel, J., Dubreuil, J. D., & Souza, E. L. De. (2019). A review of the current evidence of fruit phenolic compounds as potential antimicrobials against pathogenic bacteria. *Microbial Pathogenesis*. https://doi.org/10.1016/j.micpath.2019.03.025

Machado, N. F. L., & Domínguez-Perles, R. (2017). Addressing facts and gaps in the phenolics chemistry of winery by-products. *Molecules*, 22(2), 1–48. https://doi.org/10.3390/molecules22020286

Makris, D. P., Boskou, G., & Andrikopoulos, N. K. (2007). Polyphenolic content and in vitro antioxidant characteristics of wine industry and other agri-food solid waste extracts. *Journal of Food Composition and Analysis*, 20(2), 125–132. https://doi.org/10.1016/j.jfca.2006.04.010

Mateo, J. J., & Maicas, S. (2015). Valorization of winery and oil mill wastes by microbial technologies. *Food Research International*, 73, 13–25. https://doi.org/10.1016/j.foodres.2015.03.007

Mattos, G. N., Tonon, R. V., Furtado, A. A. L., & Cabral, L. M. C. (2017). Grape by-product extracts against microbial proliferation and lipid oxidation: a review. *Journal of the Science of Food and Agriculture*, 97(4), 1055–1064. https://doi.org/10.1002/jsfa.8062

Mir-Marqués, A., Cervera, M. L., & Guardia, M. De. (2016). Mineral analysis of human diets by spectrometry methods. *Trends in Analytical Chemistry*, 82(September), 457–467. https://doi.org/10.1016/j.trac.2016.07.007

Nosratpour, M., Jafari, S. M., & Resources, N. (2018). Bioavailability of Minerals (Ca, Mg, Zn, K, Mn, Se) in Food Products. *Encyclopedia of Food Chemistry*. Elsevier. https://doi.org/10.1016/B978-0-08-100596-5.21618-1

Nunes, M. A., Rodrigues, F., & Oliveira, M. B. P. P. (2017). Grape Processing By-Products as Active Ingredients for Cosmetic Proposes. *Handbook of Grape Processing By-Products: Sustainable Solutions*. Elsevier Inc. https://doi.org/10.1016/B978-0-12-809870-7.00011-9

OIV. (2018). International Organisation of Vine and Wine. OIV Statistical Report on World Vitiviniculture. Retrieved December 15, 2018, from http://www.oiv.int/public/medias/5479/oiv-en-bilan-2017.pdf

Oliveira, D. A., Salvador, A. A., Smânia, A., Smânia, E. F. A., Maraschin, M., & Ferreira, S. R. S. (2013). Antimicrobial activity and composition profile of grape (*Vitis vinifera*) pomace extracts obtained by supercritical fluids. *Journal of Biotechnology*, 164(3), 423–432. https://doi.org/10.1016/j.jbiotec.2012.09.014

Panche, A. N., Diwan, A. D., & Chandra, S. R. (2017). Flavonoids: an overview. *Journal of Nutritional Science*, 5(47), 1–15. https://doi.org/10.1017/jns.2016.41

Pantelić, M. M., Dabi, D. Č., Ivanka, Ž. Ć., Pergal, M. V, Reli, D. J., Todi, S. R., & Nati, M. M. (2017). Phenolic profiles, antioxidant activity and minerals in leaves of different grapevine varieties grown in Serbia. *Journal of Food Composition and Analysis*, 62(January), 76–83. https://doi.org/10.1016/j.jfca.2017.05.002

Pascual-Teresa, S. de, Moreno, D. A., & García-Viguera, C. (2010). Flavanols and anthocyanins in cardiovascular health: A review of current evidence. *International Journal of Molecular Sciences*, 11(4), 1679–1703. https://doi.org/10.3390/ijms11041679

Peixoto, C. M., Dias, M. I., Alves, M. J., Calhelha, R. C., Barros, L., Pinho, S. P., & Ferreira, I. C. F. R. (2018). Grape pomace as a source of phenolic compounds and diverse bioactive properties. *Food Chemistry*, 253(November 2017), 132–138. https://doi.org/10.1016/j.foodchem.2018.01.163

Pisoschi, A. M., Pop, A., Georgescu, C., Turcuş, V., Olah, K., & Mathe, E. (2017). An overview of natural antimicrobials role in food. *European Journal of Medicinal Chemistry*. https://doi.org/10.1016/j.ejmech.2017.11.095

Queiroz, M., Oppolzer, D., Gouvinhas, I., Silva, A. M., Barros, A. I. R. N. A., & Domínguez-Perles, R. (2017). New grape stems' isolated phenolic compounds modulate reactive oxygen species, glutathione, and lipid peroxidation in vitro: Combined formulations with vitamins C and E. *Fitoterapia*, 120, 146–157. https://doi.org/10.1016/j.fitote.2017.06.010

Ribeiro, D., Freitas, M., Tomé, S. M., Silva, A. M. S., Laufer, S., Lima, J. L. F. C., & Fernandes, E. (2014). Flavonoids Inhibit COX-1 and COX-2 Enzymes and

Cytokine/Chemokine Production in Human Whole Blood. *Inflammation*, 38(2), 858–870. https://doi.org/10.1007/s10753-014-9995-x

Rodrigues, F., Pinto, D., Vinha, A. F., & Carretero, A. S. (2018). Cosmetics. *In Polyphenols: Properties, Recovery, and Applications* (pp. 393–427). https://doi.org/10.1016/B978-0-12-813572-3.00012-9

Rodríguez-Solana, R., Salgado, J. M., Domínguez, J. M., & Cortés, S. (2014). Assessment of minerals in aged grape marc distillates by FAAS/FAES and ICP-MS. Characterization and safety evaluation. *Food Control*, 35(1), 49–55. https://doi.org/10.1016/j.foodcont.2013.06.031

Ross, J. A., & Kasum, C. M. (2002). DIETARY FLAVONOIDS: Bioavailability, Metabolic Effects, and Safety. *Annual Review of Nutrition*, 22(1), 19–34. https://doi.org/10.1146/annurev.nutr.22.111401.144957

Ruiz-Moreno, M. J., Raposo, R., Cayuela, J. M., Zafrilla, P., Piñeiro, Z., Moreno-Rojas, J. M., … Cantos-Villar, E. (2015). Valorization of grape stems. *Industrial Crops and Products*, 63, 152–157. https://doi.org/10.1016/j.indcrop.2014.10.016

Sahpazidou, D., Geromichalos, G. D., Stagos, D., Apostolou, A., Haroutounian, S. A., Tsatsakis, A. M., … Kouretas, D. (2014). Anticarcinogenic activity of polyphenolic extracts from grape stems against breast, colon, renal and thyroid cancer cells. *Toxicology Letters*, 230(2), 218–224. https://doi.org/10.1016/j.toxlet.2014.01.042

Shahidi, F., & Ambigaipalan, P. (2015). Phenolics and polyphenolics in foods , beverages and spices : Antioxidant activity and health effects –. *Journal of Functional Foods*, 18, 820–897. https://doi.org/10.1016/j.jff.2015.06.018

Singh, M., Kaur, M., & Silakari, O. (2014). Flavones: An Important Scaffold for Medicinal Chemistry. *European Journal of Medicinal Chemistry*, 84(September), 206–239. https://doi.org/10.1016/j.ejmech.2014.07.013

Soto, M. L., Falqué, E., & Domínguez, H. (2015). Relevance of Natural Phenolics from Grape and Derivative Products in the Formulation of Cosmetics. *Cosmetics*, 2(3), 259–276. https://doi.org/10.3390/cosmetics2030259

Spatafora, C., Barbagallo, E., Amico, V., & Tringali, C. (2013). Grape stems from Sicilian Vitis vinifera cultivars as a source of polyphenol-enriched fractions with enhanced antioxidant activity. *LWT - Food Science and Technology*, 54(2), 542–548. https://doi.org/10.1016/j.lwt.2013.06.007

Taghouti, M., Martins-Gomes, C., Schäfer, J., Félix, L. M., Santos, J. A., Bunzel, M., … Silva, A. M. (2018). Thymus pulegioides L. as a rich source of antioxidant, anti-proliferative and neuroprotective phenolic compounds. *Food and Function*, 9(7), 3617–3629. https://doi.org/10.1039/c8fo00456k

Teixeira, Ana, 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. *International Journal of Molecular Sciences*, 15(9), 15638–15678. https://doi.org/10.3390/ijms150915638

Teixeira, António, Eiras-Dias, J., Castellarin, S. D., & Gerós, H. (2013). Berry phenolics of grapevine under challenging environments. *International Journal of Molecular Sciences*, 14(9), 18711–18739. https://doi.org/10.3390/ijms140918711

Teixeira, N., Mateus, N., de Freitas, V., & Oliveira, J. (2018). Wine industry by-product: Full polyphenolic characterization of grape stalks. *Food Chemistry*, 268, 110–117. https://doi.org/10.1016/j.foodchem.2018.06.070

Tsao, R. (2010). Chemistry and biochemistry of dietary polyphenols. *Nutrients*, 2(12), 1231–1246. https://doi.org/10.3390/nu2121231

Vaquero, M. J. R., Alberto, M. R., & de Nadra, M. C. M. (2007). Antibacterial effect of phenolic compounds from different wines. *Food Control*, 18(2), 93–101. https://doi.org/10.1016/j.foodcont.2005.08.010

Versari, A., Du Toit, W., & Parpinello, G. P. (2013). Oenological tannins: A review. *Australian Journal of Grape and Wine Research*, 19(1), 1–10. https://doi.org/10.1111/ajgw.12002

Vuolo, M. M., Lima, V. S., Roberto, M., & Junior, M. (2019). Phenolic Compounds: Structure, Classification, and Antioxidant Power. *In Bioactive Compounds: Health Benefits and Potential Applications* (pp. 33–50). Elsevier Inc. https://doi.org/10.1016/B978-0-12-814774-0.00002-5

Vystavna, Y., Rushenko, L., Diadin, D., Klymenko, O., & Klymenko, M. (2014). Trace metals in wine and vineyard environment in southern Ukraine. *Food Chemistry*, 146, 339–344. https://doi.org/10.1016/j.foodchem.2013.09.091

Wittenauer, J., Mäckle, S., Sußmann, D., Schweiggert-weisz, U., & Carle, R. (2015). Inhibitory effects of polyphenols from grape pomace extract on collagenase and elastase activity. *Fitoterapia*, 1–9. https://doi.org/10.1016/j.fitote.2015.01.005

Xia, E., He, X., Li, H., Wu, S., Li, S., & Deng, G. (2013). Biological Activities of Polyphenols from Grapes. *In Polyphenols in Human Health and Disease* (Vol. 1, pp. 47–58). Elsevier Inc. https://doi.org/10.1016/B978-0-12-398456-2.00005-0

Chapter II:

Essential minerals and toxic metals present in grape stems

Essentials minerals and toxic metals present in grape stems

Abstract

The production of grape (*Vitis vinifera* L.) is one of the main economic activities of the agri-food sector, where millions of tons of by-products are produced annually, being the grape stems one of these by-products. Currently, there is a growing interest in the application of grape stem as source of phenolic compounds, for its use in the cosmetic, pharmaceutical and food industries. Thus, the main objective of this study is to determine the content of essential minerals and toxic metals present in grape stems, to understand if this by-product can be applied in the development of new products as a source of mineral nutrients and if its use is safe for consumers. Six varieties of grape stem were analyzed spectrometrically and fluorimetrically concerning their metal composition.

The results demonstrated that grape stems presented a high content of essential minerals, being the most abundant Na, Mg, Ca, and K, ranging from 0.65, 1.34, and 1.41 to 24.45 g/Kg dw, respectively, on average. Concerning the toxic metals analyzed, Al and As presented the highest concentrations, namely, 200.80 and 0.23 mg/Kg dw, respectively, on average. In fact, grape stems are richer in essential minerals than the other by-products of the winery industry and may even have higher mineral content than some food matrices consumed in our diet, such as olive oil or beans. In this sense, since consumers are increasingly searching for products from natural sources, grape stems can be a good bet in the production of value added products for several industries. However, it is necessary to ensure that this matrix is safe, and although some varieties may exceed the legal maximum limits for certain toxic metals, there are other varieties where their use is safe. In conclusion, for grape stems to become a source of essential minerals in industries one hundred per cent safe, greater care is needed in the use of chemicals by producers, and a reduction in air pollution, thus helping the environment.

Keywords: Grape stem; By-products; Essential minerals; Toxic metals; Human diet.

2. Introduction

The grape is one of the largest cultures in the world, being part of the human diet since ancient times (Apostolou *et al*., 2013). Currently, millions of tons are produced worldwide (Southern Europe, South Africa, Australia, and North and South America), generating amounts of by-products, such as grape stems (Gouvinhas *et al.*, 2019). This by-product is usually discarded in open areas or used for composting, causing environmental problems due to its content in polyphenols (compounds known to have antimicrobial activity), thus preventing its deterioration (Teixeira *et al*., 2014). Since the accumulation of these by-products causes serious environmental problems, recycling strategies need to be found (Deiana *et al*., 2009) in order to achieve environmentally friendly production (Karvela *et al*., 2011), and therefore, there has been a growing interest from the scientific community in using the by-products from the wine industry as a cheap source of phenolic compounds to be applied in several areas (cosmetic, pharmaceutical and food industries) (Gouvinhas *et al*., 2018; Piñeiro *et al*., 2013).

In grapes and wine, several mineral elements can be found. The presence of these minerals, and consequently their concentrations, can be influenced by several factors, namely, the uptake characteristics of the rootstock, climatic influences on the rate of transpiration, accumulation by the scion and fruit, and the characteristics of the soil mineral content of the vine (Jackson, 2014). These minerals are essentials for the human diet, and therefore, are designated as essential minerals. This group is formed by several elements, such as Na, K, Mg, Ca, Zn, and Fe, as well as others, which are only needed in small quantities such as, Se, Co, Ni, Cr, Mn, and Cu (Mir-Marqués *et al*., 2016; Rodríguez-Solana *et al*., 2014). Essential minerals play important roles in the proper functioning of our body, for example, Ca, Mg, K, Mn, and Se, contribute to the health of bones and teeth, and structural parts of enzymes (Nosratpour *et al.*, 2018). In the case of Cu, Zn, Fe, and Co elements, they are important for cell growth, enzyme reactions, and body immunity, among others (Kim *et al*., 2019).

However, other elements can be found in the vineyard soils, and consequently, in the vines, namely toxic or heavy metals (As, Al, Cd, Pb). These elements appear in the soils, mainly due to the fertilizers and chemical pesticides used, and due to industrial activities or traffic. Thus, in the last years there has been an growing concern by the population about the increase in the quantity of toxic elements in plants (Milićević *et al*., 2018), since these metals, when accumulated in the human body, can have negative effects, causing damage, for example, in the kidneys, nervous and immune systems, and even having carcinogenic effects

(Volpe *et al*., 2009). For these reasons, maximum levels of toxic metals in food has been set in most countries to prevent possible poisoning (Edelstein & Ben-hur, 2017).

In this way, in order to verify the possibility to use grape stems in distinct industry areas, their content in essential minerals and toxic metals need to be determined to find out, on the one hand, if it will be a potential source of minerals, and on the other, if these by-product can be used safely in new and innovative products on food, cosmetic, and pharmaceutical industries.

2.1. Material and methods

2.1.1. Chemicals

Hydrogen peroxide (H_2O_2, 30% p.a.), hydrocloridric acid (HCl) and concentrated nitric acid (HNO_3, 65% p.a.) were obtained from Merck (Darmstadt, Germany). 2,3-diaminonaphthalene (DAN), cyclohexane and EDTA were purchase from Sigma-Aldrich (Steinheim, Germany). Ultrapure water was obtained using a Millipore water purification system (Millipore, Bedford, MA, USA). The stock solutions (1000 mg/L) required for the dilutions of standards used for calibration and quantification of elements were acquired from Merck (Darmstadt, Germany) and the stock solution (1000 mg/L) of Se was purchase from Fluka Chemika (Neu-Ulm, Switzerland).

2.1.2. Sampling

For the accomplishment of this work the sampling was constituted by six grape (*Vitis vinifera* L.) stems varieties: Tinta Roriz, Touriga Nacional, Castelão, Syrah (red varieties), Arinto, and Fernão Pires (white varieties). These varieties were cultivated in the Spring-Autumn season (2018), in Quinta do Pinto, Alenquer (Lisbon). The Syrah sample was collected on the 28 of September and the remaining samples were collected on the 3 of October.

2.1.3. Sample preparation

For the preparation of the sample, the grape stem was washed in water, and posteriorly, were dried in oven (Memmert, Schwabach, Germany) for 72 hours at 40 ºC. Then, the samples were ground and stored at room temperature protected from light until the analysis.

2.1.4. Quantification of essential minerals/toxic metals

2.1.4.1. Sample preparation

Sixteen elements (Al, As, Ca, Co, Cd, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, Pb, Se, and Zn) were determined for the six variety samples of grape stems. For each sample 0.5 g of grape stem were weighed, in triplicate, for digestion tubes. The digestion of the samples was performed with the method previously described by Gouvinhas *et al*. (2015). To the samples, 2 mL of concentrated HNO_3 and 1 mL of H_2O_2 (30 %) were added. Thereafter, the tubes were shaken and allowed to pre-digest overnight at room temperature. Subsequently, they were placed in a block heater with a glass sphere in each tube, thus avoiding evaporation. Initially, the samples were heated to 60 ºC and the temperature was gradually increased to 150 ºC. After reaching 150 ºC, the samples were left to digest to release all the organic matter, and when the mixture become clear and colorless, the glass spheres were removed, demonstrating that the HNO_3 was completely evaporated. Afterward cooling to room temperature, 10 mL of HNO_3 matrix solution (1.5 mL of acid was added to 1000 mL of distillated water) was added and the samples were shaken.

The determination of Se was performed using the method described by Costa-Silva *et al.* (2011). The digestion method was the same, however, after cooling the samples, 0.5 mL of HCl (5 M) was added, and these were placed back into the bock heater at 100 ºC for 30 minutes. After cooling, 10 mL of EDTA (0.01 M) and 2 mL of DAN (0.1 % in 0.1 M HCl) were added, and the tubes, this time closed, were placed in the block heater for 30 minutes at 60 ºC. When samples were again cooled, 5 mL of cyclohexane was added and stirred, then allowing the separation of the organic phase from the remaining mixture.

2.1.4.2. Instrumentalization

In this work, Na and K quantification was performed by Flame Atomic Emission Spectrometry (FAES), while the quantification of Ca, Mg, Cu, Fe, Zn, Mn, Al, and Ni was determined by Flame Atomic Absorption Spectrometry (FAAS) (Thermo scientific ICE 3000). The quantification of Cd, Cr, As, Pb, and Co was performed by Graphite Furnace Atomic Absorption Spectrometry (GFAAS) (Unicam 939 AA spectrometer, GF90 furnace). Before the quantification of each element, calibration was carried out using mixed standards prepared in HNO_3 (1.0 M), being selected five concentrations, according to the expected for each element, plus the blank.

For the quantification of Se, 3 mL of the organic phase were removed and then analysed fluorimetrically (FP-77 spectrofluorometer, Jasco) with excitation wavelength of 375 nm and emission wavelength of 525 nm. Standards solutions of Se (12.5-1000 µg/mL) prepared in HCl (1.0 M) were also used for calibration, along with the blank.

2.1.5. Statistical analysis

All data were subjected to the IBM SPSS 22.0 statistical software (SPSS Inc., Chicago, IL, USA), using analysis of variance (ANOVA) and a multiple range test (Tukey's test), for a p value < 0.05. The results of the samples are presented as mean values ± standard deviation (n = 3).

2.2. Results and Discussion

2.2.1. Essential minerals in grape stems

The results obtained are shown in Table 2.1. Grape stems presented a high content of essential minerals, being the most abundant the K, ranging from 18.10 ± 0.71 (Fernão Pires) to 39.36 ± 0.38 (Arinto) g/Kg dw. Since K is necessary for the growth and development of plants, this may be the main reason why it is the most abundant element in grape stems (Pantelić *et al*., 2017). The least abundant element is Co, varying between 10.28 ± 0.30 (Syrah) and 106.08 ± 2.48 (Arinto) µg/Kg dw, not being detected in the Tinta Roriz variety. However, in Fernão Pires variety, Se is the element that presents the lowest concentration (50.12 ± 0.39 µg/Kg dw). Analyzing the results, it can be verified that there are significant differences between the varieties for all quantified essential minerals, and since all samples were taken from the same place, external conditions (variations in the soil nature, viticulture practices and climatic conditions) are the same for all samples. Therefore, genotypic differences in varieties are the main reason for these significant differences (Fabani *et al*., 2017).

Table 2.1. Essential minerals content in grape stems.

Essential minerals	Tinta Roriz	Touriga Nacional	Castelão	Syrah	Arinto	Fernão Pires	*p*-value
Ca (g/Kg dw)	1.79 ± 0.10^{c}	1.00 ± 0.01^{a}	1.14 ± 0.04^{b}	2.30 ± 0.03^{d}	1.13 ± 0.03^{ab}	1.09 ± 0.06^{ab}	***
Co (µg/Kg dw)	ND	21.37 ± 1.74^{b}	20.87 ± 3.61^{ab}	10.28 ± 0.30^{a}	106.08 ± 2.48^{d}	58.74 ± 4.88^{c}	***
Cr (mg/Kg dw)	1.41 ± 0.25^{b}	1.28 ± 0.08^{b}	0.53 ± 0.04^{a}	0.29 ± 0.01^{a}	1.35 ± 0.01^{b}	0.60 ± 0.01^{a}	***
Cu (mg/Kg dw)	16.00 ± 0.49^{a}	33.88 ± 2.52^{c}	20.65 ± 1.17^{ab}	24.92 ± 1.36^{b}	69.09 ± 1.07^{d}	156.25 ± 5.70^{e}	***
Fe (mg/Kg dw)	31.69 ± 1.52^{a}	84.15 ± 2.16^{c}	20.12 ± 3.63^{a}	17.51 ± 1.19^{a}	80.84 ± 8.99^{c}	47.44 ± 3.93^{b}	***
K (g/Kg dw)	19.39 ± 0.38^{a}	29.38 ± 1.48^{c}	19.07 ± 0.35^{a}	21.42 ± 0.44^{b}	39.36 ± 0.38^{d}	18.10 ± 0.71^{a}	***
Mg (g/Kg dw)	0.88 ± 0.05^{a}	1.40 ± 0.05^{b}	0.75 ± 0.03^{a}	1.79 ± 0.03^{c}	1.42 ± 0.05^{b}	1.81 ± 0.06^{c}	***
Mn (mg/Kg dw)	6.42 ± 0.56^{a}	13.63 ± 0.87^{cd}	10.82 ± 0.29^{b}	7.10 ± 0.40^{a}	14.94 ± 0.21^{d}	13.16 ± 0.64^{c}	***
Na (g/Kg dw)	0.43 ± 0.02^{a}	1.06 ± 0.03^{e}	0.77 ± 0.01^{d}	0.60 ± 0.05^{c}	0.48 ± 0.03^{ab}	0.56 ± 0.03^{bc}	***
Ni (mg/Kg dw)	1.47 ± 0.02^{c}	0.61 ± 0.05^{ab}	0.77 ± 0.03^{b}	0.46 ± 0.04^{a}	0.77 ± 0.08^{b}	1.53 ± 0.08^{c}	***
Se (µg/Kg dw)	36.00 ± 1.55^{bc}	29.19 ± 1.42^{ab}	30.20 ± 2.33^{ab}	16.56 ± 4.58^{a}	115.50 ± 7.97^{d}	50.12 ± 0.39^{c}	***
Zn (mg/Kg dw)	9.23 ± 0.18^{a}	14.85 ± 0.76^{b}	11.12 ± 0.49^{a}	20.75 ± 0.94^{c}	15.20 ± 1.19^{b}	16.41 ± 0.64^{b}	***

ND: not detected. The values are presented as mean ± standard deviation (n = 3). Different letters indicate significantly different results (ANOVA, $p < 0.05$). Significance: non-significant, N.S. ($p > 0.05$); * significant at $p < 0.05$; ** significant at $p < 0.01$; *** significant at $p < 0.001$.

In the literature, Bustamante *et al*. (2008) quantified the mineral content of grape stems. In this study, thirteen grape stems samples were collected and analyzed, showing to be more abundant in K, presenting an average value of 30.00 g/Kg dw. The same happened in the present work, where the average concentration of K was 24.45 g/Kg dw, being lower. Concerning other essential minerals, in this study were found higher average concentrations than those obtained in the present work, namely for the elements Ca (9.50 g/Kg dw), Mg (2.10 g/Kg dw), Fe (128.00 mg/Kg dw), Mn (25.00 mg/Kg dw), Zn (26.00 mg/Kg dw), Co (2.00 mg/Kg dw), Ni (8.70 mg/Kg dw), and Cr (1.40 mg/Kg dw). In contrast, the average concentration of Na (19.00 mg/Kg dw) and Cu (22.00 mg/Kg dw) were lower, being that in present work the average content obtained in the six varieties was 0.65 g/Kg dw and 53.47 mg/Kg dw, respectively. In addition, Deiana *et al*. (2009) also analyzed the K, Na, Fe, Ca, and Mg contents in grape stems of five cultivars (Torrontés, Syrah, Chardonnay, Merlot, and Malbec). The results were presented as mass percentage, and for the K, Na, Fe, Ca, and Mg elements the respective concentrations were obtained: 4.46, 1.42, 0.02, 1.53, and 0.37%. These contents were higher than those achieved in the present study, since, for the six varieties, concentrations of 2.45 (K), 0.07 (Na), <0.01 (Fe), 0.14 (Ca), and 0.13 (Mg) % were obtained. Also, in the Prozil *et al*. (2012) study, concentrations of K, Ca, Mg, Zn, and Na in grape stem are presented as mass percentage, obtaining the following results: 0.90, 0.15, 0.02, 0.01, and <0.01%, respectively. These results, when compared to those of the present work, were lower in the cases of K (2.45%), Mg (0.13%), and Na (0.07%), and higher in relation to Ca (0.14%) and Zn (<0.01%). The differences observed in the essential mineral contents in grape stems between the above studies and the present work may be due to several factors, namely, the growing conditions, the genetic characteristics, the soil properties, the harvest time, and the climatic conditions (Ozcan, 2010).

Nevertheless, there are some studies concerning other by-products of the wine industry. Regarding to the grape pomace, Sousa *et al*. (2014) presented in their work the composition of minerals in grape pomace of the Benitaka cultivar. The values obtained for Ca (0.44 ± 0.72 mg/100 g), Mg (0.13 ± 0.26 mg/100 g), Na (0.04 ± 0.06 mg/100 g), and K (1.40 ± 0.31 mg/100 g) are lower than the concentrations reached in grape stems, but for the Fe element, the opposite happens (18.08 ± 0.03 mg/100 g). The content of Mn in grape pomace (0.82 ± 0.55 mg/100 g) is only higher than Tinta Roriz and Syrah varieties, while the Zn content (0.98 ± 0.702 mg/100 g) is only higher than the Tinta Roriz variety (Table 2.1). Concerning to grape skins, Nisco *et al*. (2013) presented in their work the elemental

composition of grape skin of the Aglianico cultivar. The content of Ca (332.99 ± 1.12 mg/Kg dw), K (62.40 ± 0.21 mg/Kg dw), Mg (60.40 ± 2.53 mg/Kg dw), Mn (0.81 ± 0.03 mg/Kg dw), and Na (1.16 ± 0.91 mg/Kg dw) found in grape skin are lower than those obtained in the present work. However, the concentrations of Co (0.51 ±0.07 mg/Kg dw), Se (0.58 ± 0.09 mg/Kg dw), and Zn (47.11 ± 1.11 mg/Kg dw) are higher than those presented for grape stem. Regarding the concentrations of Cu and Fe, these reached until 23.82 ± 0.79 and 28.59 ± 0.73 mg/Kg dw in grape skin, respectively.

Vystavna *et al*. (2014) quantified, in their study, Cu and Zn in leaves of the Muscat white and Chardonnay cultivars. For Cu, a concentration of 9.91 ± 0.90 mg/Kg was found in leaves, showing that the analyzed grape stems had a higher concentration, while the concentration of Zn in leaves reached to 28.00 ± 4.00 mg/Kg, being at least 26% higher than the concentrations found in grape stems. For last, in the work of Lachman *et al.* (2013), the essential minerals in grape seeds were quantified, more precisely in twenty-three samples of red and white varieties. They presented concentrations of K, Na, and Cu lower than the levels found in grape stems. Contrarily, the Ca content in grape seeds was higher. In relation to the Mg, Fe, Zn, and Mn contents of grape seeds, these varied between 0.72 ± 0.05 and 1.71 ± 0.09 g/Kg dm, 25.38 ± 5.47 and 88.53 ± 7.28, 5.50 ± 1.10 and 14.18 ± 0.40, and 7.00 ± 1.11 and 23.24 ± 0.11 mg/Kg dm, respectively.

In general, grape stem presents higher concentrations of essential minerals when compared to other by-products of the same industry. These divergences are mainly cause by genetic characteristics, soil composition and different affinities of the plant parts (stems, seeds, skins) with the minerals (Milićević *et al*., 2018). Thus, grape stem proves to be a rich source of these nutrients necessary for the maintenance of human health. This by-product has a higher content in essential minerals than certain foods, such as, for example, olive oil (Gouvinhas *et al*., 2015), beans (Bella *et al*., 2016), coffee (Gogoasa *et al*., 2013), and raisins (Fabani *et al*., 2017), proving that it is a matrix that can be used in various food products, having the advantage of being a cheap product, and most of the time it is discarded by the wineries.

2.2.2. Toxic metals in grape stems

The contents of Al, As, Cd, and Pb in grape stems are presented in Table 2.2. The most abundant toxic metal in grape stems was Al, with concentrations ranging from 46.81 ± 8.73 mg/Kg dw (Tinta Roriz) to 496.35 ± 1.87 mg/Kg dw (Arinto), while Cd showed to be the

metal with the lowest concentration, reaching contents varying from 3.77 ± 0.47 µg/Kg dw (Tinta Roriz) to 22.93 ± 1.04 µg/Kg dw (Arinto). Significant differences between the varieties are observed for all metals, and as referred above, all these varieties were collected from the same place, and therefore, share the same external conditions (variations in the soil nature, viticulture practices and climatic conditions). Thus, these significant differences may be mainly due to the genetic characteristics of each variety (Fabani *et al*., 2017), the de-stemming process (Vystavna *et al*., 2014), and the pollution, caused by compounds present in chemical fertilizers and pesticides, and by other sources such as industrial activities and traffic (Milićević *et al*., 2018).

Table 2.2. Toxic metals content in grape stems.

Samples	Toxic metals			
	Al (mg/Kg dw)	**As (mg/Kg dw)**	**Cd (µg/Kg dw)**	**Pb (µg/Kg dw)**
Tinta Roriz	46.81 ± 8.73^{a}	0.11 ± 0.00^{a}	3.77 ± 0.47^{a}	19.76 ± 1.32^{a}
Touriga Nacional	145.43 ± 5.52^{b}	0.25 ± 0.00^{b}	7.22 ± 0.63^{b}	41.95 ± 1.81^{b}
Castelão	60.35 ±8.91^{a}	0.12 ± 0.02^{ab}	9.68 ± 1.56^{b}	67.10 ± 10.15^{c}
Syrah	65.36 ± 1.28^{a}	0.16 ± 0.00^{ab}	3.97 ± 0.29^{a}	41.72 ± 3.31^{b}
Arinto	496.35 ± 1.87^{d}	0.79 ± 0.06^{d}	22.93 ± 1.04^{c}	105.59 ± 9.01^{d}
Fernão Pires	390.49 ± 23.11^{c}	0.53 ± 0.06^{c}	8.76 ± 0.17^{b}	45.71 ± 2.56^{b}
p-value	***	***	***	***

The values are presented as mean ± standard deviation (n = 3). Different letters indicate significantly different results (ANOVA, $p < 0.05$). Significance: non-significant, N.S. ($p > 0.05$); * significant at $p < 0.05$; ** significant at $p < 0.01$; *** significant at $p < 0.001$.

Concerning the literature, Bustamante *et al.* (2008) also quantified the elements Cd and Pb in grape stems, obtaining average concentrations of 0.80 mg/Kg dw and 26.2 mg/Kg dw, respectively. These concentrations, when compared to the present study, are much higher, since the average value obtained for the six varieties analysed were 9.39 µg/Kg dw for Cd and 53.64 µg/Kg dw for Pb. These differences between studies can be justified by the above mentioned, namely, genetic characteristics, pollution and de-stemming process.

In relation to other by-products, Chikwanha *et al*. (2018) also analysed the Al content in three grape pomace varieties, namely, Pinotage, Shiraz, and Sauvignon Blanc, through three different drying methods. However, only the oven drying values are compared with those obtained in the present study with values ranging from 53.20 to 219.00 mg/Kg dm, while in grape stems a larger range of values was obtained, as shown in Table 2.2. Regarding

to grape skins, Canizo *et al*. (2019) obtained As content ranging from 0.08 ± 0.06 to 0.17 ± 0.17 mg/Kg dm, much lower than the concentrations achieved by Touriga Nacional, Arinto, and Fernão Pires varieties in the present study. Also, Nisco *et al*. (2013) quantified the Al, Cd, and Pb contents in grape skins of Aglianico cultivar, with concentrations of 8.10 ± 0.94, 0.02 ± 0.06 and 1.76 ± 0.09 mg/Kg dw, respectively, revealing lower values than those found for Al, and higher than those obtained for Pb in grape stems. And for the Cd, only the Arinto variety presented a higher concentration (Table 2.2).

In the study of Pantelić *et al*. (2017), the Al, As, Cd, and Pb contents in grape leaves were analyzed. The Al (0.06 – 0.21 mg/Kg, approximately), As (0.06 – 0.46 µg/Kg) and Pb (0.46 – 2.23 µg/Kg) levels found in the leaves were lower than those obtained in grape stems. In contrast the Cd content, ranged from 9.40 to 29.40 µg/Kg in grape leaves, being Castelão and Arinto varieties in the same range of concentrations, as can be seen in Table 2.2. Also, Cugnetto *et al.* (2014) analyzed the Al content in grape leaves of Nebbiolo and Barbera cultivars, where concentrations ranging from 104.00 to 176.00 mg/Kg dm were found, presenting to be within the concentration range found in grape stems. In grape seeds, the Al content was analyzed by Ozcan (2010), obtaining concentrations ranging from 4.60 to 120.04 mg/Kg, and comparatively to grape stems, only Touriga Nacional, Arinto, and Fernão Pires presented higher contents (Table 2.2).

In general, grape stems presented a lower toxic metal content when compared to other by-products, however in some cases there may be higher concentrations due to differences between varieties. In addition to genotypic characteristics, the differences in concentrations observed between the different grape by-products can be explained by several factors: (i) soil composition; (ii) plant bioaccumulation of elements (grape and vine parts have different affinities with the elements); and (iii) different sources of metals, namely atmospheric deposition (pesticides/fertilizers use and anthropic activities) and geological processes (Cugnetto *et al*., 2014; Milićević *et al*., 2018).

In the previous point it is mentioned that grape stem may be a source of essential minerals, leading to the production of new products, however its necessary to ensure the safety of this matrix for its consumption, comparing the levels found in grape stem to the established maximum limits. Thus, in CODEX STAN 193-1995 (2015) the maximum food concentrations for As, Cd, and Pb are presented, being 0.50, 0.50, and 0.20 mg/Kg, respectively. The concentrations achieved in grape stem when compared to these, present to be below the established limits, except for Arinto and Fernão Pires varieties for As

(0.79 ± 0.06 and 0.53 ± 0.06 mg/Kg DW, respectively). Regarding to Al, the EFSA (European Food Safety Authority, 2008) reports that in some European countries (for example Hungary, Germany, Sweden, Italy, France, and Finland) non-occupationally exposed adults have an average Al intake, derived from food, between 1.60 to 13.00 mg per day, equivalent to a dietary exposure of 0.20 to 1.50 mg/Kg body weight/week in a 60 Kg adult, however the EFSA recommends a maximum intake of 1.00 mg/Kg body weight/week. Although Al concentrations found in grape stem showed a high variation (46.81 ± 8.73 − 496.35 ± 1.87 mg/Kg dw), there is no certainty on the toxic concentration that can cause damage to the organism. Since, in the present work, it is proposed that grape stem should not be consumed as food, but as a supplement function in the production of new products, being used in small quantities, consumer safety may not be compromised. Nevertheless, the toxic metal content in grape stems may be reduced if there is a greater control in the use of chemical fertilizers/pesticides and a decrease in pollution caused by nearby human activities.

2.3. Conclusions

The results obtained in this work proved that grape stem, besides to be a source of bioactive compounds, is also a matrix rich in essential minerals, being the most abundant K, Ca, Mg, and Na. Furthermore, taking into account that consumers increasingly seek for their diet essential minerals from plant sources, the demand for natural products also increased due also to their medicinal properties. In this sense, grape stems revealed to have a high content of mineral nutrients needed for the normal growth and for the essential biochemical functions and enzyme systems of humans (Çetin *et al*., 2011), whereby the production of new products using the grape stems as an ingredient should be thought in order to make this by-product profitable, benefiting also the consumer health, through the application in food, pharmaceutical or cosmetic industries, for instance, as food supplements and vitamins, once their safety has been ensured. And for that, it is necessary to make producers aware of a reduction in the use of chemicals, as well as, to demonstrate that the by-products of the wine industry can be profitable due to their use in several sectors. In addition, it is necessary to reduce pollution due to industrial activities and traffic in order to diminish atmospheric deposition, also improving the quality of the environment, and thus, of the by-products resulting from the food industry activities.

2.4. References

Apostolou, A., Stagos, D., Galitsiou, E., Spyrou, A., Haroutounian, S., Portesis, N., … Kouretas, D. (2013). Assessment of polyphenolic content, antioxidant activity, protection against ROS-induced DNA damage and anticancer activity of Vitis vinifera stem extracts. *Food and Chemical Toxicology*, *61*, 60–68. https://doi.org/10.1016/j.fct.2013.01.029

Bella, G. Di, Naccari, C., Bua, G. D., Rastrelli, L., Turco, V. Lo, Potortì, A. G., … Potortı, G. (2016). Mineral composition of some varieties of beans from Mediterranean and Tropical areas. *International Journal of Food Sciences and Nutrition*, *7486*(March). https://doi.org/10.3109/09637486.2016.1153610

Bustamante, M. A., Moral, R., Paredes, C., Pe, A., & Pe, M. D. (2008). Agrochemical characterisation of the solid by-products and residues from the winery and distillery industry. *Waste Management*, *28*, 372–380. https://doi.org/10.1016/j.wasman.2007.01.013

Canizo, B. V, Escudero, L. B., Pellerano, R. G., & Wuilloud, R. G. (2019). Data mining approach based on chemical composition of grape skin for quality evaluation and traceability prediction of grapes. *Computers and Electronics in Agriculture*, *162*(September 2018), 514–522. https://doi.org/10.1016/j.compag.2019.04.043

Çetin, E. S., Altinöz, D., Tarçan, E., & Baydar, N. G. (2011). Chemical composition of grape canes. *Industrial Crops & Products*, *34*, 994–998. https://doi.org/10.1016/j.indcrop.2011.03.004

Chikwanha, O. C., Raffrenato, E., Muchenje, V., Musarurwa, H. T., & Mapiye, C. (2018). Varietal differences in nutrient, amino acid and mineral composition and in vitro rumen digestibility of grape (Vitis vinifera) pomace from the Cape Winelands vineyards in South Africa and impact of preservation techniques. *Industrial Crops and Products*, *118*(November 2017), 30–37. https://doi.org/10.1016/j.indcrop.2018.03.026

CODEX STAN 193-1995. (2015). Codex General Standard For Contaminants And Toxins In Food And Feed. Retrieved from http://www.fao.org/fileadmin/user_upload/livestockgov/documents/1_CXS_193e.pdf

Costa-silva, F., Marques, G., Matos, C. C., Barros, A. I. R. N. A., & Nunes, F. M. (2011). Selenium contents of Portuguese commercial and wild edible mushrooms. *Food Chemistry*, *126*(1), 91–96. https://doi.org/10.1016/j.foodchem.2010.10.082

Cugnetto, A., Santagostini, L., Rolle, L., Guidoni, S., Gerbi, V., & Novello, V. (2014). Tracing the " terroirs " via the elemental composition of leaves , grapes and derived wines in cv Nebbiolo (Vitis vinifera L .). *Scientia Horticulturae*, *172*, 101–108. https://doi.org/10.1016/j.scienta.2014.03.055

Deiana, A. C., Sardella, M. F., Silva, H., Amaya, A., & Tancredi, N. (2009). Use of grape stalk, a waste of the viticulture industry, to obtain activated carbon. *Journal of Hazardous Materials*, *172*, 13–19. https://doi.org/10.1016/j.jhazmat.2009.06.095

Edelstein, M., & Ben-hur, M. (2017). Heavy metals and metalloids : Sources, risks and strategies to reduce their accumulation in horticultural crops. *Scientia Horticulturae*, *234*(June), 1–14. https://doi.org/10.1016/j.scienta.2017.12.039

EFSA. (2008). Safety of aluminium from dietary intake. *The EFSA Journal*, 1–34.

Fabani, M. P., Baroni, M. V, Luna, L., Lingua, M. S., Monferran, M. V, Paños, H., … Egly, G. (2017). Journal of Food Composition and Analysis Changes in the phenolic pro fi le of Argentinean fresh grapes during production of sun-dried raisins. *Journal of Food Composition and Analysis*, *58*, 23–32. https://doi.org/10.1016/j.jfca.2017.01.006

Gogoasa, I., Alexandra, P., Maria, A. L., Ariana, V., Maria, R., Maria, D., … Gergen, I. (2013). The Mineral Content of Different Coffee Brands. *Journal of Horticulture, Forestry and Biotechnology*, *17*(4), 68–71.

Gouvinhas, I., Machado, N., Cunha, M., Pereira, M., Matos, C., Gomes, S., … Barros, A. I. R. N. A. (2015). Trace element content of monovarietal and commercial Portuguese olive oils. *Journal of Oleo Science*, *64*(10). https://doi.org/10.5650/jos.ess15101

Gouvinhas, I., Santos, R. A., Queiroz, M., Leal, C., Saavedra, M. J., Domínguez-Perles, R., … Barros, A. I. R. N. A. (2018). Monitoring the antioxidant and antimicrobial power of grape (Vitis vinifera L.) stems phenolics over long-term storage. *Industrial Crops and Products*, *126*. https://doi.org/10.1016/j.indcrop.2018.10.006

Gouvinhas, Irene, Queiroz, M., Rodrigues, M., & Barros, A. I. R. N. A. (2019). *Evaluation of the Phytochemistry and Biological Activity of Grape (Vitis vinifera L.) Stems: Toward a Sustainable Winery Industry*. *Polyphenols in Plants* (2nd ed.). Elsevier Inc. https://doi.org/10.1016/B978-0-12-813768-0.00023-2

Jackson, R. S. (2014). *Chemical Constituents of Grapes and Wine*. (W. Science, Ed.) (Fourth Edi). https://doi.org/10.1016/B978-0-12-381468-5.00006-3

Karvela, E., Makris, D. P., Kalogeropoulos, N., & Karathanos, V. T. (2011). Deployment of response surface methodology to optimise recovery of grape (Vitis vinifera) stem polyphenols. *Talanta*, *79*(5), 1311–1321. https://doi.org/10.1016/j.talanta.2009.05.042

Kim, J., Kim, Y., & Kumar, V. (2019). Heavy metal toxicity: an update of chelating therapeutic strategies. *Journal of Trace Elements in Medicine and Biology*. https://doi.org/10.1016/j.jtemb.2019.05.003

Lachman, J., Hejtmánková, A., Pivec, V., Dˇ, M., & Pˇ, J. (2013). Towards complex utilisation of winemaking residues: Characterisation of grape seeds by total phenols, tocols and essential elements content as a by-product of winemaking. *Industrial Crops and Products*, *49*, 445–453. https://doi.org/10.1016/j.indcrop.2013.05.022

Milićević, T., Ani, M., Reli, D., Vukovi, G., Sandra, Š., & Popovi, A. (2018). Science of the Total Environment Bioavailability of potentially toxic elements in soil – grapevine (leaf , skin , pulp and seed) system and environmental and health risk assessment. *Science of the Total Environment*, *626*, 528–545. https://doi.org/10.1016/j.scitotenv.2018.01.094

Mir-Marqués, A., Cervera, M. L., & Guardia, M. De. (2016). Mineral analysis of human diets by spectrometry methods. *Trends in Analytical Chemistry*, *82*(September), 457–467. https://doi.org/10.1016/j.trac.2016.07.007

Nisco, M. De, Manfra, M., Bolognese, A., & Sofo, A. (2013). Nutraceutical properties and polyphenolic profile of berry skin and wine of Vitis vinifera L. (cv. Aglianico). *Food Chemistry*, *140*(4), 623–629. https://doi.org/10.1016/j.foodchem.2012.10.123

Nosratpour, M., Jafari, S. M., & Resources, N. (2018). *Bioavailability of Minerals (Ca, Mg, Zn, K, Mn, Se) in Food Products*. *Encyclopedia of Food Chemistry*. Elsevier. https://doi.org/10.1016/B978-0-08-100596-5.21618-1

Ozcan, M. M. (2010). Mineral Contents of Several Grape Seeds. *Asian Journal of Chemistry*, *22*(8), 6480–6488.

Pantelić, M. M., Dabi, D. Č., Ivanka, Ž. Ć., Pergal, M. V, Reli, D. J., Todi, S. R., & Nati, M. M. (2017). Phenolic profiles, antioxidant activity and minerals in leaves of different grapevine varieties grown in Serbia. *Journal of Food Composition and Analysis*, *62*(January), 76–83. https://doi.org/10.1016/j.jfca.2017.05.002

Piñeiro, Z., Guerrero, R. F., Fernandez-Marin, M. I., Cantos-Villar, E., & Palma, M. (2013). Ultrasound-assisted extraction of stilbenoids from grape stems. *Journal of Agricultural and Food Chemistry*, *61*(51), 12549–12556. https://doi.org/10.1021/jf4030129

Prozil, S. O., Evtuguin, D. V, & Cruz, L. P. (2012). Chemical composition of grape stalks of Vitis vinifera L. from red grape pomaces. *Industrial Crops & Products*, *35*, 178–184. https://doi.org/10.1016/j.indcrop.2011.06.035

Rodríguez-Solana, R., Salgado, J. M., Domínguez, J. M., & Cortés, S. (2014). Assessment of minerals in aged grape marc distillates by FAAS/FAES and ICP-MS. Characterization and safety evaluation. *Food Control*, *35*(1), 49–55. https://doi.org/10.1016/j.foodcont.2013.06.031

Sousa, E. C., Uchôa-thomaz, A. M. A., Osvaldo, J., Carioca, B., Morais, S. M. De, Lima, A. De, … Rodrigues, L. L. (2014). Chemical composition and bioactive compounds of grape pomace (Vitis vinifera L.), Benitaka variety , grown in the semiarid region of Northeast Brazil. *Food Science and Technology*, *34*(1), 135–142.

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. *International Journal of Molecular Sciences*, *15*(9), 15638–15678. https://doi.org/10.3390/ijms150915638

Volpe, M. G., Cara, F. La, Volpe, F., Mattia, A. De, Serino, V., Petitto, F., … Stasio, M. Di. (2009). Heavy metal uptake in the enological food chain. *Food Chemistry*, *117*(3), 553–560. https://doi.org/10.1016/j.foodchem.2009.04.033

Vystavna, Y., Rushenko, L., Diadin, D., Klymenko, O., & Klymenko, M. (2014). Trace metals in wine and vineyard environment in southern Ukraine. *Food Chemistry*, *146*, 339–344. https://doi.org/10.1016/j.foodchem.2013.09.091

Chapter III:

Phenolic composition of
grape stem extracts

Phenolic composition of grape stem extracts

Abstract

Phenolic compounds are secondary metabolites produced in plant responses against stresses (biotic and abiotic) and are increasingly important in the scientific community because of their beneficial properties in fighting degenerative diseases. Grapes are a rich source of these phytochemicals, and their by-products generated by the wine industry also contain these bioactive compounds. Since these by-products cause environmental problems and compromise the sustainability of industries, it is necessary to create alternatives for their use. In the case of grape stem, its phenolic composition is mostly consituted by flavanols, flavonols, phenolic acids, and stilbenes, which can be used in the production of innovative products. Thus, the objective of this work was to determine the phenolic composition, in six grape stem varieties, by colorimetric methods (total phenols, *ortho*-diphenols, and flavonoids) and to identify and quantify the most abundant compounds by HPLC.

The results indicated significant differences between varieties, with concentration ranging from 30.91 ± 0.73 to 96.12 ± 8.14 mg GA/g dw, 32.17 ± 1.04 to 77.26 ± 5.31 mg GA/g dw, and 25.76 ± 1.14 to 65.14 ± 0.65 mg CAT/g dw, for total phenols, *ortho*-diphenols, and flavonoids, respectively. Eleven compounds were also identified by HPLC, and despite differences between varieties, catechin was the most abundant compound in all varieties (0.44 ± 0.02 to 2.03 ± 0.08 mg/g dw). The differences found are mainly due to the distinct genetic characteristics of each variety. In conclusion, grape stem confirms to be a potential source of phenolic compounds being able to be used in the projection of new products in the cosmetic, pharmaceutical and food industries.

Keywords: Grape stem; Bioactive compounds; Total phenols; *Ortho*-diphenols; Flavonoids; HPLC.

3. Introduction

Phenolic compounds are secondary metabolites produced in plant responses against biotic and abiotic stresses. These compounds arise from two main primary biosynthetic pathways, namely the shikimate and acetate pathways (Teixeira *et al*., 2014), and contain in their structure an aromatic ring with one or more hydroxyl groups, ranging from simple phenolic compounds to highly polymerized compounds (Beres *et al*., 2017). Phenolic compounds can be divided into four groups: flavonoids, phenolic acids, stilbenes (Del Rio *et al*., 2013), and tannins (Beres *et al*., 2017). Flavonoids have as their main classes flavonols (*e.g*. quercetin, kaempferol), flavanols (*e.g.* catechin, epicatechin), flavanones (*e.g.* hesperidin), flavones (*e.g.* luteolin), and anthocyanins (*e.g.* malvidin, cyanidin) (Pascual-Teresa *et al*., 2010). Phenolic acids include hydroxycinnamic (*e.g.* caftaric acid, caffeic acid) and hydroxybenzoic (*e.g.* gallic acid, protocatechuic acid) acids, while tannins can be divided into two classes, namely condensed and hydrolyzed tannins (Machado & Domínguez-Perles, 2017). Concerning to stilbenes, the most common are resveratrol, piceatannol, and viniferins (Flamini *et al*., 2013). The main sources of these bioactive compounds are fruits, cereals, vegetables and beverages such as tea, coffee, and wine.

The grape is a rich matrix of phenolic compounds, and its use in wine production leads to the accumulation of wine residues (skins, seeds, stems, pomaces), which have an impact on corporate sustainability and negative effects on the environment (Barros *et al*., 2016). Therefore, the valorization of these by-products is necessary to reduce the environmental impact of the winemaking activity (Teixeira *et al*., 2014). In this sense, grape stems are one of the most difficult by-products to treat. However, they can be used in the creation of new products due to their polyphenolic content, mainly in flavanols, flavonols, phenolic acids, and stilbenes (Gouvinhas *et al*., 2019).

The scientific community has increasingly paid attention to polyphenols, since research in recent years has shown that these phytochemicals can be used to prevent degenerative diseases (cancers, cardiovascular and neurodegenerative diseases) due to their antioxidant properties (Tsao, 2010), besides having antimicrobial, anti-inflammatory, and anti-cancer properties (Salehi *et al*., 2019). Thus, since studies show that grape stem may be a rich source of phenolic compounds, it may be, in the future, possible to apply it to the formation of innovative products in the cosmetic, pharmaceutical and food industries (Anastasiadi *et al*., 2012; Barros *et al*., 2015; Gouvinhas *et al*., 2018; Sahpazidou *et al*.,

2014). In this way, it is essential determine the phenolic composition of grape stem and its main compounds, in order to understand if there are differences between varieties, which may influence their use in different biological activities.

3.1. Material and Methods

3.1.1. Chemicals

Folin-Ciocalteu's reagent, 3,4,5-trihydroxybenzoic acid (gallic acid) extra pure (>99%), and sodium hydroxide (NaOH) were acquired from Panreac (Panreac Química S.L.U., Barcelona, Spain). Sodium nitrite ($NaNO_2$), aluminum chloride ($AlCl_3$), and sodium carbonate (Na_2CO_3), all extra pure (>99%), were purchase from Merck (Merck, Darmstadt, Germany). Sodium molybdate (Na_2MoO_4, 99.5%) was obtained from Chem-Lab (Chem-Lab N.V., Zedelgem, Belgium). Methanol (MeOH), catechin and the standards compounds for the separation chromatographic, namely, caftaric acid, resveratrol, and quercetin-3-*O*-rutinoside were obtained from Sigma-Aldrich (Steinheim, Germany), while the remaining standards compounds, ellagic acid, epicatechin, and malvidin-3-O-galactoside were acquired from Extrasynthese (Genay, France). Formic acid was purchase from VWR Chemicals and acetonitrile was acquired from Carlo Erba Reagents. Ultrapure water was obtained using a Millipore water purification system (Millipore, Bedford, MA, USA).

3.1.2. Sampling

See chapter II, section 2.1.2.

3.1.3. Sample preparation

See chapter II, section 2.1.3.

3.1.4. Grape stem extracts preparation

For the preparation of the extract, 40 mg of the previously milled grape stem was weighed, and 1.5 mL of MeOH/H_2O (70:30, v/v) mixture were added. Then, samples were vortexed and agitated for 30 min at room temperature, for the extraction of the phenolic compounds, and after centrifuged at 10000 *rpm* for 15 min, at 4 ºC (Sigma, Steinheim, Germany), collecting the supernatant. This procedure was repeated 3 times for each sample of grape stem. In addition, each sample was analyzed in triplicate (n = 3) (Domínguez-Perles *et*

al., 2014). Supernatants were filtered through a 0.45-µm PVDF filter (Millex HV13, Millipore, Bedford, MA, USA) and stored at 4 ºC until analysis.

3.1.5. Quantification of the phenolic composition

This determination was made by quantifying the content of total phenols, *ortho*-diphenols and flavonoids. These methods were adapted to 96-well microplates (Frilabo, Milheirós, Portugal).

3.1.5.1. Determination of the total phenols content

Total phenols content was determined by spectrophotometric method of Folin-Ciocalteau, according to the methodology exposed by Sousa *et al*. (2014). Folin-Ciocalteau reagent is formed by a mixture of phosphotungstic ($H_3PW_{12}O_{40}$) and phosphomolybdic ($H_3PMo_{12}O_{40}$) acids, which in alkaline medium has a yellow coloration. When in the presence of reducing agents and phenolic compounds, oxides of tungsten (W_8O_{23}) and molybdenum (Mo_8O_{23}) are produced through reducing reactions, and consequently, the formation of blue complexes occurs (Magalhães *et al*., 2008).

Initially, standards of gallic acid were prepared with different concentrations (5-200 mg/L). Then, 20 µL of standard solutions and respective samples were added to each well, plus 100 µL of the Folin-Ciocalteau reagent (1:10 H_2O) and 80 µL of sodium carbonate (Na_2CO_3) (7.5%). Afterwards, the microplate was incubated at 40 ºC, protected from light for 30 min. The absorbance was read at a wavelength of 750 nm in the microplate reader (Thermo Fisher Scientific, Lisbon, Portugal). All standards solutions and samples were analyzed in triplicate. The content in total phenols are expressed in milligrams of gallic acid per gram of sample (dry weight) (mg GA/g dw).

3.1.5.2. Determination of the *ortho*-diphenols content

Ortho-diphenols content was determined colorimetrically by the method adapted by Gouvinhas *et al*. (2015). This method is based on the complexation of *ortho*-diphenols in molybdate ions, resulting in an orange coloration.

Firstly, standards of gallic acid were prepared with different concentrations (5-200 mg/L). Then, 160 µL of standard solutions and respective samples were added to each well, and thereafter 40 µL of sodium molybdate (Na_2MoO_4) (5%) were added. After, the microplate was incubated at room temperature, protected from light for 15 min. After this time, the

absorbance was read at a wavelength of 375 nm in the microplate reader (Thermo Fisher Scientific, Lisbon, Portugal). All standards solutions and samples were analyzed in triplicate. The content in total phenols are expressed in milligrams of gallic acid per gram of sample (dry weight) (mg GA/g dw).

3.1.5.3. Determination of the flavonoids content

Flavonoids content was determined by spectrophotometric method described by Domínguez-Perles *et al*. (2014). Primarily, standards of catechin were prepared with different concentrations (5-200 mg/L). Then, 24 µL of standard solutions and respective samples were added to each well, plus 28 µL of sodium nitrite ($NaNO_2$) (50 g/L). After a wait of 5 min at room temperature, 28 µL of aluminum chloride ($AlCl_3$) (100 g/L) was added. And 6 min later, 120 µL of sodium hydroxide (NaOH) (1.0 M) was added. Immediately after, the absorbance was read at a wavelength of 510 nm in the microplate reader (Thermo Fisher Scientific, Lisbon, Portugal), with a shaking of 30 seconds. All standards solutions and samples were analyzed in triplicate. The content in total phenols are expressed in milligrams of catechin per gram of sample (dry weight) (mg CAT/g dw).

3.1.6. Qualitative and quantitative analysis of phenolic compounds by HPLC

The phenolic profile of grape stems samples was assessed by Reverse Phase - High Performance Liquid Chromatography - Diode Array Detector (RP-HPLC-DAD), with a C18 column (250 x 4.6 mm, 5 µm particle size; ACE, Aberdeen, Scotland), as the method exposed by Queiroz *et al*. (2017). In this work, the reverse phase HPLC method is based on a polar mobile phase with the mixture of solvent A: H_2O/HCOOH (99.9:0.1, v/v), and solvent B: CH_3CN/HCOOH (99.9:0.1, v/v), and a stationary phase with non-polar characteristics. Prior to placement of mobile phases in the chromatographic system, solvents A and B were filtered with a 0.2 µm membrane and degassed under vacuum.

The following linear gradient scheme was used (t in min; %B): (0; 5%), (15; 15%), (30; 30%), (40; 50%), (45; 95%), (50; 95%) and (55; 5%). At 55 minutes, return to 5% of B to stabilize and prepare the column for the next sample. The analysis was performed at room temperature, 25 ºC, with a flow rate of 1.0 mL min^{-1} and a sample injection volume of 20 µL. All samples were injected in triplicate. The equipment consisted of a LC pump (SRVYR-LPUMP), an auto-sampler (SRVYR-AS), and a photodiode array detector (SRVYR-PDA5) in series (Thermo Fisher Scientific, Inc., Waltham, USA).

For the identification of phenolic compounds, the standards and the respective retention times, the order of elution, the UV spectra were considered, as well as, the data present in the literature (Anastasiadi *et al*., 2012; Barros *et al*., 2014; Domínguez-Perles *et al*., 2016; Gouvinhas *et al*., 2018; Queiroz *et al*., 2017). Posteriorly, for the quantification of the compounds, standards were prepared at different concentrations (0.1-10 mg/L). For the compounds identified at 280 nm, standards of epicatechin, caftaric acid and ellagic acid were used to quantify flavanols, hydroxycinnamic acids, and hydroxybenzoic acids, respectively. For the compounds recognized at 330 nm, standards of caftaric acid, quercetin-3-*O*-rutinoside and resveratrol were used to quantify, respectively, hydroxycinnamic acids, flavonols, and stilbenes. Finally, for the quantification of anthocyanins at 520 nm a malvidin-3-*O*-galactoside standard was used.

3.1.7. Statistical analysis

All data were subjected to the IBM SPSS 22.0 statistical software (SPSS Inc., Chicago, IL, USA), using analysis of variance (ANOVA) and a multiple range test (Tukey's test), for a p value < 0.05. The results of the samples are presented as mean values ± standard deviation ($n = 3$).

3.2. Results and Discussion

3.2.1. Phenolic content of grape stem extracts

In Table 3.1 are presented the results regarding to the total phenols, flavonoids and *ortho*-diphenols contents of grape stem extracts. Analyzing the data, significant differences were verified for the total phenols, *ortho*-diphenols and flavonoids contents. The Touriga Nacional variety presented a higher concentration of total phenols (96.12 ± 8.14 mg GA/g dw) when compared to the other varieties, and the Arinto variety showed to be less abundant (30.91 ± 0.73 mg GA/g dw) in these bioactive compounds. The same happened in the *ortho*-diphenols content, where the Touriga Nacional variety had the highest content (77.26 ± 5.31 mg GA/g dw) and the Arinto variety had the lowest concentration (32.17 ± 1.04 mg GA/g dw). Concerning to the flavonoids content in grape stem extracts, the Fernão Pires variety showed the highest concentration (65.14 ± 0.65 mg CAT/g dw) in these compounds, while the Arinto variety showed, once again, the lowest concentration (25.76 ± 1.14 mg CAT/g dw).

Usually, the differences observed between varieties can be explained by several factors, namely, genetic and physiological characteristics of each cultivar, agroclimatic conditions (geographic location, soil composition, fertilization, and environmental conditions) and ripening stage (Dias *et al*., 2015; Garrido *&* Borges, 2013; Gouvinhas *et al*., 2018), but in this case, since all varieties were harvested from the same location, the differences in phenolic content are mainly due to genetic and physiological characteristics and ripening stage of each cultivar. However, despite differences between varieties, the results showed that grape stem can be a rich source of bioactive compounds.

Table 3.1. Phenolic content of grape stem extracts.

Samples	Total phenols (mg GA/g dw)	Flavonoids (mg CAT/g dw)	*Ortho*-diphenols (mg GA/g dw)
Tinta Roriz	79.29 ± 2.28^{d}	61.47 ± 4.43^{d}	62.22 ± 1.36^{c}
Touriga Nacional	96.12 ± 8.14^{e}	62.61 ± 4.42^{d}	77.26 ± 5.31^{e}
Castelão	50.53 ± 1.50^{b}	39.79 ± 2.45^{b}	44.52 ± 2.76^{b}
Syrah	67.09 ± 3.44^{c}	52.86 ± 1.19^{c}	55.68 ± 1.01^{c}
Arinto	30.91 ± 0.73^{a}	25.76 ± 1.14^{a}	32.17 ± 1.04^{a}
Fernão Pires	79.12 ± 4.68^{d}	65.14 ± 0.65^{d}	69.73 ± 0.47^{d}
p-value	***	***	***

The values are presented as mean ± standard deviation (n = 3). Different letters indicate significantly different results (ANOVA, $p < 0.05$). Significance: non-significant, N.S. ($p > 0.05$); * significant at $p < 0.05$; ** significant at $p < 0.01$; *** significant at $p < 0.001$.

In literature, the study of Domínguez-Perles *et al*. (2014) determined the phenolic content in two Portuguese grape stem varieties, namely, Touriga Nacional (red variety) and Viosinho (white variety), using different concentrations of solvent, time and temperature on the extraction of the phenolic compounds. The highest concentrations obtained for the total phenols, *ortho*-diphenols, and flavonoids were 67.70 mg GA/g dw, 44.33 mg GA/g dw, and 68.17 mg/CAT g dw for Touriga Nacional variety, respectively, and 48.00 mg GA/g dw, 36.54 mg GA/g dw, and 24.65 mg CAT/g dw for Viosinho variety, correspondingly. The value of total phenols obtained for the red variety when compared to those obtained in the present work, demonstrated to be lower than the Touriga Nacional, Tinta Roriz, and Fernão Pires (white) varieties, while the value presented for the white variety was only higher than that obtained for Arinto variety, as it can be seen in Table 3.1. Regarding to *ortho*-diphenols, in the present study, only the Arinto variety presented a lower concentration. In contrast, the

six varieties analyzed in the present work showed lower flavonoid concentrations (25.76 ± 1.14 – 65.14 ± 0.65 mg CAT/g dw) when compared to the Touriga Nacional variety and higher concentrations when compared to Viosinho variety. Also, Gouvinhas *et al*. (2018) presented in their study the phenolic content of the Tinta Barroca, Syrah, and Sousão cultivars during storage. For the Syrah variety, maximum contents of 93.49 mg GA/g dw, 64.67 mg GA/g dw, and 71.89 mg CAT/g dw were obtained for total phenols, *ortho*-diphenols and flavonoids, respectively, being these results greater to those presented in the present study, except for Touriga Nacional variety, which showed higher values for total phenols and *ortho*-diphenols, and Fernão Pires variety, which presented a higher content of *ortho*-diphenols (Table 3.1). Concerning to Tinta Barroca variety, Gouvinhas *et al*. (2018) revealed high concentrations of total phenols (96.29 mg GA/g dw), *ortho*-diphenols (81.11 mg GA/g dw), and flavonoids (76.20 mg CAT/g dw) when compared to those obtained in the present study. Finally, the Sousão cultivar presented a total phenols content (58.21 mg GA/g dw) only higher than the Castelão and Arinto varieties, which also happened for its flavonoid content (45.31 mg CAT/g dw), as can be seen in Table 3.1. In the case of *ortho*-diphenols, the Sousão variety showed lower concentrations (61.35 mg GA/g dw) than those exhibited in the present work for Touriga Nacional, Fernão Pires, and Tinta Roriz varieties.

In addition, Marchante *et al*. (2019) work quantified the total phenols content of the Tempranillo (or Tinta Roriz) variety, reaching a concentration of 46.79 ± 2.12 mg GA/ g dw. Llobera & Cañellas (2007) and Llobera & Cañellas (2008) studies presented the total phenols contents for Manto Negro (116 ± 1.90 g GA/g dm) and Prensal blanc (87.30 ± 1.20 mg GA/g dm) cultivars, respectively. These two varieties revealed a higher total phenols content than the varieties analyzed in the actual work, except for Touriga Nacional variety, which showed higher concentration than the Prensal blanc cultivar. Also, Anastasiadi *et al*. (2009) work quantified the total phenols content for Voidomato, Mandilaria, Asyrtiko, and Aindani cultivars, reaching concentrations ranging from 367.10 to 536.80 mg GA/g of extract. Moreover, Apostolou *et al*. (2013) work determined for white and red varieties the total phenols content. In the white varieties, concentrations ranged from 372.00 to 464.00 mg GA/g dw, while in the red varieties, contents oscillated from 345.00 to 584.00 mg GA/g dw were presented. These concentrations revealed to be much higher than those found in the present work (Table 3.1). The same behavior happened in the Sahpazidou *et al*. (2014) study, where the total phenols content ranged from 318.00 to 415.00 mg GA/g dw for the tested cultivars.

The differences verified between the above studies and the present work can be justified by several reasons. The reasons that can influence the phenolic composition are: (i) different intrinsic characteristics of each variety, namely at the genetic and physiological level; (ii) distinct extraction techniques (solvents, time, temperatures); (iii) different agroclimatic conditions, such as, climate, soil composition, and viticulture practices; and (iv) distinct stages of maturation (Barros *et al*., 2014; González-Centeno *et al*., 2012; Teixeira *et al*., 2014; Teixeira *et al*., 2018).

3.2.2. Identification and quantification of phenolic compounds by HPLC

The identification of phenolic compounds by RP-HPLC-DAD was realized by comparison with pure standards of DAD spectra, retention times, and literature data (Anastasiadi *et al*., 2009; Gouvinhas *et al*., 2018; Queiroz *et al*., 2017). In grape stem extracts, eleven phenolic compounds were identified, belonging to different classes, namely hydroxybenzoic and hydroxycinnamic acids, flavanols, flavonols, stilbenes, and anthocyanins (Table 3.2 and Figure 3.1).

Table 3.2. Compounds identified in grape stem extracts and their respective wavelengths and retention times (R_t).

Peak no.	Identified compounds	Wavelength (λ)	R_t (min)
1	Gallic acid	280 nm	10.93
2	Protocatechuic acid	280 nm	14.57
3	Catechin	280 nm	18.62
4	Epicatechin	280 nm	21.24
5	*Trans*-cinnamic acid	280 nm	31.99
6	Caftaric acid	330 nm	17.69
7	Quercetin-3-*O*-rutinoside	330 nm	25.39
8	Resveratrol	330 nm	29.02
9	Ɛ-viniferin	330 nm	32.50
10	Malvidin-3-*O*-Galactoside	520 nm	18.65
11	Malvidin-3-*O*-Glucoside	520 nm	25.38

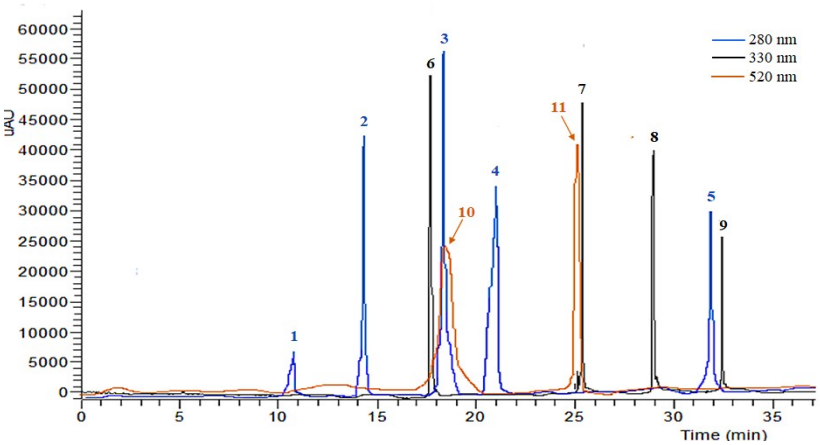


Figure 3.1. Representative chromatogram of phenolic compounds identified in grape stem extracts at 280, 330, and 520 nm. (1) Gallic acid; (2) Protocatechuic acid; (3) Catechin; (4) Epicatechin; (5) *Trans*-cinnamic acid; (6) Caftaric acid; (7) Quercetin-3-*O*-rutinoside; (8) Resveratrol; (9) Ɛ-viniferin; (10) Malvidin-3-*O*-galactoside; (11) Malvidin-3-*O*-glucoside.

Posterior to the phenolic compounds identification, their quantification was performed, being the results presented in Table 3.3. When analyzing the concentrations obtained, significant differences were found between all varieties. In all varieties, catechin revealed to be the major compound, with concentration ranging from 0.44 ± 0.02 to 2.03 ± 0.08 mg/g dw. Concerning to the minority compound found in grape stem extracts, they were different among varieties. For the Castelão and Arinto varieties, epicatechin presented to be in lower concentration than the other compounds (0.04 ± 0.00 and 0.03 ± 0.00 mg/g dw, respectively), while for the Syrah and Fernão Pires varieties, gallic acid was the minority compound (0.04 ± 0.00 and 0.05 ± 0.00 mg/g dw, correspondingly). For the Tinta Roriz and Touriga Nacional varieties, the minority compounds were resveratrol and Ɛ-viniferin (0.07 ± 0.00 mg/g dw for both) in the case of the first variety, and malvidin-3-*O*-glucoside (0.10 ± 0.01 mg/g dw) in the case of the second. It should be also noted that in Tinta Roriz, Touriga Nacional and Castelão varieties, the presence of gallic acid was not detected, whereas the protocatechuic acid was not identified in the Tinta Roriz and Syrah varieties.

As noted above, these differences between the cultivars may be due to several factors, but in this particular case, since all varieties were collected from the same location, these

Table 3.3. Quantification of phenolic compounds identified in grape stem extracts (mg/g dw).

Identified compounds	Tinta Roriz	Touriga Nacional	Castelão	Syrah	Arinto	Fernão Pires	*p*-value
Gallic acid	ND	ND	ND	0.04 ± 0.00^{b}	0.04 ± 0.00^{a}	0.05 ± 0.00^{c}	**
Protocatechuic acid	ND	0.40 ± 0.02^{c}	0.12 ± 0.00^{a}	ND	0.24 ± 0.01^{b}	0.26 ± 0.00^{b}	***
Catechin	1.62 ± 0.11^{d}	2.03 ± 0.08^{e}	0.44 ± 0.02^{a}	1.33 ± 0.02^{c}	0.66 ± 0.04^{b}	1.27 ± 0.03^{c}	***
Epicatechin	0.09 ± 0.00^{b}	0.18 ± 0.01^{d}	0.04 ± 0.00^{a}	0.11 ± 0.00^{c}	0.03 ± 0.00^{a}	0.17 ± 0.00^{d}	***
Trans-cinnamic acid	0.08 ± 0.00^{a}	0.16 ± 0.01^{c}	0.09 ± 0.01^{a}	0.08 ± 0.00^{a}	0.14 ± 0.00^{bc}	0.13 ± 0.01^{b}	***
Caftaric acid	0.43 ± 0.01^{b}	0.98 ± 0.08^{d}	0.20 ± 0.00^{a}	0.40 ± 0.01^{b}	0.22 ± 0.00^{a}	0.68 ± 0.04^{c}	***
Q-3-*O*-Rut	0.37 ± 0.01^{c}	0.44 ± 0.00^{d}	0.24 ± 0.01^{b}	0.41 ± 0.01^{cd}	0.15 ± 0.00^{a}	0.44 ± 0.03^{d}	***
Resveratrol	0.07 ± 0.00^{ab}	0.14 ± 0.00^{d}	0.07 ± 0.00^{b}	0.06 ± 0.00^{a}	0.08 ± 0.00^{c}	0.09 ± 0.00^{c}	***
Ɛ-viniferin	0.07 ± 0.00^{a}	0.11 ± 0.01^{c}	0.07 ± 0.00^{a}	0.07 ± 0.00^{a}	0.07 ± 0.00^{a}	0.08 ± 0.00^{b}	***
Mv-3-*O*-Glt	0.37 ± 0.02	ND	ND	ND	-	-	-
Mv-3-*O*-Glc	0.36 ± 0.01^{d}	0.10 ± 0.01^{b}	0.06 ± 0.00^{a}	0.19 ± 0.01^{c}	-	-	***

Q: Quercetin; Rut: Rutinoside; Mv: Malvidin; Glt: Galactoside; Glc: Glucoside; ND: Not detected. The values are presented as mean ± standard deviation (n = 3). Different letters indicate significantly different results (ANOVA, $p < 0.05$). Significance: non-significant, N.S. ($p > 0.05$); * significant at $p < 0.05$; ** significant at $p < 0.01$; *** significant at $p < 0.001$.

differences are mainly caused by the distinct genetic/physiological characteristics of each cultivar.

Regarding to other studies on phenolic compounds identified and quantified in grape stem extracts, in the work of Dias *et al*., (2015), fifteen compounds were detected and quantified in red varieties (Sousão, Touriga Nacional, Tinta Barroca, Tinta Amarela), while in white varieties (Fernão Pires, Viosinho, Rabigato) thirteen compounds were quantified. In common with the present study, five compounds were found, namely, epicatechin, caftaric acid, quercetin-3-*O*-rutinoside, Ɛ-viniferin, and malvidin-3-*O*-glucoside (this last related only to red varieties). Concerning to epicatechin, higher concentrations (2199 to 3184 mg/100 g dw) were found than those obtained in present work. In contrast, the concentrations of caftaric acid (5.28 to 13.85 mg/100 g dw), quercetin-3-*O*-rutinoside (0.27 to 1.22 mg/100 g dw), and Ɛ-viniferin (0.22 to 2.99 mg/100 g dw) presented lower concentrations than those obtained in the present study, as can be seen in Table 3.3. Relatively to the presence of anthocyanins, the contents of malvidin-3-*O*-glucoside varied between 2.38 to 8.02 mg/100 g dw, and when compared to the present work, they were lower than the concentrations obtained for the Tinta Roriz, Touriga Nacional, and Syrah varieties. In addition, Domínguez-Perles *et al*. (2016) study quantified ten phenolic compounds in the Tinto Cão, Tinta Barroca, Malvasia Fina, and Moscatel Branco cultivars, of which only four were also identified and quantified in the present study, being caftaric acid, quercetin-3-*O*-rutinoside, Ɛ-viniferin, and malvidin-3-*O*-glucoside. The concentrations achieved in the Domínguez-Perles *et al*. (2016) study for caftaric acid (2.18 ± 0.13 – 19.46 ± 1.37 mg/g dw), quercetin-3-*O*-rutinoside (1.08 ± 0.06 – 3.42 ± 0.22 mg/g dw), Ɛ-viniferin (2.94 ± 0.16 – 5.82 ± 0.37 mg/g dw), and malvidin-3-*O*-glucoside (18.17 ± 1.12 – 28.28 ± 1.80 mg/g dw) were significantly higher when compared to the results presented in Table 3.3.

Moreover, González-Centeno *et al*. (2012) quantified flavanols in grape stem extracts of ten varieties, being the catechin and epicatechin contents the relevant ones to the present study. They reached maximum concentrations of 1.34 ± 0.02 and 0.11 ± 0.02 mg/g dm for catechin and epicatechin, respectively, and these values were lower than the quantities found in Tinta Roriz and Touriga Nacional varieties for catechin, and Touriga Nacional and Fernão Pires for epicatechin. Additionally, Anastasiadi *et al*. (2012) work showed the presence of several phenolic compounds in grape stem extracts of six red and white Greek varieties. Comparing with the present study, common components were detected and quantified, namely, gallic acid, catechin, epicatechin, caftaric acid, resveratrol, and Ɛ-viniferin. The

results showed that the Greek cultivars analyzed had an average higher content of 79.91%, 35.11%, and 74,09% of gallic acid, resveratrol, and Ɛ-viniferin, respectively, than the average concentrations obtained in the present work. In contrast, the average values achieved for catechin, epicatechin, and caftaric acid were 19.76%, 34.95%, and 81.03% lower, respectively, when compared to the average contents presented for the varieties evaluated in present study. Sahpazidou *et al*. (2014) also quantified in their work the compounds gallic acid, catechin, epicatechin, resveratrol, and quercetin-3-*O*-rutinoside (or rutin), obtaining average concentrations of 19.24, 9.49, 13.05, 12.99, and 19.56 mg/g dw, being these values higher than those presented in the Table 3.3.

In the previously mentioned studies, several phenolic compounds were identified in different varieties, besides those found in present work. For example, in Portuguese grape stem varieties, different flavonols (quercetin, kaempferol and isorhamnetin) were detected (Dias *et al*., 2015; Domínguez-Perles *et al*., 2016; Queiroz *et al*., 2017), while in Greek grape stem varieties different acids were recognized, namely, ferulic, coumaric, caffeic and syringic acids (Anastasiadi *et al*., 2009; Sahpazidou *et al*., 2014).

Thus, analyzing the present work and the aforementioned studies, differences in phenolic composition were observed, in the qualitatively and quantitatively levels, proving that genetic differences between cultivars, growing conditions (soil composition and climatic conditions), ripening, and different extraction methods and drying influence the final content of phenolic compounds present in grape, and consequently in their by-products, in this case, grape stem (Salehi *et al*., 2019).

3.3. Conclusions

Grape stem had proved to be a rich matrix in phenolic compounds and reusing this by-product will help to achieve environmentally friendly production and help producers by reducing their costs in treating them. Since phenolic compounds are phytochemicals with various beneficial properties to our organism and grape stem is a rich source in these compounds, also having the advantage of being a cheap raw material, it can be used by cosmetic, pharmaceutical and food industries for extraction of these bioactive compounds, which may subsequently be applied in new products.

3.4. References

Anastasiadi, M., Chorianopoulos, N. G., Nychas, G. J. E., & Karoutounian, S. A. (2009). Antilisterial activities of polyphenol-rich extracts of grapes and vinification byproducts. *Journal of Agricultural and Food Chemistry*, *57*(2), 457–463. https://doi.org/10.1021/jf8024979

Anastasiadi, M., Pratsinis, H., Kletsas, D., Skaltsounis, A. L., & Haroutounian, S. A. (2012). Grape stem extracts: Polyphenolic content and assessment of their in vitro antioxidant properties. *LWT - Food Science and Technology*, *48*(2), 316–322. https://doi.org/10.1016/j.lwt.2012.04.006

Apostolou, A., Stagos, D., Galitsiou, E., Spyrou, A., Haroutounian, S., Portesis, N., … Kouretas, D. (2013). Assessment of polyphenolic content, antioxidant activity, protection against ROS-induced DNA damage and anticancer activity of Vitis vinifera stem extracts. *Food and Chemical Toxicology*, *61*, 60–68. https://doi.org/10.1016/j.fct.2013.01.029

Barros, A., Gironés-Vilaplana, A., Teixeira, A., Collado-González, J., Moreno, D. A., Gil-Izquierdo, A., … Domínguez-Perles, R. (2014). Evaluation of grape (Vitis vinifera L.) stems from Portuguese varieties as a resource of (poly)phenolic compounds: A comparative study. *Food Research International*, *65*(PC), 375–384. https://doi.org/10.1016/j.foodres.2014.07.021

Barros, A., Gironés-Vilaplana, A., Texeira, A., Baenas, N., & Domínguez-Perles, R. (2015). Grape stems as a source of bioactive compounds: application towards added-value commodities and significance for human health. *Phytochemistry Reviews*, *14*(6), 921–931. https://doi.org/10.1007/s11101-015-9421-5

Barros, A., Gouvinhas, I., Machado, N., Pinto, J., Cunha, M., Rosa, E., & Domínguez-Perles, R. (2016). New grape stems-based liqueur: Physicochemical and phytochemical evaluation. *Food Chemistry*, *190*, 896–903. https://doi.org/10.1016/j.foodchem.2015.06.047

Beres, C., Costa, G. N. S., Cabezudo, I., Silva-james, N. K., Teles, A. S. C., Cruz, A. P. G., … Freitas, S. P. (2017). Towards integral utilization of grape pomace from winemaking process : A review. *Waste Management*, *68*, 581–594. https://doi.org/10.1016/j.wasman.2017.07.017

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. *Antioxidants & Redox Signaling*, *18*(14), 1818–1892. https://doi.org/10.1089/ars.2012.4581

Dias, C., Domínguez-Perles, R., Aires, A., Teixeira, A., Rosa, E., Barros, A., & Saavedra, M. J. (2015). Phytochemistry and activity against digestive pathogens of grape (Vitis vinifera L.) stem's (poly)phenolic extracts. *LWT - Food Science and Technology*, *61*(1), 25–32. https://doi.org/10.1016/j.lwt.2014.11.033

Domínguez-Perles, R., Teixeira, A. I., Rosa, E., & Barros, A. I. (2014). Assessment of (poly)phenols in grape (Vitis vinifera L.) stems by using food/pharma industry compatible solvents and Response Surface Methodology. *Food Chemistry*, *164*, 339–346. https://doi.org/10.1016/j.foodchem.2014.05.020

Domínguez-Perles, Raúl, Guedes, A., Queiroz, M., Silva, A. M., & Barros, A. I. R. N. A. (2016). Oxidative stress prevention and anti-apoptosis activity of grape (Vitis vinifera L.) stems in human keratinocytes. *Food Research International*, *87*, 92–102. https://doi.org/10.1016/j.foodres.2016.06.030

Flamini, R., Mattivi, F., De Rosso, M., Arapitsas, P., & Bavaresco, L. (2013). Advanced knowledge of three important classes of grape phenolics: Anthocyanins, stilbenes and flavonols. *International Journal of Molecular Sciences*, *14*(10), 19651–19669. https://doi.org/10.3390/ijms141019651

Garrido, J., & Borges, F. (2013). Wine and grape polyphenols - A chemical perspective. *Food Research International*, *54*(2), 1844–1858. https://doi.org/10.1016/j.foodres.2013.08.002

González-Centeno, M. R., Jourdes, M., Femenia, A., Simal, S., Rosselló, C., & Teissedre, P.-L. (2012). Proanthocyanidin Composition and Antioxidant Potential of the Stem Winemaking Byproducts from 10 Different Grape Varieties (Vitis vinifera L.). *Journal of Agricultural and Food Chemistry*, *60*, 11850–11858. https://doi.org/dx.doi.org/10.1021/jf303047k

Gouvinhas, I., Santos, R. A., Queiroz, M., Leal, C., Saavedra, M. J., Domínguez-Perles, R., … Barros, A. I. R. N. A. (2018). Monitoring the antioxidant and antimicrobial power of grape (Vitis vinifera L.) stems phenolics over long-term storage. *Industrial Crops and Products*, *126*. https://doi.org/10.1016/j.indcrop.2018.10.006

Gouvinhas, Irene, De Almeida, J. M. M. M., Carvalho, T., Machado, N., & Barros, A. I. R. N. A. (2015). Discrimination and characterisation of extra virgin olive oils from three cultivars in different maturation stages using Fourier transform infrared spectroscopy in tandem with chemometrics. *Food Chemistry*, *174*, 226–232. https://doi.org/10.1016/j.foodchem.2014.11.037

Gouvinhas, Irene, Queiroz, M., Rodrigues, M., & Barros, A. I. R. N. A. (2019). *Evaluation of the Phytochemistry and Biological Activity of Grape (Vitis vinifera L.) Stems: Toward a Sustainable Winery Industry*. *Polyphenols in Plants* (2nd ed.). Elsevier Inc. https://doi.org/10.1016/B978-0-12-813768-0.00023-2

Llobera, A., & Cañellas, J. (2007). Dietary fibre content and antioxidant activity of Manto Negro red grape (Vitis vinifera): pomace and stem. *Food Chemistry*, *101*(2), 659–666. https://doi.org/10.1016/j.foodchem.2006.02.025

Llobera, A., & Cañellas, J. (2008). Antioxidant activity and dietary fibre of Prensal Blanc white grape (Vitis vinifera) by-products. *International Journal of Food Science and Technology*, *43*(11), 1953–1959. https://doi.org/10.1111/j.1365-2621.2008.01798.x

Machado, N. F. L., & Domínguez-Perles, R. (2017). Addressing facts and gaps in the

phenolics chemistry of winery by-products. *Molecules*, *22*(2), 1–48. https://doi.org/10.3390/molecules22020286

Magalhães, L. M., Segundo, M. A., Reis, S., & Lima, J. L. F. C. (2008). Methodological aspects about in vitro evaluation of antioxidant properties. *Analytica Chimica Acta*, *613*(1), 1–19. https://doi.org/10.1016/j.aca.2008.02.047

Marchante, L., Loarce, L., Izquierdo-cañas, P. M., Alañón, E., García-romero, E., Pérez-coello, M. S., & Díaz-maroto, M. C. (2019). Natural extracts from grape seed and stem by-products in combination with colloidal silver as alternative preservatives to SO2 for white wines: Effects on chemical composition and sensorial propertie. *Food Research International*, 108594. https://doi.org/10.1016/j.foodres.2019.108594

Pascual-Teresa, S. de, Moreno, D. A., & García-Viguera, C. (2010). Flavanols and anthocyanins in cardiovascular health: A review of current evidence. *International Journal of Molecular Sciences*, *11*(4), 1679–1703. https://doi.org/10.3390/ijms11041679

Queiroz, M., Oppolzer, D., Gouvinhas, I., Silva, A. M., Barros, A. I. R. N. A., & Domínguez-Perles, R. (2017). New grape stems' isolated phenolic compounds modulate reactive oxygen species, glutathione, and lipid peroxidation in vitro: Combined formulations with vitamins C and E. *Fitoterapia*, *120*, 146–157. https://doi.org/10.1016/j.fitote.2017.06.010

Sahpazidou, D., Geromichalos, G. D., Stagos, D., Apostolou, A., Haroutounian, S. A., Tsatsakis, A. M., … Kouretas, D. (2014). Anticarcinogenic activity of polyphenolic extracts from grape stems against breast, colon, renal and thyroid cancer cells. *Toxicology Letters*, *230*(2), 218–224. https://doi.org/10.1016/j.toxlet.2014.01.042

Salehi, B., Vlaisavljevic, S., Adetunji, C. O., Kregiel, D., Antolak, H., Pawlikowska, E., … Contreras, M. del M. (2019). Plants of the Genus Vitis: Phenolic compounds, anticancer properties and clinical relevance. *Trends in Food Science & Technology*, *91*(September), 362–379. https://doi.org/10.1016/j.tifs.2019.07.042

Sousa, C., Gouvinhas, I., Barreira, D., Carvalho, M. T., Vilela, A., Lopes, J., … Barros, A. I. (2014). "Cobrançosa" olive oil and drupe: Chemical composition at two ripening stages. *JAOCS, Journal of the American Oil Chemists' Society*, *91*(4), 599–611. https://doi.org/10.1007/s11746-013-2406-x

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. *International Journal of Molecular Sciences*, *15*(9), 15638–15678. https://doi.org/10.3390/ijms150915638

Teixeira, N., Mateus, N., de Freitas, V., & Oliveira, J. (2018). Wine industry by-product: Full polyphenolic characterization of grape stalks. *Food Chemistry*, *268*, 110–117. https://doi.org/10.1016/j.foodchem.2018.06.070

Tsao, R. (2010). Chemistry and biochemistry of dietary polyphenols. *Nutrients*, *2*(12), 1231–1246. https://doi.org/10.3390/nu2121231

Chapter IV:

Biological activities of
grape stem extracts

Biological activities of grape stem extracts

Abstract

Currently, by-products from the agri-food industry are being investigated so that they can be applied to new products, and at the same time, maintain the sustainability of the environment. Grape stem is one of these by-products, having in its composition different bioactive compounds, with known antioxidant, antimicrobial, anti-inflammatory, and anti-aging properties. In this sense, the objective of this work was to evaluate the biological activities of six varieties of grape stem extracts. Initially, the antioxidant capacity was tested by the ABTS, DPPH, and FRAP methods, followed by the antimicrobial activity of the extracts by disc diffusion and Minimum Inhibitory Concentration (MIC) assays against gastrointestinal and diabetic foot wound bacteria. Also, the anti-inflammatory activity of grape stem extracts was evaluated in RAW 264.7 cell line, but firstly, the toxicity of these extracts was tested. Finally, the capacity to inhibit the activity of tyrosinase and elastase enzymes, which are enzymes with an important role in aging, was analysed.

The results showed that grape stem extracts have different biological properties. Regarding to antioxidant activity, values up to 0.84 ± 0.06, 0.64 ± 0.05, and 1.03 ± 0.06 mmol T/g dw for ABTS, DPPH, and FRAP assays, respectively, were reached. While, for antimicrobial activity, grape stem extracts presented high efficacy against Gram-positive bacteria, even reaching inhibition halos superior to the tested antibiotics, also presenting MICs of 0.47 mg/mL. After testing the cellular toxicity of the extracts, their anti-inflammatory capacity exhibited inhibitions on NO production, by LPS-stimulated macrophages, ranging from 16.52% to 35.25%. Finally, grape stem extracts presented, for the first time, anti-aging effect by inhibiting anti-tyrosinase (~54%) and anti-elastase (~98%) activities.

Thus, grape stem proved to be a raw material that can be utilized in the cosmetic, pharmaceutical, and food industries, due to its great biological potential, demonstrating to have a high antioxidant power, besides being a matrix that can be applied in the fight against bacterial resistance through its use in the formulation of new antibiotics or in synergy with them. In addition, grape stem extracts, due to their anti-inflammatory power, may be used in the development of new anti-inflammatory drugs, and may also be applied in cosmetic products delaying skin wrinkling and treating/preventing pigmentation disorders due to its anti-tyrosinase and anti-elastase capabilities. However, additional studies on toxicity are

required, as well as, to discover the phenolic compounds present in grape stem mainly responsible for these properties.

Keywords: Grape stem; Biological activities; Bacterial resistance; Inflammation; Aging; Cosmetic/pharmaceutical industries.

4. Introduction

Currently, there is a growing interest from the scientific community to find new sources of functional ingredients from plant food by-products (Loizzo *et al.*, 2019), in order to achieve greater profitability and minimize the environmental impact of these residues (Taurino *et al*., 2019). Grape stem is an example of these by-products, being a waste of wine industry that has low commercial value, and although there is little information related to the actual applicability of these by-product, several studies show that it can be applied in several sectors, namely, cosmetic, pharmaceutical, and food industries, due to their content in phenolic compounds (Gouvinhas *et al*., 2019). Phenolic compounds are secondary metabolites produced by plants, which due to their several bioactivities, have been of interest. These compounds are demonstrated to have several health benefits, acting as antioxidants, antimicrobials, anticarcinogens, antidiabetics, among others (Rodrigues *et al*., 2018).

The human body, when exposed to pollutants, drugs, tobacco smoke, radiation, and toxic metals, produces a large quantity of reactive oxygen species (ROS), which causes oxidative stress, damaging vital biomolecules (lipids, proteins and DNA) (Zhang & Tsao, 2016), and may also induce inflammation as a consequence of these external attacks/injuries to cells or tissues (Shukla *et al*., 2019). By acting as antioxidants, phenolic compounds prevent or inhibit the production of ROS, suspending oxidation processes and repairing tissues deficiencies though hydrogen donations of OH groups, extinction of free radicals, chelation of metal ions (iron, cupper) or singlet oxygen inactivation (Baenas *et al*., 2018; Zhu *et al*., 2018). Moreover, while non-steroidal anti-inflammatory drugs are the most widely used in the world to fight inflammation, they have undesirable effects, for example on the kidneys and cardiovascular system, limiting their use. Thus, new studies are being performed to identify natural compounds that can be applied as anti-inflammatory agents or in the development of new drugs, being the phenolic compounds candidates (Aouey *et al*., 2016), since they can behave as anti-inflammatory, inhibiting inflammatory mediators such as NO (nitric oxide) and ROS, and regulating the activity of inflammatory enzymes (COXs and iNOS) (Shukla *et al*., 2019).

In addition, oxidative stress and inflammation are also related to aging processes (Martín-Ortega & Campos, 2019). In aging, changes in the biosynthetic activity of skin-derived cells, namely some constitutive and extracellular matrix proteins (e.g. collagen, elastin, and hyaluronic acid) arise, as well as in a simultaneous activity modulation of several aging-related enzymes (elastase, tyrosinase, collagenase, and hyaluronidase) (Aguilar-Toalá *et*

al., 2019). Reactive oxygen species (ROS) can cause, for example, a huge production of elastases, a reduction and degeneration of collagen, and an increase in melanin biosynthesis. The tyrosinase enzyme (also called monophenol monooxygenases), in humans, is responsible for melanin synthesis in melanocytes, but when overproduction of melanin occurs in the skin, pigmentation disorder, such as hyperpigmentation, over-tanning, age spots and melasma occur (Liyanaarachchi *et al*., 2018). Elastase is an enzyme that hydrolyses elastin (a protein that maintains the elasticity and firmness of the skin), influencing the mechanical properties of connective tissues, thus when a high activity of this enzyme is observed, or less elastin is produced the skin loses firmness and elasticity (Aguilar-Toalá *et al*., 2019). Phenolic compounds can inhibit the activity of these enzymes through complexation or precipitation reactions (Kolakul *&* Sripanidkulchai, 2017).

Antibiotic resistance is a public health problem worldwide, having impact in morbidity and/or high mortality rates, especially in developing countries (Lima *et al*., 2019). Specific microorganisms such as *Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus* are present frequently in processed food, and consequently transmit diseases, constituting a health risk (Gutiérrez-del-Río *et al*., 2018). In addition, one of the main causes of hospitalization worldwide are diabetic foot ulcer infections, mostly caused by *Staphylococcus aureus*, and due to frequent use of antibiotics, *S. aureus* strains have developed resistance to treatment (Zenão *et al*., 2017). To combat bacterial resistance, alternative antibiotics should be searched and developed to overcome the effectiveness of those currently available and, in these sense, data show that phenolic compounds may be an alternative due to their antibacterial properties (Lima *et al*., 2019). Phenolic compounds, acting as antimicrobials, weaken the phospholipid bilayer of the bacterial cell membrane, causing the release of vital cytoplasmatic constituents, and damaging bacterial enzyme systems (Madureira *et al*., 2015).

Thus, in order to replace synthetic drugs/antibiotics with more effective natural compounds, there is a growing demand to find natural compounds that can be used in the development of new products. In this way, the aim of this work was to evaluate four biological activities of phenolic compounds present in grape stems, namely, antioxidant activity by $ABTS^{\bullet+}$, $DPPH^{\bullet}$, and FRAP methods; antimicrobial activity against gastrointestinal and diabetic foot wound bacteria by disc diffusion and MIC assays; anti-inflammatory activity using a mouse macrophages cell line; and antiaging activity through inhibition of tyrosinase

and elastase enzymes, allowing to prove if this by-product will be a good bet to produce new formulations.

4.1. Material and Methods

4.1.1. Chemicals

The compounds 2,2-diphenyl-1-picrylhidrazyl radical (DPPH$^{\bullet}$), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)diammonium salt (ABTS$^{\bullet+}$), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), potassium persulfate, 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), ferric chloride, and the enzymes tyrosinase, elastase, and reagents for all enzymatic activities were purchased from Sigma-Aldrich (Steinheim, Germany). Saline water (0.9% NaCl), methanol, and sulfanilamide were acquired from Merck (Merck, Darmstadt, Germany). All culture media and antibiotics used in antimicrobial activity were purchase from Oxoid (Oxoid Limited, Thermo Fisher Scientific Inc.). Ultrapure water was obtained using a Millipore water purification system. Hydrochloric acid was acquired from Fluka Chemika (Neu-Ulm, Switzerland). Dulbeco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and Alamar Blue® (AB) reagent were obtained from Invitrogen (Alfagene, Portugal).

4.1.2. Sampling

See chapter II, section 2.1.2.

4.1.3. Sample preparation

See chapter II, section 2.1.3.

4.1.4. Grape stem extracts preparation

See chapter III, section 3.1.4.

4.1.5. Antioxidant activity

4.1.5.1. ABTS$^{\bullet+}$ (2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical) scavenging capacity

In this method potassium persulfate ($K_2S_2O_8$) is added to ABTS, oxidizing it to ABTS$^{\cdot+}$, resulting in a dark bluish green color. When to this mixture is added antioxidants,

the cation is reduced to ABTS, and discoloration is observed compared to the initial solution (Mena *et al*., 2011).

Initially, 88 µL of potassium persulfate (148 mM) are added to an ABTS solution (7 mM), and the mixture is left to rest for 12-16 h protected from light and at room temperature, to reach its stable oxidative state. After this period, the working solution was prepared by diluting the $ABTS^{\cdot+}$ solution in sodium acetate buffer (20 mM, pH = 4.5) until an absorbance of 0.700 ± 0.020 is obtained at a wavelength of 734 nm. Standards of Trolox were prepared with different concentrations (0.034 – 0.140 mM). Then, 188 µL of the $ABTS^{\cdot+}$ solution is added, plus 12 µL of each standard solution and sample. Blank was also included by adding 12 µL of distilled water to the $ABTS^{\cdot+}$ solution. The reaction was incubated at room temperature protected from light for 30 min. Then, the antioxidant activity was evaluated by measuring absorbance at 734 nm, using a microplate reader (Thermo-Fisher Scientific, Oporto, Portugal). To calculate the inhibition percentage of each standard solution and sample, the following formula was used: *'% Inhibition = 100 x* $(Abs734_{BLANK} - Abs734_{SAMPLE} / Abs734_{BLANK})$*'*. All standards solutions and samples were analyzed in triplicate and the results of the radical scavenging capacity were expressed as millimoles of Trolox per gram of dry weight (mmol T/g dw).

4.1.5.2. DPPH· (2,2-diphenyl-1-picrylhidrazyl radical) scavenging capacity

In this method, DPPH· has a purple color, but in the presence of antioxidants it is reduced to diphenyl-picryl-hydrazine, presenting a discoloration compared to the initial solution (Mena *et al.*, 2011).

A mixture was prepared by diluting 0.5 mL of DPPH (8.87 mM) into 26 mL of MeOH/H_2O (70:30, v/v), adjusting its absorbance to 1.000 at a wavelength of 520 nm. Standards of Trolox were prepared with different concentrations (0.039 – 0.625 mM). Then, 190 µL of the DPPH· solution are added, plus 10 µL of each standard solution and sample. A blank was also prepared by adding 10 µL of MeOH/H_2O (70:30, v/v) to the DPPH· solution. The reaction was incubated at room temperature protected from light for 15 min, and the antioxidant activity was evaluated by measuring absorbance at 520 nm, using a microplate reader (Thermo-Fisher Scientific, Oporto, Portugal). To calculate the inhibition percentage of each standard solution and sample, the following formula was used: *'% Inhibition = 100 x* $(Abs520_{BLANK} - Abs520_{SAMPLE} / Abs520_{BLANK})$*'*. All standards solutions and samples were

analyzed in triplicate and the results of the radical scavenging capacity were expressed as millimoles of Trolox per gram of dry weight (mmol T/g dw).

4.1.5.3. FRAP (Ferric Reduction Antioxidant Power) scavenging capacity

This assay is based on the reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions at low pH which causes the formation of a violet-blue color ferrous-tripyridyltriazine complex. The absorbance obtained is proportional to the total ferric reducing/antioxidant power of the antioxidants (Vuolo *et al*., 2019).

This method was performed according to Bolanos de la Torre *et al*. (2015), adapted for 96-well microplates (Frilabo, Milheirós, Portugal). Initially, the FRAP working solution was prepared by mixing 10 volumes of acetate buffer (330 mM, pH = 3.6), 1 volume of TPTZ (2,4,6-Tripyridyl-s-Triazine) (40 mM) previously dissolved in HCl (40 mM) and 1 volume of ferric chloride (20 mM). This solution is prepared daily and heated 10 minutes before use. Standards of Trolox were prepared with different concentrations (0.039 – 0.625 mM). Then, 140 µL of the FRAP solution are added, plus 10 µL of each standard solution and sample. The reaction was incubated at 37 ºC protected from light for 30 min and the absorbance was read at 593 nm, in the microplate reader (Thermo-Fisher Scientific, Oporto, Portugal). All standards solutions and samples were analyzed in triplicate and the results of the antioxidant power capacity were expressed as millimoles of Trolox per gram of dry weight (mmol T/g dw).

4.1.6. Antimicrobial activity

4.1.6.1. Bacterial isolates

In this work, clinical isolates (Gram-positive and Gram-negative bacteria) were used, collected from biological samples of the gastrointestinal segment of humans and diabetic foot ulcers, provided by the Hospital Centre of Trás-os-Montes and Alto Douro (CHTMAD), under the protocol established since 2004, belonging to the MJS, MJMC and MJH collections, namely, *Staphylococcus aureus* MJS241, *Enterococcus faecalis* MJS257, *Staphylococcus aureus* MJMC109, *Staphylococcus aureus* MJMC534B, *Escherichia coli* MJS260, *Klebsiella pneumoniae* MJS281, *Klebsiella pneumoniae* MJH602, and *Enterobacter aerogenes* MJMC534A. Reference bacteria obtained from American Type Culture Collection (ATCC) were also used, specifically *Listeria monocytogenes* ATCC 15313 and *Pseudomonas aeruginosa* ATCC 10145, and from Spanish Type Culture Collection (CECT), namely

Staphylococcus aureus CECT 976 (TABLE 4.1). The isolate collections are stored in the medical microbiology laboratory, DCV-ECAV, and laboratory blocks of the University of Trás-os-Montes and Alto Douro.

The bacterial isolates were previously identified by biochemical methods (API 20E, API 20NE, API Staphy (BioMérieux)) and by molecular methods, namely, the partial sequencing of the 16S rRNA gene. All isolates were stored in aliquots of BHI (Brain Heart Infusion) at -70 ºC, in 15 % (v/v) of glycerol. Prior to antimicrobial activity tests, isolates were cultivated on BHI agar for 24 h at 37 ºC. The processing of the bacterial cultures used in this study followed all laboratory safety procedures, according to point 4 of the UTAD biosafety standards.

Table 4.1. Bacterial isolates tested.

Bacterial isolates	**Source**	**Class**
Listeria monocytogenes ATCC 15313	American Type Culture Collection	Gram +
Staphylococcus aureus CECT 976	Spanish Type Culture Collection	Gram +
Staphylococcus aureus MJS241	Clinical-human gastrointestinal segment	Gram +
Enterococcus faecalis MJS257	Clinical-human gastrointestinal segment	Gram +
Staphylococcus aureus MJMC109	Diabetic foot ulcers	Gram +
Staphylococcus aureus MJMC534B	Diabetic foot ulcers	Gram +
Pseudomonas aeruginosa ATCC 10145	American Type Culture Collection	Gram -
Escherichia coli MJS260	Clinical-human gastrointestinal segment	Gram -
Klebsiella pneumoniae MJS281	Clinical-human gastrointestinal segment	Gram -
Klebsiella pneumoniae MJH602	Clinical-human gastrointestinal segment	Gram -
Enterobacter aerogenes MJMC534A	Diabetic foot ulcers	Gram -

4.1.6.2. Disc diffusion assay

For the determination of the antimicrobial activity was used the disc diffusion method, described by Bauer *et al.* (1966), with some modifications. In this method the suspensions were carried out from a pure culture in 0.9% NaCl, and the turbidity was adjusted to the value of 0.5 McFarland. Then, suspensions of the bacterial isolates were cultivated with a swab in Petri dishes (90 mm diameter) containing 20 mL of Muller-Hinton agar. Afterwards, sterile white disks (6 mm diameter) were placed on the plates using a disk dispenser (OXOID) and impregnated with 10 µL of the different samples (the extracts were evaporated in nitrogen, and posteriorly, dissolved in 10% DMSO) at the same concentration (200 mg/mL). In the tests performed, commercial antibiotics, namely, Gentamicin 10 µg, Gentamicin 30 µg, and

Ciprofloxacin 10 µg, were used as positive controls and 10% DMSO (non-toxic to the bacterial cells) as a negative control. After 24 h of incubation at 37 ºC, the microbial growth inhibition halos were measured around the disks impregnated with the samples and antibiotics.

The antimicrobial activity was calculated according to the following equation: *'% RIZD = (IZD_{sample} – $IZD_{negative\ control}$) / $IZD_{antibiotic}$ x 100%'*, where IZD corresponds to inhibition halos (mm) and RIZD to the percentage of the relative diameter of the inhibition halo (Aires *et al*., 2009). This equation considers, and compensates, the possible effect of solvent (blank) other than water. Additionally, the antimicrobial activity of each extract was also classified according to the following:

- No effect (-): inhibition halo = 0;
- Moderate efficacy (+): 0 < inhibition halo < antibiotic inhibition halo;
- Good efficacy (++): antibiotic inhibition halo < inhibition halo < 2 x antibiotic inhibition halo;
- High efficacy (+++): inhibition halo > 2 x antibiotic inhibition halo.

4.1.6.3. Minimum Inhibitory Concentration (MIC) assay

The Minimum Inhibitory Concentration (MIC) method was performed, with some modifications, as reported by Sarker *et al*. (2007). Initially, isolates were cultivated on BHI agar for 24 h at 37 ºC. After this period, the bacterial cultures were passed into flasks with Muller-Hinton broth and incubated at 37 ºC for 12-18 h. Then, the absorbance of bacterial suspension was adjusted at 0.100 with a wavelength of 625 nm in a microplate reader (Thermo-Fisher Scientific, Oporto, Portugal), to obtain a concentration of 1 x 10^8 cfu/mL. In the first row of the 96-well microplates (Frilabo, Milheirós, Portugal), 200 µL of each sample (30 mg/L), previously evaporated in nitrogen and dissolved in 10% DMSO, and the positive and negative controls were added. In the remaining wells, 100 µL of Muller-Hinton broth were added, and ½ serial dilutions were performed for all samples and controls. Muller-Hinton broth and DMSO solution were used as negative controls, and the gentamicin and ciprofloxacin antibiotics were used as positive controls, having the same concentration of samples. Then, 20 µL of bacterial suspension (2 x 10^6 cfu/well) were added. Finally, 20 µL of resazurin indicator solution, prepared by dissolving 270 mg tablet in 40 mL of sterile water, were added to each well. The microplates were incubated at 37 ºC for 18 h, and then the color

change was evaluated visually. The lowest concentration where there was no color change (purple) was considered the minimum inhibitory concentration (MIC).

Afterwards, from each well containing the minimum inhibitory concentration of the samples, a volume of 100 µL was taken and spread in Petri dishes with Muller-Hinton agar. The Petri dishes were incubated at 37 ºC for 24 h, and at the end of this period, it was observed if there was bacterial growth. In case of growth, the minimum inhibitory concentration was considered to have a bacteriostatic action, but if there was no growth, it was considered to have a bactericidal action.

4.1.7. Cell culture and cell viability assay

The Raw 264.7 cell line (Mouse macrophages, Abelson murine leukemia virus-induced tumor, CLS, Germany) was cultured in flasks (Orange Scientific, Frilabo, Portugal) with DMEM culture medium, supplemented with 10 % FBS (v/v), and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) at 37 ºC in an atmosphere of 5 % CO_2/95% air with controlled humidity as described by Queiroz *et al*. (2017). Then, the cells were detached from the culture flasks, counted, diluted at density of 1.00 x 10^5 cells/mL and plated in 96-well culture plates (100 µL/well), obtaining a density of 1.00 x 10^4 cells/well. For the next 24 hours, the cells were allowed to stabilize to adhere to the walls.

For the cell viability assay, cells were incubated in fresh FBS-free medium (control) and in fresh FBS-free medium containing the grape stem extracts (the extracts were evaporated in nitrogen, and posteriorly, resuspended in phosphate-buffered saline solution (20 mg/mL)) with final concentrations ranging from 1.00 to 50.00 µg/mL for Touriga Nacional, Syrah and Fernão Pires extracts, 5.00 to 75.00 µg/mL for Tinta Roriz and Castelão extracts, and 25.00 to 150.00 µg/mL for Arinto extract. The cells were exposed to extracts for 24 h and 48 h, and after the exposure time, the culture media was replaced with FBS-free medium supplemented with 10% (v/v) of Alamar Blue (AB). After 4 h, the absorbance was read at 570 and 620 nm using a Multiskan EX microplate reader (MTX Labsystems, USA). The percentage of AB reduction was calculated according the following equation, previously described by Andreani *et al*. (2014): *'% AB reduction = $((\varepsilon_{OX}\lambda_2)(A\lambda_1) - (\varepsilon_{OX}\lambda_1)(A\lambda_2))/((\varepsilon_{RED}\lambda_1)(Å\lambda_2) - (\varepsilon_{RED}\lambda_2)(Å\lambda_1))$*100'*, where, $\varepsilon_{OX}\lambda_1$ correspond to the molar extinction coefficient of oxidized AB at 570 nm, $\varepsilon_{OX}\lambda_2$ to the molar extinction coefficient of oxidized AB at 620 nm, $\varepsilon_{RED}\lambda_1$ to the molar extinction coefficient of reduced AB at 570 nm, $\varepsilon_{RED}\lambda_2$ to the molar extinction coefficient of reduced AB at 620 nm, $A\lambda_1$ and $A\lambda_2$ to the absorbance of

test wells at 570and 620 nm, respectively, and Åλ$_1$ and Åλ$_2$ to the absorbance of the negative control wells (no cells) at 570 and 620 nm, respectively. The results were expressed as percentage of control (non-exposed cells). All samples and negative control were analyzed in quadruplicate.

4.1.8. Anti-inflammatory activity

The anti-inflammatory activity of grape stem extracts was performed as report by Queiroz *et al*. (2017). The Raw 264.7 cell line was cultured as mentioned above. The cells were incubated, during 24 h, in fresh FBS-free medium containing grape stem extracts, in the absence and in the presence of 1µg/mL LPS (lipopolysaccharide), to induce the nitrite (NO) production. Grape stem extracts were applied at different concentrations, namely, 5.00, 12.50 and 25.00 µg/mL for Tinta Roriz, Touriga Nacional, Castelão, Syrah, and Fernão Pires extracts, and 12.50, 25.00, 50.00 and 75.00 µg/mL for Arinto extract.

NO production was measured colorimetrically with the Griess reagent, which detects the accumulated nitrites in the cell supernatants, after the 24h incubation. From each well, 50 µL of supernatant, was transferred to a new 96-well plate, and 50 µL of Griess reagent (0.1% (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride in water and 1% (w/v) sulfanilamide prepared in 5.0% (w/v) H_3PO_4 (v/v)) was added. The plates were incubated at room temperature protected from light for 10 min, and the absorbance was read at 550 nm using a Multiskan EX microplate reader (MTX Labsystems, USA). For the quantification of NO production, a sodium nitrite (Na_2NO_3) standard curve (0 to 100 µM) was used, and the results were expressed as percentage of NO production, in relation to control.

4.1.9. Anti-aging activity

4.1.9.1. Tyrosinase inhibition assay

In this assay, reported by Taghouti *et al*. (2018), 25 µL of each sample (1 mg/mL), plus 80 µL of phosphate buffer (50 mM, pH 6.8) and 40 µL of L-DOPA (2.50 mM) were added, and the plate was incubated for 2 min at 37º C. Then, 40 µL of tyrosinase (40 U/mL, in phosphate buffer (50 mM, pH 6.5)) were added to initiate the reaction. The reaction was incubated for 10 min at 37º C, and after this time, the absorbance was read at 492 nm, in the microplate reader (Thermo-Fisher Scientific, Oporto, Portugal). Methanol was used as positive control. The tyrosinase inhibition was calculated according to the following equation:

'% Inhibition = ($Abs_{control}$ – Abs_{sample}) / $Abs_{control}$ x 100%'. All samples were analyzed in triplicate.

4.1.9.2. Elastase inhibition assay

For elastase inhibition, the Taghouti *et al*. (2018) method was used. Initially, 50 µL of each sample (1 mg/mL), plus 160 µL of Tris-HCl buffer (0.20 mM, pH 8) and 20 µL of N-(methoxysuccinyl)-ala-ala-pro-val-4-nitroanilide (0.80 mM, in buffer) were added, and incubated for 10 min at room temperature. Then, 20 µL of elastase (0.40 U/mL, in Tris-HCl buffer) were added to initiate the reaction, this was incubated for 20 min at room temperature. After, the absorbance was read at 410 nm using a microplate reader (Thermo-Fisher Scientific, Oporto, Portugal). Methanol was used as positive control. The elastase inhibition was calculated according to the following equation: *'% Inhibition = ($Abs_{control}$ – Abs_{sample}) / $Abs_{control}$ x 100%'*. All samples were analyzed in triplicate.

4.1.10. Statistical analysis

The antioxidant and anti-aging activities data were subjected to the IBM SPSS 22.0 statistical software (SPSS Inc., Chicago, IL, USA), using analysis of variance (ANOVA) and a multiple range test (Tukey's test), for a p-value <0.05. The results of the samples are presented as mean values ± standard deviation (n = 3). The cell viability and anti-inflammatory activity data were subjected to the GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA), using one-way and two-way ANOVA along with a multiple comparison test (Tukey's test), for a p-value <0.05. The results of the samples are presented as mean values ± standard deviation (n = 4).

4.2. Results and Discussion

4.2.1. Antioxidant activity of grape stem extracts

The results regarding to antioxidant activity by ABTS, DPPH, and FRAP methods are presented in Table 4.2. Analyzing the results, significant differences between varieties were verified for the three methods. In the three assays, the Touriga Nacional variety demonstrated the highest antioxidant power, reaching values of 0.84 ± 0.06, 0.64 ± 0.05, and 1.03 ± 0.06 mmol T/g dw, for ABTS, DPPH, and FRAP methods, respectively, while the Arinto variety exhibited lower power antioxidant, presenting values of 0.35 ± 0.00, 0.15 ± 0.01, and 0.35 ± 0.02 mmol T/g dw for ABTS, DPPH, and FRAP methods, correspondingly.

Table 4.2. Scavenging capacity (mmol T/g dw) of grape stem extracts by ABTS, DPPH, and FRAP methods.

Samples	ABTS	DPPH	FRAP
Tinta Roriz	0.70 ± 0.02^{d}	0.61 ± 0.03^{de}	0.94 ± 0.02^{cd}
Touriga Nacional	0.84 ± 0.06^{e}	0.64 ± 0.05^{e}	1.03 ± 0.06^{e}
Castelão	0.46 ± 0.01^{b}	0.31 ± 0.01^{b}	0.56 ± 0.01^{b}
Syrah	0.59 ± 0.01^{c}	0.44 ± 0.04^{c}	0.85 ± 0.02^{c}
Arinto	0.35 ± 0.00^{a}	0.15 ± 0.01^{a}	0.35 ± 0.02^{a}
Fernão Pires	0.69 ± 0.02^{d}	0.55 ± 0.01^{d}	0.99 ± 0.02^{de}
p-value	***	***	***

The values are presented as mean ± standard deviation (n = 3). Different letters indicate significantly different results (ANOVA, $p < 0.05$). Significance: non-significant, N.S. ($p > 0.05$); * significant at $p < 0.05$; ** significant at $p < 0.01$; *** significant at $p < 0.001$.

In general, the antioxidant power of the different grape stem varieties was as follows: Touriga Nacional > Tinta Roriz > Fernão Pires > Syrah > Castelão > Arinto, except for Fernão Pires and Tinta Roriz varieties on the FRAP assay. Comparing the values obtained in the antioxidant activity with the results presented in the phenolic composition (see chapter III, section 3.2.1), it was concluded that the polyphenolic content of each variety is correlated with the antioxidant activity achieved (Barros *et al*., 2014), and therefore the varieties with highest concentration of phenols (Touriga Nacional > Tinta Roriz > Fernão Pires > Syrah > Castelão > Arinto) were the ones with the greatest reducing power. Also, the presence of specific compounds, such as gallic acid, catechin, epicatechin, resveratrol, and quercetin-3-*O*-rutinoside (or rutin) found in the present work in grape stem extracts (see chapter III, section 3.2.2) is associated with a high antioxidant capacity (Gouvinhas *et al*., 2018).

Moreover, when comparing the ABTS and DPPH methods, the first presented higher values, this is explained by the fact that in the ABTS assay, hydrophilic and lipophilic antioxidants were quantified (Kusumawati & Indrayanto, 2013), while in DPPH assay only lipophilic antioxidants were quantified. Regarding the FRAP assay, this cannot be compared with the other assay performed in the present study, since the ABTS and DPPH methods have radicals in their systems and the FRAP method does not, making it impossible to compared the results obtained (Vuolo *et al*., 2019).

In literature data, Domínguez-Perles *et al*. (2014) evaluated the antioxidant capacity of the Touriga Nacional (red) and Viosinho (white) varieties, by the ABTS method, using different extractions to understand the effects of solvent concentrations, temperature, and extraction time. They reached maximum scavenging capacity values of 0.18 ± 0.00 and

0.05 ± 0.00 mmol T/g dw for the Touriga Nacional and Viosinho varieties, respectively. These results showed a much lower scavenging capacity than those obtained for the six grape stem varieties (Table 4.2). In other work, Domínguez-Perles *et al*. (2016) quantified the antioxidant capacity of grape stem extracts by the ABTS and DPPH assays, using two red (Tinto Cão, Tinta Barroca) and two white (Malvasia Fina, Moscatel) varieties, and reported lower antioxidant activity than the one in the current study. In addition, Gouvinhas *et al*., (2018) study quantified the scavenging capacity of red grape stem extracts of the Syrah, Tinta Barroca, and Sousão varieties by the ABTS and DPPH assays during 64 days of storage. Regarding the ABTS assay, average values of 4.77, 7.76, and 6.44 mmol T/g dw for Sousão, Tinta Barroca, and Syrah varieties, respectively, were obtained, which proved to be much higher than those achieved in the present study (0.35 ± 0.00 – 0.84 ± 0.06 mmol T/g dw). In the DPPH assay, they presented average values of 0.54, 0.90, and 0.84 mmol T/g dw for Sousão, Tinta Barroca, and Syrah varieties, respectively, being the results shown in Table 4.2 lower than those reached for Tinta Barroca and Syrah varieties.

Moreover, in the Poveda *et al*. (2018) study the antioxidant capacity by ABTS and DPPH assays of Tempranillo (or Tinta Roriz) grape stem extracts was analyzed. Two extraction methods were performed, with values of 0.02 ± 0.00 and 0.03 ± 0.00 mmol T/g of wet weight for ABTS method, and 0.01 ± 0.00 and 0.02 ± 0.00 mmol T/g of wet weight for DPPH method. These results, although presented by wet weight, demonstrated to be much lower than those presented in Table 4.2 for the six varieties. Anastasiadi *et al*. (2012) work also determined the antioxidant activity of grape stem extracts by the FRAP assay, using white (Asyrtiko, Athiri, and Aidani) and red (Mandilaria, Mavrotragano, and Voidomatis) cultivars, reporting higher values, on average, 64.92% for white varieties, when compared to the average value obtained in the present work (0.67 mmol T/g dw). In the case of the red varieties, they presented higher values, on average 59.52%, when compared to the average value determined for the red varieties tested in present study (0.85 mmol T/g dw).

Other by-products of the wine industry have also been shown to have an antioxidant capacity. However, the grape stem extracts used in the present work, when compared to some studies that determine the antioxidant activity of grape pomace, seeds, and skins, have shown to have an antioxidant capacity equal to or greater than theses by-products, depending on the variety (Jara-Palacios *et al*., 2014; Jara-Palacios *et al*., 2014; Ky *et al*., 2014; Melo *et al*., 2015; Poveda *et al*., 2018; Rockenbach *et al*., 2011; Teles *et al*., 2018), thus proving to be a matrix with a high antioxidant power. Nevertheless, the differences between the cited studies

and the by-products can be explained by the fact that there are variations in the execution of antioxidant activity assays, and by a possible correlation between phenolic composition and the antioxidant capacity, where varieties with higher phenolic content generally have higher antioxidant activity (Katalinić *et al*., 2010). However, the phenolic composition between varieties may differ due to genetic characteristics of each variety, different extraction methods, distinct agroclimatic conditions, and maturity at harvest time, as mentioned in chapter III.

4.2.2. Antimicrobial activity of grape stem extracts

4.2.2.1. Disc diffusion

The results of antimicrobial activity of grape stem extracts by the disc diffusion assay are presented in Table 4.3. Concerning to Gram-positive bacteria, the inhibition halos ranged from 8 to 11 mm for the grape stem extracts tested. The varieties that achieved the greatest inhibition of bacterial growth were Tinta Roriz, Touriga Nacional, and Syrah, against *S. aureus* CECT 976, *E. faecalis* MJS257, and *S. aureus* MJMC109 isolates, respectively. It should also be noted that although the tested antibiotics showed greater inhibition halos, they did not have any effect on growth inhibition in *S. aureus* MJS241 and *E. faecalis* MJS257 isolates, unlike grape stem extracts. However, in the case of Gram-negative bacteria, the grape stem extracts only inhibited the growth of *P. aeruginosa* ATCC 10145 isolate, with inhibition halos ranging from 8 to 10 mm. In contrast, all antibiotics tested did not allow bacterial growth, except for ciprofloxacin in *K. pneumoniae* MJH602 isolate.

In Table 4.4 are exhibited the %RIZD of each grape stem extract in relation to the antibiotics tested. These results showed that percentages higher than 100, indicate that the extracts have a higher bacterial growth inhibiting power than antibiotics, as can be seen for *P. aeruginosa* ATCC 10145 isolate, where the Syrah and Fernão Pires varieties presented a %RIZD of 111 relatively to the gentamicin antibiotic (10 µg). Analyzing the results, it was also possible to observe that for the *S. aureus* MJS241 and *E. faecalis* MJS257 isolates no value was presented (#), this happened because, although the grape stem extracts inhibited the growth of the isolates, the antibiotics did not achieved it, making it impossible to calculate the %RIZD. However, the %RIZD for extracts tested in these isolates was significantly higher when compared to the other isolates, demonstrating the potential of this matrix to inhibit bacterial growth. Regarding to the extracts that presented a %RIZD equal to 100, such as, the Touriga Nacional and Fernão Pires varieties in relation to the ciprofloxacin antibiotic for

S. aureus MJMC534B isolate, and the Tinta Roriz, Touriga Nacional, and Castelão varieties in relation to the gentamicin antibiotic (10 µg) for *P. aeruginosa* ATCC 10145 isolate, this indicate that these extracts had the same inhibition of bacterial growth as the antibiotic tested. The extracts that did not exhibit any inhibition of bacterial growth presented a %RIZD of 0.

In addition, antimicrobial activity was also classified as: (i) no effect; (ii) moderate efficacy; (iii) good efficacy; and (iv) high efficacy, being the results shown in Table 4.5. For Gram-positive bacteria all grape stem extracts presented moderate efficacy compared to antibiotics, except for *S. aureus* MJS241 and *E. faecalis* MJS257 isolates, where extracts exhibited a high efficacy. In relation to Gram-negative bacteria, the extracts indicated no effect on microbial activity, except for *P. aeruginosa* ATCC 10145 isolate, where extracts of the varieties Tinta Roriz, Touriga Nacional, Castelão, Syrah, and Fernão Pires demonstrated a good efficacy in inhibiting bacterial growth when compared to the gentamicin antibiotic (10 µg), but when compared to the remaining antibiotics, the six grape stem extracts showed only moderate efficacy.

Grape stem extracts showed to inhibit bacterial growth due to the accumulation of phenolic compounds (especially those with OH groups) present in grape stems, in the lipid bilayer cell, thus causing changes in the membrane structure and function. Phenolic compounds infiltrate the bacterial cell, applying inhibitory activity in the cellular cytoplasm, leading to lysis and release of intracellular ATP, and may also cause loss of cellular components, increasing the permeability of the cytoplasmic membrane (Gyawali & Ibrahim, 2014; Mattos *et al*., 2017). However, it was observed that grape stem extracts were more effective in inhibiting the growth of Gram-positive than Gram-negative isolates. This happened because Gram-negative bacteria have a lipopolysaccharide layer on the outer membrane, being a physical barrier that impedes the entry of phenolic compounds (Pisoschi *et al*., 2017).

Table 4.3. Inhibition halos (mm) obtained for grape stem extracts, and positive and negative controls tested.

Bacterial isolates	TR	TN	CT	SH	AR	FP	CN10	CN30	CIP10	DMSO
L. monocytogenes ATCC 15313	9	8	10	10	8	10	20	22	23	ND
S. aureus CECT 976	11	10	9	10	8	10	18	22	29	ND
S. aureus MJS241	9	9	10	10	8	10	ND	ND	ND	ND
E. faecalis MJS257	10	11	10	10	9	10	ND	ND	ND	ND
S. aureus MJMC109	10	10	10	11	9	10	19	21	27	ND
S. aureus MJMC534B	9	10	9	9	9	10	19	22	10	ND
P. aeruginosa ATCC 10145	9	9	9	10	8	10	9	12	21	ND
E. coli MJS260	ND	ND	ND	ND	ND	ND	15	17	9	ND
K. pneumoniae MJS281	ND	ND	ND	ND	ND	ND	17	19	25	ND
K. pneumoniae MJH602	ND	ND	ND	ND	ND	ND	18	20	ND	ND
E. aerogenes MJMC534A	ND	ND	ND	ND	ND	ND	16	18	27	ND

TR: Tinta Roriz; TN: Touriga Nacional; CT: Castelão; SH: Syrah; AR: Arinto; FP: Fernão Pires; CN10: gentamicin (10 µg per disc); CN30: gentamicin (30 µg per disc); CIP10: ciprofloxacin (10 µg per disc); DMSO: dimethyl sulfoxide; ND: Not detected.

Table 4.4. Percentages of Relative Inhibition Zone Diameter (%RIZD) relative to antibiotics tested.

Antibiotics	**CN10**						**CN30**						**CIP10**					
Bacterial isolates	**TR**	**TN**	**CT**	**SH**	**AR**	**FP**	**TR**	**TN**	**CT**	**SH**	**AR**	**FP**	**TR**	**TN**	**CT**	**SH**	**AR**	**FP**
L. monocytogenes ATCC 15313	45	40	50	50	40	50	41	36	45	45	36	45	39	35	43	43	35	43
S. aureus CECT 976	61	56	50	56	44	56	50	45	41	45	36	45	37	34	31	34	28	34
S. aureus MJS241	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#
E. faecalis MJS257	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#	#
S. aureus MJMC109	53	53	53	58	47	53	48	48	48	52	43	48	37	37	37	41	33	37
S. aureus MJMC534B	47	53	47	47	47	53	41	45	41	41	41	45	90	100	90	90	90	100
P. aeruginosa ATCC 10145	100	100	100	111	89	111	75	75	75	83	67	83	43	43	43	48	38	48
E. coli MJS260	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
K. pneumoniae MJS281	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
K. pneumoniae MJH602	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. aerogenes MJMC534A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

TR: Tinta Roriz; TN: Touriga Nacional; CT: Castelão; SH: Syrah; AR: Arinto; FP: Fernão Pires; CN10: gentamicin (10 µg per disc); CN30: gentamicin (30 µg per disc); CIP10: ciprofloxacin (10 µg per disc).

Table 4.5. Classification of antimicrobial activity of grape stem extracts.

Antibiotics	**CN10**						**CN30**						**CIP10**					
Bacterial isolates	**TR**	**TN**	**CT**	**SH**	**AR**	**FP**	**TR**	**TN**	**CT**	**SH**	**AR**	**FP**	**TR**	**TN**	**CT**	**SH**	**AR**	**FP**
L. monocytogenes ATCC 15313	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S. aureus CECT 976	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S. aureus MJS241	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
E. faecalis MJS257	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
S. aureus MJMC109	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S. aureus MJMC534B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
P. aeruginosa ATCC 10145	++	++	++	++	+	++	+	+	+	+	+	+	+	+	+	+	+	+
E. coli MJS260	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
K. pneunomiae MJS281	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
K. pneumoniae MJH602	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E. aerogenes MJMC534A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

TR: Tinta Roriz; TN: Touriga Nacional; CT: Castelão; SH: Syrah; AR: Arinto; FP: Fernão Pires; CN10: gentamicin (10 µg per disc); CN30: gentamicin (30 µg per disc); CIP10: ciprofloxacin (10 µg per disc); No effect: -; Moderate efficacy: +; Goof efficacy: ++; High efficacy: +++.

The results obtained in the present work are in agreement with those reported by Gouvinhas *et al*. (2018), where the antibacterial activity of grape stem extracts of Syrah, Tinta Barroca, and Sousão varieties was tested against the same isolates used in the present work. In relation to Gram-positive bacteria, the same behavior was observed for *S. aureus* MJS241 and *E. faecalis* MJS257 isolates, since antibiotics had no effect on bacterial growth inhibition, unlike grape stem extracts. In the case of *L. monocytogenes* ATCC 15313 isolate, they presented a %RIZD that ranged between 47 and 58. Regarding Gram-negative bacteria, extracts also exhibited an inhibition of bacterial growth of *P. aeruginosa* ATCC 10145 isolate, with a %RIZD that oscillated from 50 to >100, and for *E. coli* MJS260 and *K. pneumoniae* MJS281 isolates, no inhibition of bacterial growth by the grape stem extracts was detected, as also shown in the present work (Table 4.4).

Concerning to other by-products, Corrales *et al*. (2010) evaluated the potential antimicrobial activity of grape skin extracts (Riesling cultivar). Different bacterial strains were used and, in common with the present study, they utilized *L. monocytogenes*, *S. aureus*, *E. faecalis*, and *E. coli* strains. For Gram-positive isolates (*L. monocytogenes*, *S. aureus*, *E. faecalis*) they presented inhibitions ranging from 1 to 6 mm, which were lower than those obtained in the present work (Table 4.3), whereas for Gram-negative isolate (*E. coli*) no inhibition of bacterial growth was found, observing equal behavior for grape stem extracts. In contrast, the study of Butkhup *et al*. (2010) showed inhibition of bacterial growth of *E. coli* strain using grape skins and seeds extracts of the Shiraz (or Syrah) cultivar, achieving inhibition halos of 4 ± 0.06 and 7 ± 0.12 mm, respectively. And for a *S. aureus* strain, they presented inhibition halos varying from 11 ± 0.23 to 12 ± 0.10 mm for grape skins and seeds. In addition, Zambrano *et al*. (2019) work evaluated the antimicrobial activity of grape pomace extracts of the Othello cultivar against different strains of *L. monocytogenes*, *S. aureus*, *E. coli*, and *P. aeruginosa*. Regarding to Gram-positive isolates (*L. monocytogenes* and *S. aureus*), they reached maximum inhibition halos of 5 mm, showing that the grape pomace extracts showed lower antibacterial activity than the grape stem extracts, as can be noticed in Table 4.3, whereas for Gram-negative isolates (*E. coli* and *P. aeruginosa*) they achieved a maximum inhibition of 3 mm.

Grape stems, when compared with other by-products, presented a higher antimicrobial power against Gram-positive bacteria, proving to be an option in the fight against these pathogens. However, the antimicrobial potential of each extract may depend on the extraction method, the microorganism tested (Zambrano *et al*., 2019), and its polyphenolic content, since

compounds belonging to the classes of flavanols, flavonols, and tannins have a higher antimicrobial activity when compared to other polyphenols (Baenas *et al*., 2018).

4.2.2.2. Minimum Inhibitory Concentration (MIC)

The antimicrobial activity of grape stem extracts was also evaluated by the Minimum Inhibitory Concentration assay, being the results presented in Table 4.6. In addition, it was also tested if these concentrations had a bacteriostatic or bactericidal effect (Table 4.6).

Table 4.6. Minimum Inhibitory Concentration (MIC) (mg/mL) of grape stem extracts against bacterial isolates tested.

Bacterial isolates	TR	TN	CT	SH	AR	FP
L. monocytogenes ATCC 15313	1.88○	1.88○	1.88○	1.88○	1.88○	1.88○
S. aureus CECT 976	0.94■	0.47■	0.94■	0.94■	0.94■	0.94■
S. aureus MJS241	0.94■	0.94■	0.94■	0.94■	0.94■	0.94■
E. faecalis MJS257	0.94■	0.94■	0.94○	0.94○	1.88○	0.94○
S. aureus MJMC109	1.88■	0.94■	0.94■	0.94■	1.88■	1.88■
S. aureus MJMC534B	0.94■	0.47○	0.94■	0.94■	0.94■	0.94■
P. aeruginosa ATCC 10145	1.88■	1.88■	1.88○	3.75○	3.75■	1.88○
E. coli MJS260	ND	ND	ND	ND	ND	ND
K. pneumoniae MJS281	ND	ND	ND	ND	ND	ND
K. pneumoniae MJH602	ND	ND	ND	ND	ND	ND
E. aerogenes MJMC534A	ND	ND	ND	ND	ND	ND

TR: Tinta Roriz; TN: Touriga Nacional; CT: Castelão; SH: Syrah; AR: Arinto; FP: Fernão Pires; Bacteriostatic concentration: ○; Bactericidal concentration: ■; ND: Not detected.

Analyzing the results presented in Table 4.6, it is possible to verify that the MIC of grape stem extracts against Gram-positive isolates ranged from 0.47 and 1.88 mg/mL, depending on variety and strain. Concerning to *S. aureus* CECT 976, *S. aureus* MJS241, and *S. aureus* MJMC109 isolates, all the concentrations of grape stem extracts exhibited bactericidal effect (Table 4.6). With respect to Gram-negative isolates, the results obtained presented the same behavior observed in the disc diffusion assay, only inhibiting the growth of *P. aeruginosa* ATCC 10145 isolate with MIC ranging from 1.88 to 3.75 mg/mL, depending on variety. The MIC of grape stem extracts in Gram-negative isolate was higher than the achieved for Gram-positive isolates, possibly because Gram-negative bacteria have a lipopolysaccharide layer on the outer membrane that difficult the penetration of the phenolic compounds, as mentioned in the previous point. Nevertheless, for the remaining

Gram-negative bacteria the concentrations tested were not sufficient to inhibit their bacterial growth.

There are few data in literature about the antimicrobial activity of grape stems by the MIC method, however the Dias *et al*. (2015) determined the antimicrobial activity of grape stem extracts of seven Portuguese varieties (Sousão, Touriga Nacional, Tinta Barroca, Tinta Amarela, Fernão Pires, Viosinho, and Rabigato), using the same isolates used in the present work, and reported that both Gram-positive (*L. monocytogenes* ATCC 15313, *S. aureus* MJS241, and *E. faecalis* MJS257) and Gram-negative (*P. aeruginosa* ATCC 10145, *E. coli* MJS260, and *K. pneumoniae* MJS281) isolates had MICs ranging from 66.70 to >134.00 mg/mL, depending on the cultivars tested. These concentrations are very high, compared to those presented in Table 4.6, but in the case of *E. coli* MJS260, *K. pneumoniae* MJS281, *K. pneumoniae* MJH602, and *E. aerogenes* MJMC534A isolates it may mean that higher concentrations than those tested in the present work may be necessary to inhibit their growth.

In relation to other by-products of wine industry, in the study of Katalinić *et al*. (2010), the antimicrobial activity of fourteen cultivars of grape skins was tested against different bacterial strains, of which *S. aureus* and *E. coli*. They obtained average MIC of 0.27 and 0.24 mg/mL for *S. aureus* and *E. coli*, respectively, being these extracts more effective than grape stem extracts. Shrestha *et al*. (2012) work analyzed the antibacterial potential of grape seeds extracts against different strains of *S. aureus*, presenting a MIC of 0.63 mg/mL. Also, grape pomace extracts of the Syrah and Merlot cultivars were analyzed regarding their antimicrobial activity in the study of Oliveira *et al*. (2013), against different bacterial species. They tested the antimicrobial power of the extracts (with different extractions methods) and for the Merlot variety obtained a MIC of 0.63 ± 0.38 mg/mL against *S. aureus* strain, and 1.00 mg/mL against *P. aeruginosa* and *E. coli* strains. With respect to Syrah extracts, they considered that they were not as effective, since they presented MICs of 1.60 mg/mL against the isolates tested. These results exhibited a better inhibition of bacterial growth of Gram-positive than Gram-negative isolates, as in the present work (Table 4.6). In addition, Peixoto *et al*. (2018) study used bacterial isolates of *L. monocytogenes*, *S. aureus*, *E. faecalis*, *P. aeruginosa*, *E. coli*, and *K. pneumoniae* species to determine the antibacterial activity of grape pomace, skins, and seeds extracts. In the case of Gram-positive isolates (*L. monocytogenes*, *S. aureus*, and *E. faecalis*), they reached MICs ranging from 2.50 to >20.00 mg/mL, with better results for the grape seeds. For Gram-negative isolates

(*P. aeruginosa*, *E. coli* and *K. pneumoniae*), MICs varying from 10.00 to >20.00 mg/mL were presented, again with better results for grape the seeds.

Once again, the differences observed between studies and by-products are mainly due to distinct preparations of the extracts, the performance of the microbiology assays (Katalinić *et al*., 2010), the distinct bacterial strains (Zambrano *et al*., 2019), and the differences in phenolic composition of each extract, as mentioned above.

Grape stems have proven to be an effective matrix in inhibiting Gram-positive bacteria, namely, bacterial pathogens of gastrointestinal segment and diabetic foot ulcers, being a by-product that could be applied in the future to replace antibiotics or in synergy with them, fighting against the antibiotic resistance that currently exists, and may also be a matrix used in the development of innovate and efficient product dressing the diabetic foot wounds. Despite grape stems are not effective against Gram-negative bacteria, due to their lipopolysaccharide layer on the outer membrane, literature data show that testing higher concentrations in the future may lead to better inhibition of Gram-negative isolates (Dias *et al.*, 2015; Peixoto *et al*., 2018). However, although it is considered that flavanols, flavonols, and anthocyanins have greater antimicrobial potential, it is possible that they may also be a synergistic and antagonistic effect between phenolic compounds (Dias *et al*., 2015), therefore it is necessary in the future to evaluate the antimicrobial activity of compounds isolated from grape stems and to test possible synergies between them, in order to obtain the most effective antimicrobial possible.

4.2.3. Cell toxicity and anti-inflammatory activity of grape stem extracts

Before performing the anti-inflammatory assay, an effect of grape stem extracts on cell viability/toxicity was performed. The toxicity of the extracts was tested so that the cell viability is not compromised, and therefore cannot interfere with the results regarding the anti-inflammatory activity. In this sense, different concentrations of extracts were tested using final concentrations ranging from 1.00 to 50.00 µg/mL for Touriga Nacional, Syrah, and Fernão Pires varieties, 5.00 to 75.00 µg/mL for Tinta Roriz and Castelão varieties, and 25.00 to 150.00 µg/mL for Arinto variety, being the results (expressed as percentage of control) presented in Figure 4.1.

Observing the results, significant differences were found, principally between the highest concentration tested and controls, and between 24 and 48h of exposure at the highest applied concentration. As expected, the extracts were more toxic at higher concentrations,

both 24h and 48h exposure, showing a concentration and time-dependent effect.

For the Tinta Roriz and Castelão varieties, which were applied at the same concentrations, the lowest cell viability obtained was 68.16% and 70.10% at 24h, and 52.24% and 55.65% at 48h, respectively, at a concentration of 75.00 µg/mL (Figure 4.1A and C), however for Tinta Roriz variety, we observe a non-linear effect of concentration, which may reflect the fact that, as we are working with extracts (complex mixtures of bioactive compounds), different type of antagonistic and synergistic molecules, on cell viability/proliferation mechanisms, may be present in this extract. This effect was observed at both incubation periods, as the assays were performed separately, it reinforces the theory explained above. Regarding the Touriga Nacional, Syrah, and Fernão Pires varieties, their extracts showed lower cell viability at a concentration of 50.00 µg/mL, with values of 84.52%, 62.72%, and 83.46%, at 24h, and 73.21%, 51.40% and 82.02% at 48h, respectively (Figure 4.1B, D and F). The Arinto variety was the least toxic to cells, exhibiting a lower cell viability of 75.54% at 24h, and 53.16% at 48h, at the concentration of 150.00 µg/mL (Figure 4.1E).

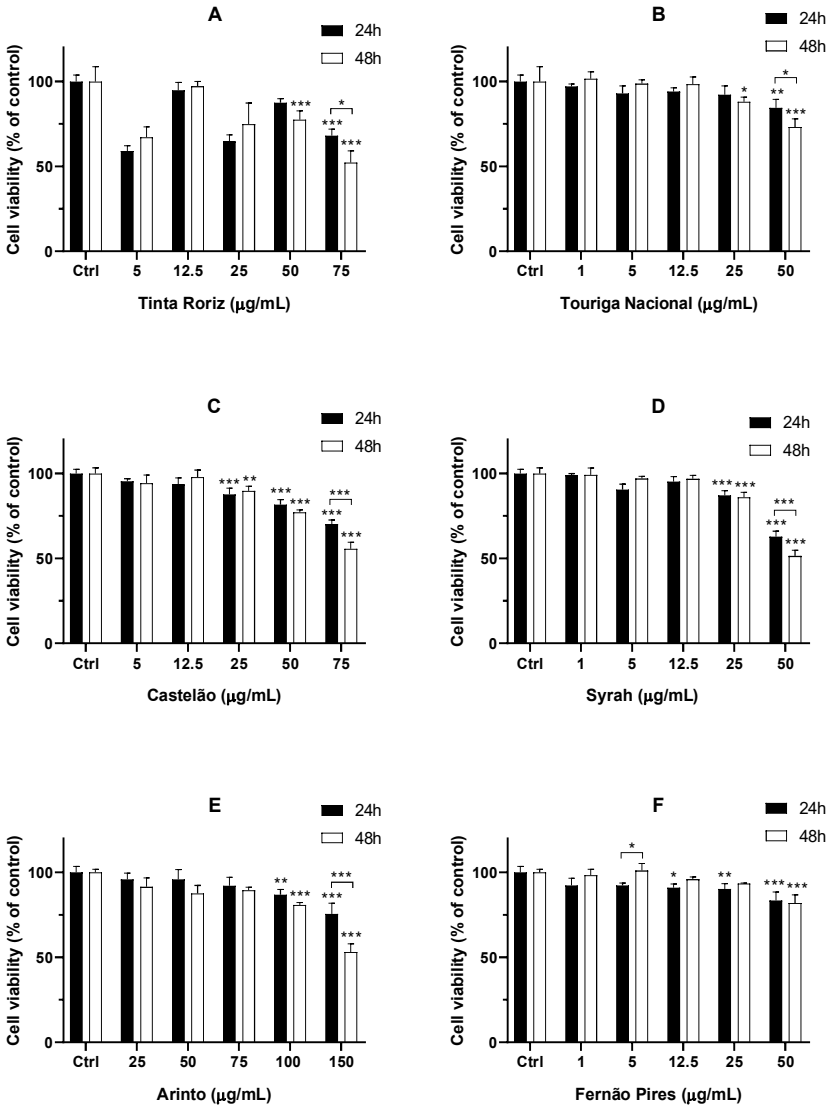


Figure 4.1. Effect of grape stem extracts on mouse macrophages (Raw 264.7 cell line) cell viability at 24h and 48h of exposure. (A) Tinta Roriz; (B) Touriga Nacional; (C) Castelão; (D) Syrah; (E) Arinto; (F) Fernão Pires. Results are presented as mean ± standard deviation (n=4). Significance: non-significant, N.S. ($p > 0.05$); * significant at $p < 0.05$; ** significant at $p < 0.01$; *** significant at $p < 0.001$.

In addition, through the results reached it was possible to calculate an extrapolation of the data, finding the IC_{50} of each variety, that is, the concentration at which 50% of cell growth is inhibited, being the results presented in the Table 4.7. As can be observed, there are significant differences between the varieties at both 24h and 48h, being the extract of Arinto variety that showed higher IC_{50}, meaning that it was the least toxic for the cells.

Table 4.7. IC_{50} values of grape stem extracts obtained from an extrapolation of results at 24h and 48h exposure.

Extracts IC_{50} (µg/mL)		
	Exposure Time	
	24h	**48h**
Tinta Roriz	136.80 ± 38.45^{a}	99.81 ± 19.44^{a}
Touriga Nacional	259.50 ± 34.23^{a}	163.10 ± 19.23^{ab}
Castelão	188.90 ± 9.84^{a}	134.90 ± 12.32^{a}
Syrah	108.00 ± 9.98^{a}	85.53 ± 10.57^{a}
Arinto	600.80 ± 63.69^{b}	295.70 ± 32.77^{c}
Fernão Pires	220.80 ± 30.67^{a}	260.40 ± 26.17^{bc}
p-value	***	***

The values are presented as mean ± standard deviation (n = 4). Different letters indicate significantly different results (ANOVA, $p < 0.05$). Significance: non-significant, N.S. ($p > 0.05$); * significant at $p < 0.05$; ** significant at $p < 0.01$; *** significant at $p < 0.001$.

Following testing the cellular toxicity of each variety, concentrations were chosen for the evaluation of anti-inflammatory activity of grape stem, namely concentrations where cell viability was above 90%. In this way, grape stem extracts were applied at concentrations of 5.00, 12.50, and 25.00 µg/mL, except for Arinto variety extract where concentrations of 12.50, 25.00, 50.00, and 75.00 µg/mL were tested.

The data obtained for the anti-inflammatory activity of grape stem extracts are presented in Figure 4.2. For all extracts, significant differences were presented when compared to the controls, and between the concentrations tested. Contrary to controls, where the NO production was 100%, grape stem extracts were able to inhibit LPS-stimulated NO production at all tested concentrations. In the presence of the extracts, the NO production varied between 64.75 and 83.48% of control, meaning that they induced NO production inhibitions between 16.52 and 35.25%, indicating anti-inflammatory activity.

In general, analyzing each variety, it can be verified that NO production inhibition was concentration-dependent, except for Fernão Pires variety, where 25.00 µg/mL produced a NO

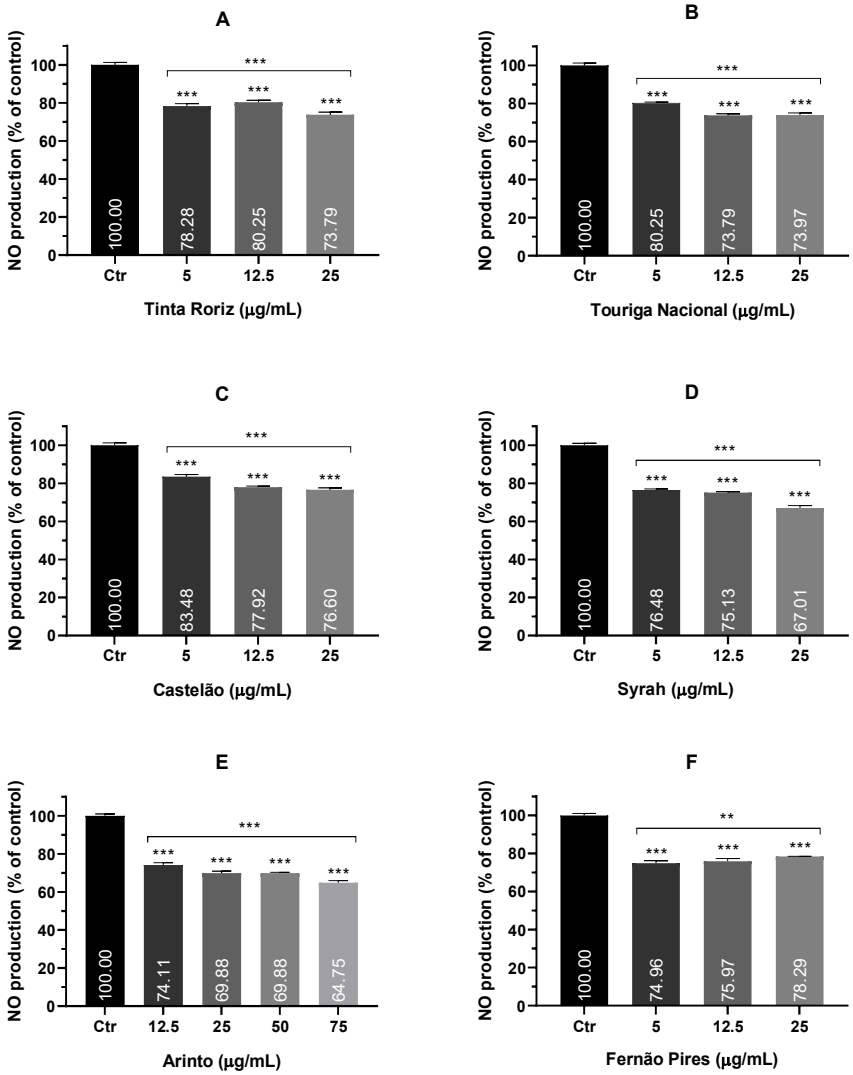


Figure 4.2. Anti-inflammatory activity of grape stem extracts on mouse macrophages (Raw 264.7 cell line). (A) Tinta Roriz; (B) Touriga Nacional; (C) Castelão; (D) Syrah; (E) Arinto; (F) Fernão Pires. Results are presented as mean ± standard deviation (n=4). Significance: non-significant, N.S. ($p > 0.05$); * significant at $p < 0.05$; ** significant at $p < 0.01$; *** significant at $p < 0.001$.

production (78.29%) slightly higher than 12.50 µg/mL (75.97%) (Figure 4.2F). The reason for this behavior can be explained, again, by the different compounds present in the extract and in their capacity to modulate the LPS-induced NO production pathways.

Concerning to the most effective grape stem extracts, the Arinto variety exhibited a greater capacity in inhibiting NO production with 35.25% inhibition (Figure 4.2E); however, extracts of this variety were tested at higher concentrations. Therefore, the results obtained for the extracts of the six varieties at 25.00 µg/mL, when compared, showed that at this concentration it is the Syrah variety that presents the greatest capacity of NO production inhibition (32.99%) (Figure 4.2D).

The distinct efficiencies in inhibiting NO production may be explained by the differences in phenolic content of the six varieties, and although the Syrah and Arinto varieties obtained the best results (Figure 4.2D and E), their phenolic contents are not the highest among the six varieties (see chapter III, section 3.2.2), suggesting that certain phenolic compounds when present in lower concentrations may have a better synergistic effect, exhibiting superior anti-inflammatory activity.

In this work, NO production was stimulated by the addition of LPS (lipopolysaccharide), since LPS causes macrophages to activate the production of excessive amounts of pro-inflammatory mediators, such as NO (via iNOS (inducible nitric oxide synthase, enzyme responsible for the NO synthesis) modulation) and pro-inflammatory cytokines (TNF-α (Tumor necrosis factor-α), Interleukins (ILs), namely IL-1β, IL-6) (Harbeoui *et al*., 2019). Nevertheless, grape stem extracts were able to inhibit the production of NO due to its polyphenolic content, since these bioactive compounds have been indicated as promising compounds in resolving the inflammatory process by intervening with the enzymes and signaling cascades activated during this process (Ribeiro *et al*., 2014). Some compounds, for example quercetin, kaempferol, resveratrol, epicatechin, and luteolin, have been shown the capacity to inhibit pro-inflammatory molecules (NO, TNF-α, IL-1β, IL-6) production by macrophages, to stimulate the expression of anti-inflammatory markers and inhibit pro-inflammatory enzymes (iNOS and COX-2 (cyclooxygenase-2)), thus causing a decrease in NO production (Zhang *et al*., 2019).

Regarding literature data, as far as we know, only Queiroz *et al*. (2017) tested the anti-inflammatory activity of grape stem extracts in the Raw 264.7 cell line, using extracts from the Sousão variety and isolated bioactive compounds from them (malvidin-3-*O*-glucoside, quercetin-3-*O*-glucoside and malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside). As regard to

whole extract, it presented an inhibition in NO production of 37.20% (at 2.00 μM), and for the isolated compounds under the same conditions, inhibition values ranging from 47.20% to 52.40% were exhibited, showing that the isolated compounds have a higher anti-inflammatory capacity, unlike the whole extract, where possible antagonistic combinations between the phenolic compounds causes a lower anti-inflammatory activity (Queiroz *et al*., 2017). The differences observed between this study and the present work (Figure 4.2), may be due to the different concentrations of extracts applied, but mainly to the different phenolic composition in the extracts.

With respect to other by-products, in Harbeoui *et al*. (2019) work analyzed the NO inhibition capacity of grape seeds of the Syrah, Merlot, and Carignan cultivars (at 5.00 μg/mL) in the Raw 264.7 cell line, presenting inhibition values of 37.05%, 35.12%, and 31.32%, respectively. Additionally, the Rebelo *et al*. (2014) study demonstrated the anti-inflammatory power of different wines, presenting as main reasons for this ability, the presence of anthocyanins, but also the synergistic effects among the compounds present in wine.

Environmental, immune and chronic diseases, such as diabetes and cancer all have inflammation in common (Arulselvan *et al*., 2016). Therefore, the development of drugs more effective in eliminating NO radicals and inhibiting iNOS enzyme activity and/or iNOS gene expression are required (Queiroz *et al*., 2017). In this sense, grape stem extracts are good candidates for the development of new drugs that can reduce or eliminate tissue inflammation, since they have the capacity to inhibit the production of NO radicals. However, further studies should be performed to ensure the safety of this by-product, and also understand which compounds present in the extracts have the highest anti-inflammatory potential.

4.2.4. Anti-aging activity of grape stem extracts

In order to understand if grape stems can be used in anti-aging products, the present work evaluated the capacity of grape stem extracts (1 mg/mL) to inhibit the activity of two enzymes, namely tyrosinase and elastase. The results obtained are presented in Table 4.8.

Table 4.8. Anti-tyrosinase and anti-elastase activities of grape stem extracts.

	Enzymatic Inhibition (%)	
Samples	**Tyrosinase**	**Elastase**
Tinta Roriz	41.47 ± 1.05^{a}	78.30 ± 2.72^{b}
Touriga Nacional	46.29 ± 2.00^{a}	86.84 ± 0.42^{c}
Castelão	44.38 ± 2.05^{a}	88.71 ± 1.68^{c}
Syrah	53.83 ± 0.84^{b}	98.02 ± 1.96^{d}
Arinto	44.07 ± 0.49^{a}	67.98 ± 1.32^{a}
Fernão Pires	44.83 ± 2.99^{a}	72.93 ± 2.33^{ab}
p-value	***	***

The values are presented as mean ± standard deviation (n = 3). Different letters indicate significantly different results (ANOVA, $p < 0.05$). Significance: non-significant, N.S. ($p > 0.05$); * significant at $p < 0.05$; ** significant at $p < 0.01$; *** significant at $p < 0.001$.

There are significant differences between the extracts of the six varieties. For tyrosinase, the inhibition percentages ranged from 41.47 to 53.83%, and for elastase, they ranged from 67.98 to 98.02%, thus showing that regardless of the varieties of grape stem extracts, there was a better inhibition of elastase activity. The obtained results showed that the Syrah variety had a better inhibition activity of the enzymes analyzed, with 53.83 and 98.02% for tyrosinase and elastase, respectively, while the Tinta Roriz variety demonstrated to have a lower ability to inhibit tyrosinase activity (41.47%), and the Arinto variety presented the least inhibition of elastase activity (67.98%).

Grape stem extracts exhibited a better effect on inhibiting elastase activity, as noted above. While tyrosinase is an enzyme containing at its active center copper (Aguilar-Toalá *et al*., 2019), elastase is a metalloproteinase, specifically an endopeptidase, which contains zinc in its catalytic domain (Taofiq *et al*., 2016). Phenolic compounds, such as quercetin or gallic acid (Kolakul & Sripanidkulchai, 2017), have the potential to sequester metal ions, which are cofactors or catalytic factors at the active center of an enzyme (Ratz-Łyko *et al*., 2015). Thus, the results presented (Table 4.8) suggest that the phenolic compounds in the extracts have a better efficacy in blocking the active center of the elastase and/or in chelating the zinc ions present in this enzyme. Moreover, although the extract of Syrah variety has demonstrated better results in inhibiting the two enzymes, this is not the variety with the highest phenolic content (see chapter III, section 3.2.2), indicating, once again, that the fact that phenolic compounds of this variety are present in lower concentrations, may contribute to more effective synergies.

To the best of our knowledge, to date no studies on anti-tyrosinase and anti-elastase

activities of grape stem extracts have been published, being here presented for the first time. Nevertheless, the Wittenauer *et al*. (2015) study proved that grape pomace extracts (Weisser Riesling cultivar) have anti-elastase activity, presenting 73.00% inhibition (at 35.3 µg/mL) of this enzyme activity. They also tested fractions of the identified compounds in the extracts, where the fraction containing gallic and caftaric acids showed the best inhibition (47.00%), and finally, the inhibitory capacity of the isolated compounds was also analyzed, with catechin obtaining better inhibition (12.00%). Although this study cannot be compared to the present work due mainly to different conditions to the assays and distinct extraction methods (Wittenauer *et al*., 2015), it suggests the next steps to be taken to find out which compounds present in grape stem extracts are responsible for inhibiting the activities of the tested enzymes, being them, to study different synergies between the phenolic compounds and to test the inhibitory power of each isolated compound. In addition, Fujimaki *et al*. (2017) work exhibited the anti-tyrosinase activity of 15 different red wines, with inhibitions ranging from 24.00% to 46.70%.

The present study is the first to report the anti-tyrosinase and anti-elastase activities of grape stem extracts, proving that this raw material may, in future, be applied in cosmetic products to combat skin wrinkling and pigmentations disorders. As the grape stem extracts also comprise antioxidant and anti-inflammatory activity, together with anti-aging properties, affirm the grape-stem by-product of great added-value to cosmeceutical industry. But first, finding the compounds responsible for these activities and ensuring consumer safety are vital to the success of this by-product.

4.3. Conclusions

The biological potential of grape stems was proven, once again, in this study. This by-product showed antioxidant activity, revealing a good scavenging capacity; antimicrobial activity, which showed high efficacy against Gram-positive bacteria, especially *S. aureus* and *E. faecalis*; anti-inflammatory activity, in which all extracts were shown to inhibit the NO production at non-toxic cellular concentrations, and anti-aging activity, where important results were obtained for the inhibition of the activity of tyrosinase and elastase enzymes, increasing the cosmetic potential of this by-product.

Grape stem presented to be a matrix with potential biological activities, and since its production endangers the sustainability of the environment, the present work proves that this by-product can be reused for cosmetic, pharmaceutical and food industries, thus avoiding its

accumulation, and reducing the costs for its treatment. However, further studies are needed to evaluate the toxicity of this by-product, as well as, to analyze the biological properties of each bioactive compounds present in grape stem in order to obtain the highest possible efficacy, so that the extracts can finally be tested in *in vivo* systems.

4.4. References

Aguilar-Toalá, J. E., Hernández-Mendoza, A., González-Córdova, A. F., Vallejo-Cordoba, B., & Liceaga, A. M. (2019). Potential role of natural bioactive peptides for development of cosmeceutical skin products. *Peptides*, *122*(July), 170170. https://doi.org/10.1016/j.peptides.2019.170170

Aires, A., Mota, V. R., Saavedra, M. J., Rosa, E. A. S., & Bennett, R. N. (2009). The antimicrobial effects of glucosinolates and their respective enzymatic hydrolysis products on bacteria isolated from the human intestinal tract. *Journal of Applied Microbiology*, *106*(6), 2086–2095. https://doi.org/10.1111/j.1365-2672.2009.04180.x

Anastasiadi, M., Pratsinis, H., Kletsas, D., Skaltsounis, A. L., & Haroutounian, S. A. (2012). Grape stem extracts: Polyphenolic content and assessment of their in vitro antioxidant properties. *LWT - Food Science and Technology*, *48*(2), 316–322. https://doi.org/10.1016/j.lwt.2012.04.006

Andreani, T., Kiill, C. P., Luiza, A., Souza, R. De, Fangueiro, J. F., Fernandes, L., … Silva, A. M. (2014). Surface engineering of silica nanoparticles for oral insulin delivery: Characterization and cell toxicity studies. *Colloids and Surfaces B: Biointerfaces*. https://doi.org/10.1016/j.colsurfb.2014.10.047

Aouey, B., Mahjoubi, A., Fetoui, H., & Simmonds, M. S. J. (2016). Anti-oxidant , anti-inflammatory, analgesic and antipyretic activities of grapevine leaf extract (Vitis vinifera) in mice and identi fi cation of its active constituents by LC–MS/MS analyses. *Biomedicine et Pharmacotherapy*, *84*, 1088–1098. https://doi.org/10.1016/j.biopha.2016.10.033

Arulselvan, P., Fard, M. T., Tan, W. S., Gothai, S., Fakurazi, S., Norhaizan, M. E., & Kumar, S. S. (2016). Role of Antioxidants and Natural Products in Inflammation. *Oxidative Medicine and Cellular Longevity*, *2016*. https://doi.org/http://dx.doi.org/10.1155/2016/5276130

Baenas, N., Abellán, Á., Rivera, S., Moreno, D. A., García-Viguera, C., & Domínguez-Perles, R. (2018). *Foods and supplements*. *Polyphenols: Properties, Recovery, and Applications*. Elsevier. https://doi.org/10.1016/B978-0-12-813572-3.00010-5

Barros, A., Gironés-Vilaplana, A., Teixeira, A., Collado-González, J., Moreno, D. A., Gil-Izquierdo, A., … Domínguez-Perles, R. (2014). Evaluation of grape (Vitis vinifera L.) stems from Portuguese varieties as a resource of (poly)phenolic compounds: A comparative study. *Food Research International*, *65*(PC), 375–384.

https://doi.org/10.1016/j.foodres.2014.07.021

Bauer, A. W., Kirby, W. M., Sherris, J. C. & Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol*, *45*, 493–496.

Bolanos de la Torre, A. A. S., Henderson, T., Nigam, P. S., & Owusu-apenten, R. K. (2015). A universally calibrated microplate ferric reducing antioxidant power (FRAP) assay for foods and applications to Manuka honey. *Food Chemistry*, *174*, 119–123. https://doi.org/10.1016/j.foodchem.2014.11.009

Butkhup, L., Chowtivannakul, S., Gaensakoo, R., Prathepha, P., & Samappito, S. (2010). Study of the Phenolic Composition of Shiraz Red Grape Cultivar (Vitis vinifera L .) Cultivated in North-eastern Thailand and its Antioxidant and Antimicrobial Activity. *South African Journal for Enology and Viticulture*, *31*(2), 89–98.

Corrales, M., Fernandez, A., Vizoso, M. G., Butz, P., Franz, C. M. A. P., Schuele, E., & Tauscher, B. (2010). Characterization of phenolic content , in vitro biological activity , and pesticide loads of extracts from white grape skins from organic and conventional cultivars. *Food and Chemical Toxicology*, *48*(12), 3471–3476. https://doi.org/10.1016/j.fct.2010.09.025

Dias, C., Domínguez-Perles, R., Aires, A., Teixeira, A., Rosa, E., Barros, A., & Saavedra, M. J. (2015). Phytochemistry and activity against digestive pathogens of grape (Vitis vinifera L.) stem's (poly)phenolic extracts. *LWT - Food Science and Technology*, *61*(1), 25–32. https://doi.org/10.1016/j.lwt.2014.11.033

Domínguez-Perles, R., Teixeira, A. I., Rosa, E., & Barros, A. I. (2014). Assessment of (poly)phenols in grape (Vitis vinifera L.) stems by using food/pharma industry compatible solvents and Response Surface Methodology. *Food Chemistry*, *164*, 339–346. https://doi.org/10.1016/j.foodchem.2014.05.020

Domínguez-Perles, Raúl, Guedes, A., Queiroz, M., Silva, A. M., & Barros, A. I. R. N. A. (2016). Oxidative stress prevention and anti-apoptosis activity of grape (Vitis vinifera L.) stems in human keratinocytes. *Food Research International*, *87*, 92–102. https://doi.org/10.1016/j.foodres.2016.06.030

Fujimaki, T., Mori, S., Horikawa, M., & Fukui, Y. (2017). Isolation of proanthocyanidins from red wine, and their inhibitory effects on melanin synthesis in vitro. *Food Chemistry*. https://doi.org/10.1016/j.foodchem.2017.12.024

Gouvinhas, I., Santos, R. A., Queiroz, M., Leal, C., Saavedra, M. J., Domínguez-Perles, R., … Barros, A. I. R. N. A. (2018). Monitoring the antioxidant and antimicrobial power of grape (Vitis vinifera L.) stems phenolics over long-term storage. *Industrial Crops and Products*, *126*. https://doi.org/10.1016/j.indcrop.2018.10.006

Gouvinhas, Irene, Queiroz, M., Rodrigues, M., & Barros, A. I. R. N. A. (2019). *Evaluation of the Phytochemistry and Biological Activity of Grape (Vitis vinifera L.) Stems: Toward a Sustainable Winery Industry*. *Polyphenols in Plants* (2nd ed.). Elsevier Inc. https://doi.org/10.1016/B978-0-12-813768-0.00023-2

Gutiérrez-del-Río, I., Fernández, J., & Lombó, F. (2018). Plant nutraceuticals as antimicrobial agents in food preservation : terpenoids , polyphenols and thiols. *International Journal of Antimicrobial Agents*, *52*, 309–315. https://doi.org/10.1016/j.ijantimicag.2018.04.024

Gyawali, R., & Ibrahim, S. A. (2014). Natural products as antimicrobial agents. *Food Control*. https://doi.org/10.1016/j.foodcont.2014.05.047

Harbeoui, H., Hichami, A., Wannes, W. A., Lemput, J., Tounsi, M. S., & Khan, N. A. (2019). Anti-inflammatory effect of grape (Vitis vinifera L.) seed extract through the downregulation of NF- κ B and MAPK pathways in LPS-induced RAW264 . 7 macrophages. *South African Journal of Botany*, *125*, 1–8. https://doi.org/10.1016/j.sajb.2019.06.026

Jara-Palacios, M. J., Hernanz, D., Escudero-Gilete, M. L., & Heredia, F. J. (2014). Antioxidant potential of white grape pomaces: Phenolic composition and antioxidant capacity measured by spectrophotometric and cyclic voltammetry methods. *Food Research International*, *66*(December), 150–157. https://doi.org/10.1016/j.foodres.2014.09.009

Jara-Palacios, M. J., Hernanz, D., González-Manzano, S., Santos-Buelga, C., Escudero-Gilete, M. L., & Heredia, F. J. (2014). Detailed phenolic composition of white grape by-products by RRLC / MS and measurement of the antioxidant activity. *Talanta*, *125*, 51–57. https://doi.org/10.1016/j.talanta.2014.02.065

Katalinić, V., Možina, S. S., Skroza, D., Generalić, I., Abramovič, H., Miloš, M., … Boban, M. (2010). Polyphenolic profile, antioxidant properties and antimicrobial activity of grape skin extracts of 14 Vitis vinifera varieties grown in Dalmatia (Croatia). *Food Chemistry*, *119*(2), 715–723. https://doi.org/10.1016/j.foodchem.2009.07.019

Kolakul, P., & Sripanidkulchai, B. (2017). Phytochemicals and anti-aging potentials of the extracts from Lagerstroemia speciosa and Lagerstroemia fl oribunda. *Industrial Crops & Products*, *109*, 707–716. https://doi.org/10.1016/j.indcrop.2017.09.026

Kusumawati, I., & Indrayanto, G. (2013). *Natural Antioxidants in Cosmetics*. *Studies in Natural Products Chemistry* (1st ed., Vol. 40). Copyright © 2013 Elsevier B.V. All rights reserved. https://doi.org/10.1016/B978-0-444-59603-1.00015-1

Ky, I., Lorrain, B., Kolbas, N., Crozier, A., & Teissedre, P. (2014). Wine by-Products: Phenolic Characterization and Antioxidant Activity Evaluation of Grapes and Grape Pomaces from Six Different French Grape Varieties. *Molecules*, *19*, 482–506. https://doi.org/10.3390/molecules19010482

Lima, M. C., Sousa, C. P. De, Harel, J., Dubreuil, J. D., & Souza, E. L. De. (2019). A review of the current evidence of fruit phenolic compounds as potential antimicrobials against pathogenic bacteria. *Microbial Pathogenesis*. https://doi.org/10.1016/j.micpath.2019.03.025

Liyanaarachchi, G. D., Samarasekera, J. K. R. R., Mahanama, K. R. R., & Hemalal, K. D. P. (2018). Tyrosinase, elastase, hyaluronidase, inhibitory and antioxidant activity of Sri

Lankan medicinal plants for novel cosmeceuticals. *Industrial Crops & Products*, *111*(August 2017), 597–605. https://doi.org/10.1016/j.indcrop.2017.11.019

Loizzo, M. R., Sicari, V., Pellicanò, T., Xiao, J., Poiana, M., & Tundis, R. (2019). Comparative analysis of chemical composition , antioxidant and anti- proliferative activities of Italian Vitis vinifera by-products for a sustainable agro-industry. *Food and Chemical Toxicology*, *127*(March), 127–134. https://doi.org/10.1016/j.fct.2019.03.007

Madureira, A. R., Pereira, A., & Pintado, M. (2015). Current state on the development of nanoparticles for use against bacterial gastrointestinal pathogens. Focus on chitosan nanoparticles loaded with phenolic compounds. *Carbohydrate Polymers*, *130*(October), 429–439. https://doi.org/10.1016/j.carbpol.2015.05.030

Martín-Ortega, A. M., & Campos, M. R. S. (2019). *Bioactive Compounds as Therapeutic Alternatives*. *Bioactive Compounds*. Elsevier Inc. https://doi.org/10.1016/B978-0-12-814774-0.00013-X

Mattos, G. N., Tonon, R. V., Furtado, A. A. L., & Cabral, L. M. C. (2017). Grape by-product extracts against microbial proliferation and lipid oxidation: a review. *Journal of the Science of Food and Agriculture*, *97*(4), 1055–1064. https://doi.org/10.1002/jsfa.8062

Melo, P. S., Massarioli, A. P., Denny, C., Santos, L. F. dos, Franchin, M., Pereira, G. E., … Alencar, S. M. de. (2015). Winery by-products : Extraction optimization, phenolic composition and cytotoxic evaluation to act as a new source of scavenging of reactive oxygen species. *Food Chemistry*, *181*, 160–169. https://doi.org/10.1016/j.foodchem.2015.02.087

Mena, P., García-Viguera, C., Navarro-Rico, J., Moreno, D. A., Bartual, J., Saura, D., & Martí, N. (2011). Phytochemical characterisation for industrial use of pomegranate (Punica granatum L.) cultivars grown in Spain. *Journal of the Science of Food and Agriculture*, *91*(10), 1893–1906. https://doi.org/10.1002/jsfa.4411

Oliveira, D. A., Salvador, A. A., Smânia, A., Smânia, E. F. A., Maraschin, M., & Ferreira, S. R. S. (2013). Antimicrobial activity and composition profile of grape (Vitis vinifera) pomace extracts obtained by supercritical fluids. *Journal of Biotechnology*, *164*(3), 423–432. https://doi.org/10.1016/j.jbiotec.2012.09.014

Peixoto, C. M., Dias, M. I., Alves, M. J., Calhelha, R. C., Barros, L., Pinho, S. P., & Ferreira, I. C. F. R. (2018). Grape pomace as a source of phenolic compounds and diverse bioactive properties. *Food Chemistry*, *253*(November 2017), 132–138. https://doi.org/10.1016/j.foodchem.2018.01.163

Pisoschi, A. M., Pop, A., Georgescu, C., Turcuş, V., Olah, K., & Mathe, E. (2017). An overview of natural antimicrobials role in food. *European Journal of Medicinal Chemistry*. https://doi.org/10.1016/j.ejmech.2017.11.095

Poveda, J. M., Loarce, L., Alarcón, M., Díaz-maroto, M. C., & Alañón, M. E. (2018). Revalorization of winery by-products as source of natural preservatives obtained by means of green extraction techniques. *Industrial Crops & Products*, *112*(October), 617–

625. https://doi.org/10.1016/j.indcrop.2017.12.063

Queiroz, M., Oppolzer, D., Gouvinhas, I., Silva, A. M., Barros, A. I. R. N. A., & Domínguez-Perles, R. (2017). New grape stems' isolated phenolic compounds modulate reactive oxygen species, glutathione, and lipid peroxidation in vitro: Combined formulations with vitamins C and E. *Fitoterapia*, *120*, 146–157. https://doi.org/10.1016/j.fitote.2017.06.010

Ratz-Łyko, A., Arct, J., Majewski, S., & Pytkowska, K. (2015). Influence of Polyphenols on the Physiological Processes in the Skin. *Phytotherapy Research*, *29*(4), 509–517. https://doi.org/10.1002/ptr.5289

Rebelo, M. J., Sousa, C., Valentão, P., Rego, R., & Andrade, P. B. (2014). Phenolic profile of Douro wines and evaluation of their NO scavenging capacity in LPS-stimulated RAW 264.7 macrophages. *Food Chemistry*, *163*(November), 16–22. https://doi.org/10.1016/j.foodchem.2014.04.070

Ribeiro, D., Freitas, M., Tomé, S. M., Silva, A. M. S., Laufer, S., Lima, J. L. F. C., & Fernandes, E. (2014). Flavonoids Inhibit COX-1 and COX-2 Enzymes and Cytokine/Chemokine Production in Human Whole Blood. *Inflammation*, *38*(2), 858–870. https://doi.org/10.1007/s10753-014-9995-x

Rockenbach, I. I., Rodrigues, E., Gongaza, L. V., Caliari, V., Genovese, M. I., Gonçalves, A. E. de S. S., & Fett, R. (2011). Phenolic compounds content and antioxidant activity in pomace from selected red grapes (Vitis vinifera L . and Vitis labrusca L .) widely produced in Brazil. *Food Chemistry*, *127*(1), 174–179. https://doi.org/10.1016/j.foodchem.2010.12.137

Rodrigues, F., Pinto, D., Vinha, A. F., & Carretero, A. S. (2018). Cosmetics. In *Polyphenols: Properties, Recovery, and Applications* (pp. 393–427). https://doi.org/10.1016/B978-0-12-813572-3.00012-9

Sarker, S. D., Nahar, L., & Kumarasamy, Y. (2007). Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth , and its application in the in vitro antibacterial screening of phytochemicals. *Methods*, *42*, 321–324. https://doi.org/10.1016/j.ymeth.2007.01.006

Shrestha, B., Theerathavaj, M. L. S., Thaweboon, S., & Thaweboon, B. (2012). In vitro antimicrobial effects of grape seed extract on peri-implantitis microflora in craniofacial implants. *Asian Pacific Journal of Tropical Biomedicine*, *2*(10), 822–825. https://doi.org/10.1016/S2221-1691(12)60236-6

Shukla, R., Pandey, V., Vadnere, G. P., & Lodhi, S. (2019). Role of Flavonoids in Management of Inflammatory Disorders. In *Bioactive Food as Dietary Interventions for Arthritis and Related Inflammatory Diseases* (2nd ed., pp. 293–322). Elsevier Inc. https://doi.org/10.1016/B978-0-12-813820-5.00018-0

Taghouti, M., Martins-Gomes, C., Schäfer, J., Félix, L. M., Santos, J. A., Bunzel, M., … Silva, A. M. (2018). Thymus pulegioides L. as a rich source of antioxidant, anti-proliferative and neuroprotective phenolic compounds. *Food and Function*, *9*(7), 3617–

3629. https://doi.org/10.1039/c8fo00456k

Taofiq, O., González-paramás, A. M., Martins, A., Filomena, M., & Ferreira, I. C. F. R. (2016). Mushrooms extracts and compounds in cosmetics, cosmeceuticals and nutricosmetics — A review. *Industrial Crops & Products*, *90*, 38–48. https://doi.org/10.1016/j.indcrop.2016.06.012

Taurino, R., Ferretti, D., Cattani, L., Bozzoli, F., & Bondioli, F. (2019). Lightweight clay bricks manufactured by using locally available wine industry waste. *Journal of Building Engineering*, *26*(October 2018), 100892. https://doi.org/10.1016/j.jobe.2019.100892

Teles, A. S. C., Chávez, D. W. H., Oliveira, R. A., Bon, E. P. S., Terzi, S. C., Souza, E. F., … Tonon, R. V. (2018). Use of grape pomace for the production of hydrolytic enzymes by solid-state fermentation and recovery of its bioactive compounds. *Food Research International*, *120*(June), 441–448. https://doi.org/10.1016/j.foodres.2018.10.083

Vuolo, M. M., Lima, V. S., Roberto, M., & Junior, M. (2019). Phenolic Compounds: Structure, Classification, and Antioxidant Power. In *Bioactive Compounds: Health Benefits and Potential Applications* (pp. 33–50). Elsevier Inc. https://doi.org/10.1016/B978-0-12-814774-0.00002-5

Wittenauer, J., Mäckle, S., Sußmann, D., Schweiggert-weisz, U., & Carle, R. (2015). Inhibitory effects of polyphenols from grape pomace extract on collagenase and elastase activity. *Fitoterapia*, 1–9. https://doi.org/10.1016/j.fitote.2015.01.005

Zambrano, C., Kerekes, E. B., Kotogán, A., Papp, T., Vágvölgyi, C., Krisch, J., & Takó, M. (2019). Antimicrobial activity of grape, apple and pitahaya residue extracts after carbohydrase treatment against food-related bacteria. *LWT - Food Science and Technology*, *100*(February), 416–425. https://doi.org/10.1016/j.lwt.2018.10.044

Zenão, S., Aires, A., Dias, C., Saavedra, M. J., & Fernandes, C. (2017). Antibacterial potential of Urtica dioica and Lavandula angustifolia extracts against methicillin resistant Staphylococcus aureus isolated from diabetic foot ulcers. *Journal of Herbal Medicine*. https://doi.org/10.1016/j.hermed.2017.05.003

Zhang, H., & Tsao, R. (2016). Dietary polyphenols , oxidative stress and antioxidant and anti-inflammatory effects. *Current Opinion in Food Science*, *8*, 33–42. https://doi.org/10.1016/j.cofs.2016.02.002

Zhang, L., Virgous, C., & Si, H. (2019). Synergistic anti-inflammatory effects and mechanisms of combined phytochemicals. *The Journal of Nutritional Biochemistry*, *69*(July), 19–30. https://doi.org/10.1016/j.jnutbio.2019.03.009

Zhu, M., Huang, Y., Wang, Y., Shi, T., & Zhang, L. (2018). Comparison of (Poly)phenolic compounds and antioxidant properties of pomace extracts from kiwi and grape juice. *Food Chemistry*, *271*(January), 425–432. https://doi.org/10.1016/j.foodchem.2018.07.151

Chapter V:

Conclusions and future perspectives

5. Conclusions

Grape stem revealed to be a raw material rich in essential minerals, such as K, Ca, Mg, and Na, and phenolic compounds, for instance, catechin and caftaric acid. Consumers are increasingly looking for natural products with health beneficial properties, and since these essential nutrients, which are indispensable for the normal functioning of the organism, and these bioactive compounds, with several beneficial biological activities, are present in grape stem, this by-product can be a bet for the cosmetic, pharmaceutical, and food industries.

Moreover, it has been proved in this work that grape stem extracts have significant biological activities, namely antioxidant, antimicrobial, anti-inflammatory, and anti-aging. Grape stem has shown to have antioxidant power (higher than other by-products of wine industry in some cases), being able to be applied in new products for this propose. In addition, it demonstrated great efficacy in inhibiting the growth of Gram-positive bacteria, especially *Staphylococcus aureus* and *Enterococcus faecalis*, being its use promising against bacterial resistance. Also, the anti-inflammatory and anti-aging properties of grape stem were exhibited, where all tested varieties inhibited the NO production (an inflammatory marker) up to 35.25% in the RAW 264.7 cell line, and reduced the activities of enzymes responsible for skin pigmentation disorders and aging, up to 53.83% for tyrosinase, and up to 98.02% for elastase.

The results achieved help to reinforce the application of grape stem in several sectors, more precisely in new antibiotic/drugs, as well as, in food supplements, vitamins, and cosmetic creams. Nevertheless, for the use of this by-product in new formulations, consumer safety must be established, since the presence of toxic metals can be hazardous to the organism, along with the toxicity of extracts/phenolic compounds that can cause cell damage. Thus, in order to become profitable and to be applied in several industries, measures must be taken regarding grape stems, starting with a reduction in pollution and fertilizer/pesticide application in the vineyards, and ending with further testing on the toxicity of extracts/phenolic compounds in *in vivo* systems to, subsequently, use this by-product safely in new products.

5.1. Future perspectives

In order to apply grape stems in the cosmetic, pharmaceutical, and food industries in the future, the following steps have to be taken into account:

- To determine the mineral composition of grape stems of other varieties and from different wine regions, comparing conventional productions with biological productions, to understand if pesticides/fertilizers are mainly responsible for the presence of toxic metals;
- To evaluate the phenolic composition of grape stems of other varieties and from different wine regions, in order to identify which phenolic compounds are present;
- To study the biological activities of possible synergies between the phenolic compounds present in grape stems, and also each one independently;
- To test, more precisely, the toxicity of grape stem extracts to obtain concentrations that are non-toxic to the organism without losing their effectiveness;
- To evaluate the biological activities in *in vivo* systems after the ensuring of the safety of the extracts, and the finding of the phenolic compounds with higher biological power.