Universidade de Trás-os-Montes e Alto Douro

Evaluation of the phenolic profile, antioxidant and antibacterial activities of grape (*Vitis vinifera* L.) stems extracts

Dissertação de Mestrado em Biotecnologia e Qualidade Alimentar

Nome do candidato: Irene Pereira Gouvinhas
Nome do Orientador: Professora Doutora Ana Isabel Novo de Barros

Vila Real, 2018

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Irene Pereira Gouvinhas

Tese apresentada à Universidade de Trás-os-Montes e Alto Douro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia e Qualidade Alimentar, realizada sob a orientação científica da Doutora Ana Isabel Amorim Novo de Barros, Professora Auxiliar com Agregação do Departamento de Química da Universidade de Trás-os-Montes e Alto Douro.

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- Grape (*Vitis vinifera* L.) stems as valuable candidates for food, cosmetic, and pharmaceutical industries: towards a circular and sustainable bioeconomy through waste valorization. Ana I.R.N.A Barros**, Irene Gouvinhas**. 3rd Edition of International Conference on Agriculture & Food Chemistry. 23-24th July **2018**, Rome (Italy).
- Storage effect on polyphenolic composition and biological activities of winery by-products against human clinical pathogens. **Irene Gouvinhas**. II International Symposium on Water, Soils and Geotechnology (SASGEO)/3rd Interact Meeting. 17-18th May **2018**, Vila Real (Portugal).
- Evaluation of the antioxidant and antimicrobial activities of grape (*Vitis vinífera* L.) stem extracts. Carla Leal, Bárbara Teixeira, Marcelo Queiroz, **Irene Gouvinhas**, Rafaela Santos, Maria José Saavedra, Ana Barros. XI Biochemistry Seminary integrated in the Week of Science and Technology of UTAD. 21-24th November **2017**, UTAD, Vila Real (Portugal).
- Long-term storage effect on the antimicrobial activity of winery by-products against multidrug resistant bacteria. Rafaela Santos, **Irene Gouvinhas**, Carla Leal, Marcelo Queiroz, Eduardo Rosa, Miguel Rodrigues, Ana Barros, Maria José Saavedra. XI Biology Seminary integrated in the Week of Science and Technology of UTAD. 21-24th November **2017**, UTAD, Vila Real (Portugal).
- Evaluation of the phenolic profile and antioxidant activity of extracts and isolate compounds of winery by-products. **Irene Gouvinhas**. 2nd Interact Meeting. 12-13th July **2017**, Vila Real (Portugal).

- Evaluation of the phenolic profile and antioxidant activity of extracts and isolate compounds of winery by-products. **Irene Gouvinhas**, Miguel Rodrigues, Carla Leal, Marcelo Queiroz, Rafaela Santos, Maria José Saavedra, Ana Barros. 11th World Congress on Polyphenols Aplications, 21-22th June **2017**, Vien (Austria).
- Evaluation of the phytochemistry and biological activity of grape (*Vitis vinífera* L.) Stems: towards a sustainable winery industry. Ana Barros, **Irene Gouvinhas**, Miguel Rodrigues. 11th World Congress on Polyphenols Aplications, 21-22th June **2017**, Vien (Austria).
11 - Evaluation of winery by-products to develop optimal valorisation procedures. **Irene Gouvinhas**. 1st Interact Meeting. 16th November **2016**, Vila Real (Portugal).

Poster Communications

- **Irene Gouvinhas**, Rafaela A. Santos, Maria José Saavedra, Raúl Domínguez-Perles, Miguel Rodrigues, Ana Barros. Effect of storage on polyphenolic composition and biological activities of grape (*Vitis vinifera* L.) stems. Encontro Ciência 2018, 2-4th July **2018**, Lisbon (Portugal).
- **Irene Gouvinhas**, Rafaela Santos, Marcelo Queiroz, Maria José Saavedra, Miguel Rodrigues, Ana Barros. Phenolic profile and biological activities of winery by-products: sustainable wineries through waste valorization.1st International Meeting on I&D In The Food Sector. 5th June **2018**. Auditorium ESTGV, Viseu (Portugal).
- 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).
- **Irene Gouvinhas**, Rafaela A. Santos, Marcelo Queiroz, Carla Leal, Maria José Saavedra, Raúl Domínguez-Perles, Miguel Rodrigues, Ana I.R.N.A Barros. Impact of grape (*Vitis vinifera* L.) stems storage on their polyphenolic composition and biological activities. Infowine - 6th edition. 23-24th May **2018**, Theater of Vila Real (Portugal).
- **Irene Gouvinhas**, Marcelo Queiroz, Rafaela A. Santos, Carla Leal, Maria José Saavedra, Raúl Domínguez-Perles, Miguel Rodrigues, Ana Barros. Evaluation of the phenolic profile, antioxidant and antimicrobial activities of winery by-products. XI Biology Seminary integrated in the Week of Science and Technology of UTAD. 21-24th November **2017**, UTAD, Vila Real (Portugal).

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- Adriana Silva, **Irene Gouvinhas**, Patrícia Lage, Luís Ferreira, Alexandra Mendes-Ferreira, Ana Barros. Identificação dos compostos fenólicos presentes no engaço da casta Rabigato por HPLC. XI Biochemistry Seminary integrated in the Week of Science and Technology of UTAD. 21-24th November **2017**, UTAD, Vila Real (Portugal).

Outreach Activities

- Organization of the Workshop: Chemistry - "Valorization of by-products of the winery industry", within the scope of the II Bioengineering Conference. Phytochemical Lab. 24th April - 5th May **2017**, UTAD, Vila Real (Portugal).

Publications

- **Irene Gouvinhas**, Rafaela A. Santos, Marcelo Queiroz, Carla Leal, Maria José Saavedra, Raúl Domínguez-Perles, Miguel Rodrigues, Ana I.R.N.A Barros. Monitoring the antioxidant and antimicrobial power of grape (*Vitis vinifera* L.) stems phenolics over long-term storage. *Industrial Crops & Products* (**2018**) 126C: 83-91.
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- **Irene Gouvinhas**, Marcelo Queiroz, Miguel Rodrigues, Ana Barros. Evaluation of the phytochemistry and biological activity of grape (*Vitis vinifera* L.) stems: towards a sustainable winery industry. In: Watson, Ronald (ed.) Polyphenols in Plants, 2nd Edition, 2019 Elsevier. Chapter 23, *pp. 381-394. UK: Academic Press* Elsevier. ISBN: 978-0-12-813768-0.

RESUMO

A uva tem sido cada vez mais apontada como uma matriz rica em compostos bioativos, e a sua produção é uma das principais atividades económicas do setor agro-alimentar em todo o mundo, com mais de 60 milhões de toneladas produzidas anualmente. Estima-se que na Europa se obtenham cerca de 14,5 milhões de toneladas de subprodutos provenientes da produção de uva por ano. Um desses subprodutos é o engaço, e atualmente estima-se que pode existir a capacidade de se desenvolverem produtos inovadores e de valor acrescentado usando esta matriz, devido aos seus efeitos potencialmente benéficos para a saúde humana como a sua capacidade antioxidante. Efetivamente, existe um interesse cada vez mais crescente na comunidade científica na aplicação deste material como fonte de compostos fenólicos, para o uso como suplementos alimentares e/ou compostos bioativos para as indústrias de cosmética e farmacêutica. No entanto, devido ao alto teor de humidade dos engaços, seria necessário um consumo elevado de energia para eliminar o teor de água para outras aplicações. Deste modo, a alternativa é armazenar este resíduo durante dias e/ou meses de forma a verificar este efeito na sua composição. Tendo este aspeto como principal objetivo deste trabalho, as amostras em estudo foram imediatamente recolhidas após o desengace, lavadas e armazenadas durante 64 dias, de forma a obter mais informações sobre o potencial deste subproduto, nomeadamente de amostras cultivadas numa das regiões de maior produção em Portugal (Douro). Para tal, este trabalho foi conduzido através da identificação e quantificação dos compostos fenólicos presentes em extratos de engaços provenientes de castas tintas produzidas na Região Demarcada do Douro durante o seu armazenamento, procedendo-se posteriormente à determinação da respetiva atividade antioxidante. Além disso, a capacidade de inibição dos extratos fenólicos contra várias bactérias Gram positivas e Gram negativas, tendo como termo de comparação antibióticos de primeira escolha médica, foi ainda explorada a fim de encontrar usos industriais promissores para acrescentar valor a esses resíduos.

Os resultados demonstraram que, após 64 dias de armazenamento à temperatura ambiente, algumas castas não apresentaram diferenças significativas em termos de composição fenólica, enquanto outras apresentaram um decréscimo máximo de 35% no seu conteúdo. Além disso, a atividade antibacteriana e antioxidante permaneceu intacta no último dia de estudo, revelando o grande potencial desta matriz ser armazenada durante pelo menos 2 meses, sendo sempre uma grande fonte de compostos bioativos.

Palavras-chave: Subprodutos; Engaço; Composição fenólica; Atividade antioxidante; Atividade antibacteriana.

ABSTRACT

The winery industry represent an important economical and social impact in several regions in the world, with large quantities of by-products generated annually, causing economical and environmental problems. Thus, a growing interest on recycling or reuse the winery wastes have been paid due to their antioxidant properties and putative health-promoting effects. Grape stems are one of the by-products generated seasonally in high quantities, being stressed as a valuable candidate to be used by various industrial sectors due to its constitution rich in phenolic compounds with remarkable biological activities, such as antioxidant, antibacterial and anti-inflammatory, which would allow to replace synthetic antioxidants currently used. However, due to the high moisture content in grape stems, it would be necessary an high energy consumption to eliminate the water content for further applications. So, the alternative is to store this residue during some time (days, months) and verify the effect in its composition after that.

In this study, the grape stem samples were collected immediately after destemming, washed and stored during 64 days, in order to obtain more information about the potential of grape stems, namely concerning samples varieties cultivated in the highest production region in Portugal (Douro). This work was carried out to generate data on the phenolic composition of stems during their storage, as well as their antioxidant activity *in vitro*. Also, information about the potential antibacterial activity of the phenolic extracts present in this winery by-product against several Gram positive and Gram negative bacteria was explored to find promising industrial uses to add value to this waste.

The results demonstrated that after 64 days of storage at room temperature, some varieties did not present significant differences in terms of phenolic composition, while others presented a maximum decrease of 35% in their polyphenolic content. Furthermore, the antibacterial and antioxidant activity remained intact at the last day of study, revealing the great potential of this matrix to be stored at least 60 days being always a great source of bioactive compounds.

Keywords: Winery by-products; Grape stems; Phenolic composition; Antioxidant activity; Antibacterial activity.

CONTENTS

TABLES INDEX

FIGURES INDEX

ABBREVIATIONS LIST

Acronym	
ABTS	2,2-azino-bis-3-ethylbenzothiazoline-6 sulphonic acid
CAT	Catechin
DAD	Diode Array Detector
DMSO	Dimethyl Sulfoxide
$DPPH^{\bullet}$	2,2-diphenyl-1-picrylhidrazyl radical
DW	Dry Weight
EU	European Union
FW	Fresh Weight
GA	Gallic Acid
HVED	High Voltage Electrical Discharges
LC	Liquid Chromatography
MS	Mass Spectrometry
Nd	Not detected
ROS	Reactive Oxygen Species
RP-HPLC-DAD	Reverse Phase-High Performance Liquid Chromatography-Diode Array Detector
RSM	Response Surface Methodology
SC CO_2	Supercritical Carbon Dioxide
TEAC	Trolox Equivalents Antioxidant Capacity
Tr	Traces
UV	Ultraviolet

I – State of the art

1.1. Evaluation of the phytochemistry and biological activity of grape (*Vitis vinifera* L.) stems: towards a sustainable winery industry

Adapted from:

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Abstract

The winery industry is one of the most important industries worldwide, with an economical and social impact. As a consequence of this fact, the large amount of by-products that are generated annually, can cause important environmental problems or even economical ones. Thus, a growing interest on recycling or reuse the winery wastes have been paid due to their antioxidant properties and putative health-promoting effects. Grape stems are one of the by-products generated seasonally in high amounts (representing 25% of the total by-products produced by this industry), being stressed as a valuable candidate to be used by various industrial sectors due to its constitution rich in phenolic compounds with remarkable biological activities, which would allow to replace synthetic antioxidants currently used. Therefore, this chapter reviews the phytochemical composition and the health benefits by distinct phenolic compounds found in grape (*Vitis vinifera* L.) stems, further supporting their interest as potential bioactive ingredient, and, thus, acting as a value-adding by product of the winery industry.

Keywords: Winery by-products; Grape stems; Phenolic composition; Biological activity; Applications.

1.1.1 Introduction

Grapes are one of the most important and widely fruit crops produced worldwide, from which around 80% are addressed to the winery industry, representing one of the most important economical activities in several regions. The most important wine producing regions are in Europe (Italy, Spain, France, Germany and Portugal), America (USA, Argentina and Chile) and also Australia and South Africa (Hussain, Cholette, & Castaldi, 2008). This industry generates huge amounts of by-products, consisting in organic wastes (around 9 million tons generated annually), such as grape pomace, seeds, pulp, skins, grape stems, and grape leaves, wine lees, wastewater, emission of greenhouse gases (CO_2, volatile organic compounds), and inorganic wastes, namely diatomaceous earth, bentonite clay, and perlite, representing large amounts of solid wastes, reaching more than >30% (*w/w*) of the total material transformed that constitutes only in Europe up to 14.5 million Tons (Chouchouli et al., 2013; González-Centeno et al., 2012; Makris, Boskou, & Andrikopoulos, 2007). Given the relevance of the winemaking companies all over the world, and the amount of underexploited wastes produced, the development of innovative applications for these organic materials urges, retrieving added value for this industry, thus turning into sustainable this relevant socio-economic activity (Matias et al., 2010). Effectively, wine production requires high energy consumptions and a considerable amount of other resources such as water, fertilizers and organic amendments, inducing elevate environmental costs in terms of CO_2 emissions. On the other hand, a large amount of wastes is produced. However, although this production of wastes is seasonal (during grape harvest), they are distributed along the year, which can cause a several environmental problem if they are not correctly disposed. Thus, it is urgent to search for environmentally friendly and low-cost row materials as well as new methods to guarantee the sustainability of the food chain, since grape stems seem to be a potential bioactive ingredient, and, thus, acting as a value-adding by-product of the winery industry.

1.1.2. Phytochemical composition of grape stems

Grape stems are one of the most worrying winery by-products generated during the vintage process (12% of organic waste), once it can cause serious environmental problems in terms of biological and chemical oxygen demand. This residue is removed before the vinification procedure in order to avoid a negative effect on the organoleptic characteristics of

the produced wine, since its presence during fermentation increases the wine astringency, mainly due to the richness of this matrix in proanthocyanidins. Little attention has been paid to this winery by-product, however some studies revealed its great potential as a rich source not only of natural antioxidants but also of dietary fiber (Teixeira et al., 2014).

1.1.2.1. Chemical composition of grape stems

Grape stems present a high moisture percentage ranging from 56% to 77%, with the elevate variability attributed essentially to the grape variety (Hussain, Cholette, Castaldi, 2008; González-Centeno et al., 2012; Chouchouli et al., 2013; Data not published). The content of stems alcoholic insoluble residues is ranging between 60 and 83% of the dry weight, with no significant differences found between red and white varieties (González-Centeno et al., 2010).

Concerning the macromolecules, grape stems present high quantities of cellulose, hemi-cellulose, and lignins, the former being the major component (30.3%), followed by the insoluble hemi-cellulose in hot water (21%), and lignins (17.4%). Regarding the other components, this residue presents a high content of tannins (15.9%) (Ping et al., 2011).

1.1.2.1.1. Phenolic composition of grape stems

Regarding the phenolic composition, (poly)phenols from grape stems represents approximately 5.8% of the dry weight (DW) (Katalinić et al., 2010). These compounds are secondary metabolites with diverse chemical structures and functions that are synthesized via shikimate pathway and/or phenylpropanoid metabolism during the normal plant development and/or as response to stress conditions, like UV irradiation, infection or wounding. Some polyphenols are extracted during the vinification process, however the major part remains in by-products, such as in pomace, wine lees or grape stems, accounting about 13% of the processed grape weight. The main group of polyphenols presents in grape stems are phenolic acids (hydroxycinnamic and hydroxybenzoic acids), flavan-3-ols, monomeric and oligomeric flavonols, and stilbens (**Table I.1**). Concerning the last group, several studies revealed the presence of *trans*-resveratrol and its dimmer ε-viniferin in considerable amounts, that can be range from 0.09 to 0.27 mg g^{-1} DW for red varieties and from 0.07 to 0.18 mg g^{-1} DW for white varieties for the former, while ε-viniferin concentrations can vary between 0.12 and 5.82 mg g^{-1} DW for red varieties, and between 0.12 and 3.59 mg g^{-1} DW in white varieties (Anastasiadi, Pratsinis, Kletsas, Skaltsounis, & Haroutounian, 2012). Sun et al. (2006) also

found *trans*-piceid and *cis*-piceid in lower concentrations in stems of red varieties of 61.43 and 143.85 mg kg^{-1} DW, respectively (Sun, Ribes, Leandro, Belchior, & Spranger, 2006). Piceatannol and vitisin-B were identified for the first time in grape stems by Piñeiro et al. (2013) in concentration ranged from "not detected" (Nd) to 21.1 mg kg^{-1} DW and from Nd to 61.1 mg kg^{-1} DW, respectively, in white and red varieties (Piñeiro, Guerrero, Fernández-Marin, Cantos-Villar, & Palma, 2013).

Another important group of phenolic compounds found in grape stems is flavonoids (**Table I.1**), being (+)-catechin one of the most abundant polyphenol, with concentrations between 0.71 and 12.18 mg g^{-1} DW. Its isomer, (–)- epicatechin, was also found in considerably high amounts, with a maximum detected concentration of 22.00 mg g^{-1} DW (Barros et al., 2014).

Regarding the phenolic acids, the main hydroxycinnamic acids detected are caftaric, caffeic, coumaric and ferulic acids, while the most abundant hydroxybenzoic acids are gallic acid and syringic acid with high concentrations (0.39-42.29 mg g^{-1} DW and 2.85-32.23 mg g^{-1} DW, respectively) (**Table I.1**).

For the first time, our investigation group found, in Portuguese red grape stem varieties from Douro Region, three anthocyanins (malvidin-3-*O*-glucoside, malvidin-3-*O*-rutinoside, and malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside) in concentration ranged between 0.40 and 28.28 mg g^{-1} DW, on average (**Table I.1**).

The majority of these studies demonstrated significant higher values of phenolic compounds in grape stems from red varieties than white varieties, however, in some cases, the content of polyphenols in red varieties can be lower than the content in white varieties (Apostolou et al., 2013). This was also demonstrated by the total phenolic content determination of grape stem extracts, where some white varieties presented higher content than the red varieties (Pinelo, Rubilar, Jerez, Sineiro, & Núñez, 2005). However, these results are still significantly higher than those found for grape skins or grape pomace, revealing the great potential of grape stems as a natural source of bioactive compounds (Anastasiadi, Pratsinis, Kletsas, Skaltsounis, & Haroutounian, 2010).

For the assessment of this matrix, not only screening spectrophotometric methods, but also chromatographic processes can allow the determination of the chemical composition of this winery by-product.

Table I.1. Review of the (poly)phenolic compounds of grape stems from red and white varieties

Compound	Red varieties	White varieties
Phenolic acids		
Hydroxybenzoic acids		
Gallic acid	11.48-42.29 mg g^{-1} DW; 38.6-48.2 mg $100g^{-1}$ DW (Sahpazidou et al., 2014; Spatafora, Barbagallo, Amico, & Tringali, 2013)	8.38 mg g^{-1} DW (Sahpazidou et al., 2014)
Syringic acid	2.85-32.23 mg g^{-1} DW (Sahpazidou et al., 2014)	0.80 mg g^{-1} DW (Sahpazidou et al., 2014)
Hydroxycinnamic acids		
Caftaric acid	0.190-0.706 mg g^{-1} DW; 14.75-19.46 mg g^{-1} DW; 0.04-0.27 mg g^{-1} DW (Anastasiadi et al., 2012; Dias et al., 2015; Domínguez-Perles, Guedes, Queiroz, Silva, & Barros, 2016)	0.01-0.15 mg g^{-1} DW; 0.082-0.167 mg g^{-1} DW; 2.18-2.79 mg g^{-1} DW (Anastasiadi et al., 2012; Dias et al., 2015; Domínguez-Perles et al., 2016)
Caffeic acid	0.54-1.78 mg g^{-1} DW (Sahpazidou et al., 2014)	0.31 mg g^{-1} DW (Sahpazidou et al., 2014)
Coumaric acid	0.75-1.55 mg g^{-1} DW (Sahpazidou et al., 2014)	0.41 mg g^{-1} DW (Sahpazidou et al., 2014)
Ferulic acid	0.51-3.59 mg g^{-1} DW (Sahpazidou et al., 2014)	0.31 mg g^{-1} DW (Sahpazidou et al., 2014)
Coutaric acid	≤4.50 mg kg^{-1} FW (Llobera & Cañellas, 2007)	-
Flavan-3-ols and Tannins		
(+)-Catechin	0.71-1.69 mg g^{-1} DW; 9.10-12.18 mg g^{-1} DW; 12.2-126.9 mg $100g^{-1}$ DW (Anastasiadi, Pratsinis, Kletsas, Skaltsounis, & Haroutounian, 2012; Dias et al., 2015; Domínguez-Perles, Guedes, Queiroz, Silva, & Barros, 2016)	0.39-1.86 mg g^{-1} DW; 7.35 mg g^{-1} DW; 9.3-133.9 mg $100g^{-1}$ DW (Anastasiadi et al., 2012; González-Centeno et al., 2012; Sahpazidou et al., 2014)
(–)-Epicatechin	Nd -0.09 mg g^{-1} DW; 4.51-19.13 mg g^{-1} DW; 22.00 mg g^{-1} DW; 0.6-11.1 mg $100g^{-1}$ DW (Anastasiadi et al., 2012; Barros et al., 2014; González-Centeno et al., 2012; Sahpazidou et al., 2014)	Nd-0.06 mg g^{-1} DW; 0.5-5.8 mg g^{-1} DW; 15.23 mg g^{-1} DW; 22.6 mg g^{-1} DW (Anastasiadi et al., 2012; Barros et al., 2014; González-Centeno et al., 2012; Sahpazidou et al., 2014)
Epicatechin gallate	0.06-0.13 mg g^{-1} DW (Anastasiadi et al., 2012)	0.03-0.09 mg g^{-1} DW; 70.1 mg g^{-1} DW (Anastasiadi et al., 2012; Barros et al., 2014)
Epigalocatechin	1.50-5.40 mg kg^{-1} FW; 6.20-13.73 mg g^{-1} DW (Llobera & Cañellas, 2007; Ratnasooriya & Rupasinghe, 2012)	0.80-0.90 mg kg^{-1} FW (Llobera & Cañellas, 2007)
Procyanidin dimmer B1	24.6-195.8 mg $100g^{-1}$ DW; 619.8-1373.2 mg $100g^{-1}$ DW (González-Centeno et al., 2012; Spatafora, Barbagallo, Amico, & Tringali, 2013)	13.3-187.7 mg $100g^{-1}$ DW (González-Centeno et al., 2012)
Procyanidin dimmer B2	Traces-9.4 mg $100g^{-1}$ DW; Nd-0.11 mg g^{-1} DW (Anastasiadi et al., 2012; González-Centeno et al., 2012)	0.04-0.17 mg g^{-1} DW; 1.1-4.8 mg $100g^{-1}$ DW (Anastasiadi et al., 2012; González-Centeno et al., 2012)
Procyanidin dimmer B3	4.1-23.2 mg $100g^{-1}$ DW; 0.14-0.99 mg g^{-1} DW (Anastasiadi et al., 2012; González-Centeno et al., 2012)	0.16-0.65 mg g^{-1} DW; 4.5-22.2 mg $100g^{-1}$ DW (Anastasiadi et al., 2012; González-Centeno et al., 2012)

Nd - Not detected; DW - Dry Weight; FW - Fresh Weight

Table I.1. Review of the (poly)phenolic compounds of grape stems from red and white varieties (*Cont.*)

Stilbens		
Trans-Resveratrol	0.09-0.27 mg g^{-1} DW; nd-139.1 mg kg^{-1} DW (Anastasiadi et al., 2012; Piñeiro, Guerrero, Fernández-Marin, Cantos-Villar, & Palma, 2013)	0.07-0.18 mg g^{-1} DW; nd-42.2 mg kg^{-1} DW (Anastasiadi et al., 2012; Piñeiro et al., 2013)
ε-Viniferin	0.12-0.30 mg g^{-1} DW; 2.48 mg g^{-1} DW; 0.22-0.48 mg g^{-1} DW; 3.74-5.82 mg g^{-1} DW (Anastasiadi et al., 2012; Barros et al., 2014; Domínguez-Perles et al., 2016)	0.12-0.30 mg g^{-1} DW; 0.17-0.50 mg g^{-1} DW; 2.61-2.99 mg g^{-1} DW; 2.94-3.59 mg g^{-1} DW (Anastasiadi et al., 2012; Barros et al., 2014; Dias et al., 2015; Domínguez-Perles et al., 2016)
Trans-Piceid	61.43 mg kg^{-1} DW (Sun et al., 2006)	-
Cis-Piceid	143.85 mg kg^{-1} DW (Sun et al., 2006)	-
Piceatannol	Nd-21.1 mg kg^{-1} DW (Piñeiro, Guerrero, Fernández-Marin, Cantos-Villar, & Palma, 2013)	Nd (Piñeiro et al., 2013)
Vitisin-B	7.2-22.2 mg kg^{-1} DW (Piñeiro et al., 2013)	Nd-61.1 mg kg^{-1} DW (Piñeiro et al., 2013)
Flavonols		
Isorhamnetin-3-*O*-(6-*O*-feruloyl)-Glucoside	1.56-2.43 mg g^{-1} DW (Domínguez-Perles, Guedes, Queiroz, Silva, & Barros, 2016)	1.67-2.04 mg g^{-1} DW (Domínguez-Perles et al., 2016)
Kaempferol	0.67-4.08 mg g^{-1} DW (Sahpazidou et al., 2014)	1.04 mg g^{-1} DW (Sahpazidou et al., 2014)
Kaempferol- 3-*O*-Glucoside	0.48-0.74 mg g^{-1} DW (Domínguez-Perles et al., 2016)	0.89-1.09 mg g^{-1} DW (Domínguez-Perles et al., 2016)
Kaempferol- 3-*O*-Glucuronide	Traces (Llobera & Cañellas, 2007)	3.20 mg g^{-1} DW (Di Lecce et al., 2014)
Kaempferol- 3-*O*-Rutinoside	0.06-0.12 mg g^{-1} DW; 1.31-2.05 mg g^{-1} DW (Barros et al., 2014; Domínguez-Perles, Guedes, Queiroz, Silva, & Barros, 2016)	0.59-0.72 mg g^{-1} DW (Domínguez-Perles et al., 2016)
Quercetin	3.94-10.24 mg g^{-1} DW (Sahpazidou et al., 2014)	7.54 mg g^{-1} DW (Sahpazidou et al., 2014)
Quercetin-3-*O*-Galactoside	6.50-15.0 mg g^{-1} DW (Apostolou et al., 2013)	13.50-19.20 µg g^{-1} DW (Apostolou et al., 2013)
Quercetin-3-*O*-Glucoside	28.4-84.5 mg $100g^{-1}$ DW (Spatafora et al., 2013)	4.50-7.20 µg g^{-1} DW (Apostolou et al., 2013)
Quercetin-3-*O*-Glucuronide	30.44-47.39 mg g^{-1} DW; 55.2-126.8 mg $100g^{-1}$ DW (Domínguez-Perles et al., 2016; Spatafora et al., 2013)	10.14-12.38 mg g^{-1} DW (Domínguez-Perles et al., 2016)
Quercetin-3-*O*-Rhamnoside	0.30-2.80 mg g^{-1} DW (Apostolou et al., 2013)	0.30-1.90 µg g^{-1} DW (Apostolou et al., 2013)
Quercetin-3-*O*-Rutinoside	2.20-3.42 mg g^{-1} DW; 4.47-41.83 mg g^{-1} DW (Domínguez-Perles et al., 2016; Sahpazidou et al., 2014)	1.08-1.32 mg g^{-1} DW; 16 mg g^{-1} (Domínguez-Perles et al., 2016; Sahpazidou et al., 2014)
Myricetin-3-*O*-Glucoside	Traces (Llobera & Cañellas, 2007)	-
Myricetin-3-*O*-Glucuronide	Traces (Llobera & Cañellas, 2007)	-
Engeletin	Traces (Llobera & Cañellas, 2007)	-

Nd - Not detected; DW - Dry Weight; FW - Fresh Weight

Table I.1. Review of the (poly)phenolic compounds of grape stems from red and white varieties (*Cont*.)

Anthocyanins		
Malvidin-3-*O*-Glucoside	18.17-28.28 mg g^{-1} DW (Domínguez-Perles et al., 2016)	-
Malvidin-3-*O*-Rutinoside + Malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside	0.40-0.60 mg g^{-1} DW; 12.93-20.13 mg g^{-1} DW (Barros et al., 2014; Domínguez-Perles et al., 2016)	-
Flavones		
Luteolin	0.02-0.04 µg g^{-1} DW (Çetin, Altinöz, Tarçan, & Göktürk Baydar, 2011)	0.01-0.07 µg g^{-1} DW (Delgado-Torre, Ferreiro-Vera, Priego-Capote, Pérez-Juan, & Luque De Castro, 2012)
Flavanonols		
Astilbin	35.00 mg kg^{-1} FW (Llobera & Cañellas, 2007)	-

Nd - Not detected; DW - Dry Weight; FW - Fresh Weight

Concerning the phytochemical content, several extractions methods were studied using different solvents and extraction conditions (temperature and time), once the concentration of bioactive compounds in the extract can vary greatly depending on the food matrix studied and the technology employed for the extraction. In 2014, our investigation group conducted a Box–Behnken design of Response Surface Methodology (RSM) to analyze the effect of time (10–30 min), temperature (25–95 ºC), and solvents concentration (5–90%) on the extraction of phenolics from Portuguese grape stems using solvents compatible with further uses in food/pharma industries. Despite the variations of specific optimal extraction conditions according to the variety of *Vitis vinifera* L. and the agro-climatic region studied, in general, at higher temperatures, food quality ethanol possess higher capacity to solubilize phytochemicals from grape stems. Furthermore, the use of 40% food quality ethanol concentration revealed to be the most efficient condition for the extraction of phenolics from this matrix (Domínguez-Perles, Teixeira, Rosa, & Barros, 2014).

Recently, some authors have also investigated the potential of green alternative extraction procedures for the recovery of bioactive compounds from winery by-products. In these studies, the effect of supercritical carbon dioxide extraction (pressure and temperature), the addition of modifier (5% v/v of ethanol), the high voltage electrical discharges (HVED) assisted extractions, and the effect of solvent type were some of the parameters evaluated to identify optimal phenolic extraction from stems separated from grape pomace, at low costs and environmentally friendly. The best results were obtained by working at high pressure (400 bar) and low temperature (35 ºC), with an increase of extraction yields of 60% for stems of white varieties, using 5% v/v ethanol as a co-solvent (Casas et al., 2010). Bousseta et al. (2012) also found an intensification on the total polyphenols extracted from white and red stem varieties 7 times higher after HVED assisted extraction (Boussetta et al., 2012). The effect of various organic (methanol, ethyl acetate) and inorganic (aqueous KOH) solvents was also investigated by Louli et al. (2004), proving that both extracts, ethyl acetate ones and those treated with supercritical carbon dioxide (SC CO_2) at various extraction pressures, were enriched in phenolic compounds (Louli, Ragoussis, & Magoulas, 2004).

After the optimal procedures applied, the extracts can be assessed by spectrophotometric screening methods to evaluate the content in total phenols, flavonoids, anthocyanins, and *ortho*-diphenols, in addition to the antioxidant activity, which can be assessed by radical scavenging capacity methods, such as DPPH or ABTS (González-Centeno et al., 2012), since they do not require expensive equipment. However, these analytical

procedures enclose diverse constraints related with the limited information provided, the overestimation of the phenolic concentration and the lack qualitative information on individual bioactive phenolics. Thus, these extracts can be assessed resorting to Reverse Phase - High Performance Liquid Chromatography (RP-HPLC) by diverse detection systems, such as diode array detector (DAD), or tandem Mass Spectrometry (MS), in order to identify and quantify the bioactive phytochemicals (Barros et al., 2014; Queiroz et al., 2017). After the phenolic identification recorded by HPLC, they can be also isolated by semi-preparative HPLC, which allows the evaluation of the specific contribution of the major compounds present in the grape stems to the radical scavenging power or antibacterial activities, possibly leading to the discovery of new potential bio-active compounds.

The evolution undergone by winery by-products, in this case, by grape stems, during their storage is another important factor to be considered, since this material represents a large volume of waste, which can be a problem to be discarded. Furthermore, the storage of this residue can not be necessarily negative, since the microflora might be responsible for the transformation of organic acids or antinutrients, so as for the appearance of metabolites that might constitute important bioactive compounds with valuable biological and technological functions (Da Ros, Cavinato, Pavan, & Bolzonella, 2014; Mateo & Maicas, 2015; Teixeira et al., 2014). Furthermore, yeast degrades pesticides, which decrease to non-detectable quantities during storage (Cabras & Angioni, 2000). Therefore, the monitoring of these residues' contents, so as their evolution, constitutes an important challenge to find the proper application for each residue and processing conditions, accounting with its constitution and storage time. In the **Chapter III**, the study of the effect of grape stems storage of several varieties cultivated in North of Portugal, in terms of the phytochemical composition and antioxidant and antibacterial activities will be demonstrated.

These results bring other factors to be hereafter considered, namely the identification of optimal storage conditions to start the processing for dedicated applications (time, temperature, and relative humidity), to overcome the few losses verified in some varieties.

1.1.3. Biological activity of grape stem phenolic compounds

Polyphenols are one of the most important compound group in grape and in their by-products, mainly due to their high number of biological properties (Xia, Deng, Guo, & Li, 2010).

Grape stems, like the other by-products derived from the winery industry, contain a high amount of phenolic compounds that, to date, have shown an interesting amount of evidences regarding possible biological activities either *in vitro* and *in vivo*, such as antioxidant, anti-inflammatory, insulinotropic, antimicrobial, anti-apoptotic, anti-carcinogenic, among others that will be further discuss in this chapter (Barros, Gironés-Vilaplana, Texeira, Baenas, & Domínguez-Perles, 2015a; Domínguez-Perles et al., 2016; Teixeira et al., 2014; Vázquez-Armenta et al., 2017a, 2017b).

1.1.3.1. Antioxidant Activity

Even though grape stems are relatively less studied than the majority of the other grape by-products, it is well known that extracts from grape stems have significant antioxidant capacities (Anastasiadi et al., 2012).

The specific phenolic content of grape stems is related to a high radical scavenging capacity, which provides a protective capacity against oxidative stress. This capacity is commonly known as antioxidant activity and is associated to the chemical properties of the phenolic compounds present in grape stems, namely the presence of conjugated double bonds and/or the presence of functional groups bonded to the phenolic ring. The antioxidant activity of the phenolic compounds occurs through diverse mechanisms of action, like the inhibition of reactive oxygen species (ROS) formation and the decrease of ROS complexation levels, as well as the extinction of the oxygen singlet, reduction of chelated metal ions, that are responsible for catalyzing reactions leading to the formation of ROS, interrupting the cascade of free radical in lipid peroxidation, and the protection of the cell natural antioxidants (Apostolou, Stagos, & Galitsiou, 2013; Dzialo et al., 2016).

The phenolics present in grape stems act against damage dealt by ROS, that are produced in cells as consequence of the respiration chain. ROS are chemically reactive molecules that contain oxygen, including free radicals, such as superoxide radical ($O_2^{+\cdot}$), peroxyl radical ($ROO^{\bullet}$), and hydroxyl radical ($OH^{\bullet}$), and non-radical species such as hydrogen peroxide (H_2O_2) (Goutzourelas et al., 2015). These species can attack different biological macromolecules such as deoxyribonucleic acid (DNA), proteins and lipids, and their excessive accumulation can lead to the overwhelming of the natural defense mechanisms of organisms and, thus inducing oxidative stress. Therefore, this can further lead to several diseases like tumors, arteriosclerosis, diabetes and chronic inflammation (Apak, Güçlü,

Ozyürek, & Karademir, 2004; Barros, Gironés-Vilaplana, Texeira, Baenas, & Domínguez-Perles, 2015b; Ross & Kasum, 2002b).

Several studies have proved that grape stem extracts exhibit a great deal of free radical scavenging activity *in vitro* in a dose dependent manner, when low concentrations are applied. The same doesn't always happens with higher concentrations, which indicates that, perhaps, the qualitative composition of the stem extracts may be more important than their quantitative polyphenolic content for the antioxidant potency (Anastasiadi et al., 2012; Goutzourelas et al., 2015). Therefore, the presence of specific compounds is associated with high antioxidant activity, for example quercetin, *trans*-resveratrol, (–)-epicatechin, (+)- catechin, gallic acid and rutin, that have been identified as components with high scavenging potential (Goutzourelas et al., 2015).

Besides the study of the grape stem extracts as a whole, there are also studies that evaluated the antioxidant capacity of individual phenolics from grape stems, and their possible synergetic effects when mixed with vitamins C and E, some of the more recognized natural antioxidants (Queiroz et al., 2017). Queiroz et al. (2017) have shown that in human keratinocytes (HaCaT cells) under basal conditions, malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside combined with vitamin E have the capacity to decrease the basal concentration of ROS, relatively to other isolated compounds and the whole extract, as well as quercetin-3-*O*-glucoronide combined with vitamin C that decrease the ROS levels by 65.3% compared with the control group in cells exposed to H_2O_2. In general, this study proved that in oxidative environments, the individual phenolic compounds have more antioxidant capacity than the whole extract, demonstrating also synergetic effects when combined with vitamins C and E (Queiroz et al., 2017).

The antioxidant capacity of grape stem extracts can also be evaluated by their capacity to enhance natural antioxidants, like glutathione (L-ɣ-glutamyl-L-cysteinylglycine, GSH), which is one of the most important antioxidant component of the cell. The reduce form of GSH is responsible for scavenging ROS, in order to maintain the redox homeostasis in the cell (Domínguez-Perles et al., 2016; Goutzourelas et al., 2015; Queiroz et al., 2017). Grape stem extracts demonstrated the capacity to increase the GSH levels in cells, as demonstrated by Goutzourelas et al. (2015), where extracts from stems of Mandilaria cultivar promoted an increase of GSH levels, which is very important since GSH is the principal source of non-protein thiol in cells (Goutzourelas et al., 2015). Other studies also proved that individual phenolics from grape stems can prevent the decrease of the GSH concentrations in either

basal and oxidative environments (65.2% and 18.7 respectively) (Domínguez-Perles et al., 2016).

Phenolic compounds from grape stems present also the capacity to prevent lipid peroxidation (LP) in cells exposed to an oxidative environment. This capacity happens due to the reducing power of the phenolics in the extracts, once, as they are antioxidants, they donate electrons and reduce the oxidized intermediates of LP processes, and thus, acting as primary and secondary antioxidants (Goutzourelas et al., 2015).

Some studies demonstrated that synergetic effects between vitamins C and E and individual phenolics from grape stems result in a higher protection against LP, instead of the whole extract and the individual isolated compounds, and also with the vitamins by themselves (Queiroz et al., 2017). This can be explained by the fact that, unlike vitamins C and E, which are respectively concentrated in the aqueous phase and phospholipid bilayer, the phenolic compounds are essentially located between the two places, due to their amphipathic properties (Ross & Kasum, 2002a). This distribution allows to envisage a complementary work amongst these different groups of bioactive molecules.

Given the importance of intracellular oxidation reactions in the development of several pathologies and also degenerative processes, this biological activity demonstrated by grape stem extracts and their individual phenolic compounds points it to their potential application in the development of functional products focused in the prevention of diseases, like Alzheimer, skin diseases (such as skin cancer, psoriasis, and rosacea), and also in the combat against the effects of aging (Działo et al., 2016).

1.1.3.2. Anti-inflammatory capacity

Our body is in constant contact to external factors that can be the cause of different types of damages, irritations or allergies. This activates the immune system to normalize the internal environment. Natural defenses of our organism respond against those negative factors by two main mechanisms: inflammation and anti-viral response. Inflammation is a response to tissue injuries consisting in five components: tumor, redness, heat, pain and lack of functionality (*functio laesa*) (Nathan, 2002; Vane & Botting, 1987). These reactions, hemo-dynamic based, are responsible to recruit cell components to remove etiological factors and also damage cells. Neutrophils and monocytes are the main leukocytes recruited, the second ones became macrophages. The main proteins involved are released by affected cells and leukocytes, including cytokines such as interleukins and chemokines (Abbas, Lichtman, &

Pillai, 2014). During the inflammatory process, macrophages and neutrophils convert molecular oxygen into ROS, increasing the release of free radicals in the damage site. Macrophages also produce reactive nitrogen species (RNS), most of them constituted by nitric oxide (NO). These radicals are responsible to activate enzymes and transcriptional factors, like the transcription factor AP-1 and the nuclear factor kappa B (NF-kB). These factors are regulated and will further regulate the secretion of signaling molecules, such as pro-inflammatory cytokines, which lead to tissues inflammation and immune cells recruitment as activation (Działo et al., 2016).

Phenolic compounds in grape and their by-products showed significant anti-inflammatory effects. The main phenolic groups that can contribute to this capacity are flavonols, flavanols and also procyanidins (Panico et al., 2006; Xia et al., 2010).

Queiroz el al. (2017) employed cellular tests to prove that individual phenolic compounds from grape stem extracts have the ability to suppress lipopolysaccharide (LPS) induced inflammation, using murine macrophages (RAW 264.7 cell line), measuring the reduction or inhibition of NO production. The results showed that all tested combinations (whole phenolic extract, individual phenolics and combinations with vitamins E and C) decreased significantly the NO production up to 52.4%, relatively to the untreated control indicating, therefore, an effective anti-inflammatory effect of those compounds. This study also allowed to show that this anti-inflammatory activity is higher when the individual compounds are applied, relatively to what happens when the whole extract is used as treatment (Queiroz et al., 2017).

1.1.3.3. Antimicrobial action

Many types of infections and diseases are treated by antibiotics. Although effective, there is a real concern about the increasing resistance to antibiotics by several pathogens. Thus, the use of natural sources of phenolic compounds becomes very important and urgent, due to their potent antifungal, antiviral and antibacterial activities, which can make them a possible alternative to antibiotics in the combat of the increasing levels of infections, bacteria and other pathogens resistant to antibiotics (Działo et al., 2016).

Antimicrobial agents are also used in food industries to increase products stability and durability, and thus, to preserve food for longer times and to insure safety and quality. Since most of those products used to this purpose are synthetic, there is a latent urge to search for

natural food antimicrobials to reduce the intake of possible toxic substances (Mattos, Tonon, Furtado, & Cabral, 2017).

Based on this, the grape stems present a good source of phenolic compounds with antimicrobial properties (Anastasiadi, Chorianopoulos, Nychas, & Haroutounian, 2009). These compounds act on the microbial cell membrane, accumulating themselves in the lipid bilayer, causing alterations to the function and structure of the membrane, allowing the penetration into to the bacterial cell, and exerting their inhibitory effects in the cytoplasm leading to cell lysis, release of intracellular ATP, and loss of cell constituents (Mattos et al., 2017; Nazer, Kobilinsky, Tholozan, & Dubois-brissonnet, 2005).

Once again, the specific structure of the phenolic compounds is related with their antimicrobial proprieties, and is believed that hydroxyl groups (-OH) are the main responsible for this specific activity. Effectively, the number of hydroxyls and the degree of polymerization can be extremely important for antimicrobial activity of phenolic compounds (Xia et al., 2010). The antimicrobial activity is also related to pH and solubility of the phenolic extracts, once low pH seems to be more efficient in limiting the microbial growth by promoting more membrane damages by H^+ -ATPase loss (Daglia, 2012; Mattos et al., 2017).

Dias et al. (2015) studied the antibacterial potential of grape stem extracts from both red and white varieties against digestive pathogens. Data revealed that Gram positive and Gram negative bacteria react differently to distinct stem's extracts. The results showed that *Listeria monocytogenes* and *Pseudomonas aeruginosa* were more sensitive to the white variety 'Fernão Pires', while *P. aeruginosa* was more reactive to red cultivar 'Tinta Amarela'. Globally, this work showed that the antimicrobial effect of grape stems has a more efficient capacity against Gram positive strains. This occurs because Gram negative bacteria have a larger lipid barrier making it more difficult for the polyphenols to migrate through the cell membrane (Corrales, Han, & Tauscher, 2009; Dias et al., 2015).

In a different line of investigation, Ruiz-Moreno et al. (2015) studied the possibility of using grape stem extracts to substitute the use of Sulphur dioxide (SO_2) in wine due to its negative effects in human health. SO_2 is used to minimize the effects of dissolved oxygen, to inhibit oxidase enzymes endogenous to grapes, and also to prevent the growth of microorganisms such as yeasts, lactic acid bacteria's, and also acetic acid bacteria (Ruiz-Moreno et al., 2015; Santos, Nunes, & Coimbra, 2012). The data revealed that grape stem's extracts showed lower inhibition than the SO_2 for *Saccharomyces cerevisiae, Hanseniaspora uvarum, Dekkera bruxellensis* and *Pediococcus damnosus* , but in other hand, it proved to be

more efficient for *Candida stellata* and *Botryotinia fuckeliana*. However, this data demonstrated to be inconclusive due to the effectiveness of the grape stem's extracts be dependent on the microorganisms (Ruiz-Moreno et al., 2015).

1.1.3.4. Insulinotropic effect

Phenolic compounds from grape stems can also present antidiabetic activity, supported by several studies. For example, polyphenolic extracts from grape stems have shown the capacity to regulate the secretion of insulin (Barros et al., 2015b). Doshi et al. (2015) demonstrated that in the presence of grape stem extracts the amount of insulin secreted by pancreatic islets increases at different concentrations of glucose, making it potentially useful to the treatment of type II diabetes (Doshi, Adsule, Banerjee, & Oulkar, 2015).

1.1.3.5. Anti-carcinogenic properties

As stated before, the excessive production and accumulation of ROS in the organisms may lead to a series of degenerative processes, amongst those serious problems as cancer (Apostolou et al., 2013). To date, several studies have pointed to the anti-carcinogenic potential of extracts from grapes, and also, from their by-products (Xia et al., 2010).

The molecular mechanisms involved in the anti-carcinogenic properties of phenolic compounds include induction of apoptosis through modulation of cell-cycle regulators and cell signaling, inhibition of angiogenesis, and inhibition of essential enzymes for cell proliferation (Apostolou et al., 2013; Sahpazidou et al., 2014).

Apostolou et al. (2013) have shown that grape stem extracts can inhibit the growth of liver and cervical cancer cell lines, suggesting their use as chemo-preventive agents (Apostolou et al., 2013).

Other study carried out by Sahpazidou et al. (2014) proved that there is an anti-carcinogenic potential in grape stem extracts using four different varieties of grape stems from Grecia against colon, breast, renal and thyroid cancers (Sahpazidou et al., 2014). The data from this study showed that stems can successfully inhibit the proliferation of cancerous cell lines. In particular, in colon cancer this inhibition happen once grape stem extracts enhance the production of cell cycle negative regulators like Cip1/p21 and kip1/p27,by inducing the intrinsic apoptotic pathway, by increasing the levels of caspases-9,-3 and -7, and also by increasing apoptosis inducing factors(AIF) and poly-ADP-ribose polymerase (PARP) (Dinicola et al., 2017; Sahpazidou et al., 2014).

These inhibitions caused by grape stems extracts in such different types of cancer suggests that there is a huge potential for their use as beneficial products for human health, either as a treatment or as prevention method (Sahpazidou et al., 2014).

1.1.3.6. Anti-apoptotic effects

Apoptosis, which constitutes a mechanism of programmed cell death coursing without the implication of inflammatory molecules, occurs as consequence of diverse pathophysiological situations (lack of trophic factors, elimination of cells during normal development process, viral infections or deregulation of the redox balance) (Rock & Kono, 2008). There are two major pathways that are responsible for signaling apoptosis, involving the death receptor and the mitochondrial pathways, known as extrinsic and intrinsic pathway, respectively (Gupta, 2003).

This process undergoes throughout a well-organized sequence of events that ultimately leads to cell death (Karp, 2013). Hence, death by apoptosis is a well-structured process, featured by a decrease in cells' and nucleus' volume, the loss of inter-cellular connection and adhesion capacity to neighboring cells, the formation of blebs at the surface of cells, the dissection of chromatin into fragments, and the rapid engulfment of the apoptotic bodies (Karp, 2013).

During the last decades, several bioactive compounds have been assayed on their capacity to modulate apoptosis (prevent or induce), by monitoring the specific capacity to act on the triggering factors (e.g., oxidative stress, among others) (Selassie, Kapur, Verma, & Rosario, 2005). In this connection, flavonoids have been tested in cancer and normal cells (mainly resorting to *in vitro* experiments). These studies have provided experimental support on the apoptotic effects in a selective way, on malignant cells, whilst the cell viability of the normal ones was kept unaffected by these compounds (Ramos, 2007). For instance, quercetin has been demonstrated as exerting apoptotic effects by inhibiting aggressive and moderately aggressive tumor cells' growth from prostate cancer (PC-3 and DU-145 cell lines, respectively), whilst this flavonol did not affect poorly aggressive LNCap prostate cancer cells or normal cells (Nair et al., 2004). This information reinforces the relevance of further evaluation of these compounds as 'therapeutic' drugs that could provide valuable biological effects on target cells without toxic side-effect on normal cells.

Domínguez et al. (2016) proved that grape stem polyphenols have anti-apoptotic effects in HaCaT cells. To prove this, the expression of several apoptotic markers was tested, such as

annexin-V/PI, cleaved caspase-3 and apoptotic bodies. The data indicated that apoptosis could be mediated by a cell cycle arrest at G_1/S phase, that way suppressing cell cycle progression, preventing apoptosis caused by oxidative stress (Domínguez-Perles et al., 2016). The stem's extracts also were responsible to reduce the expression of activated (cleaved) caspase-3, which is a pro-apoptotic caspase.

These biological properties make grape stems a valuable alternative as a source of phenolic compounds with potential applications, in the direction of developing new and valuable products that are beneficial for human health and also not armful for the environment, as it will be discussed hereafter (Barros et al., 2015b; Domínguez-Perles et al., 2016; Queiroz et al., 2017).

1.1.4. Industrial applications: challenges for the winery sector

1.1.4.1. Food, cosmetic and pharmaceutical applications of phenolic grape stems

In our days, the main strategies for the valorization of food wastes concern their biotechnological transformation into chemical or biofuels, or even the recovery of important substances, such as polyphenols, that typically appear in these wastes. Therefore, the tendency is to take advantage of some residual phenolic compounds in order to exploit their potential use in cosmetic, nutraceutical, food conservation, packaging, pharmaceutical and medical industrial fields. Furthermore, the European Commission pretends, until 2020, to promote a global target of 20% of renewable energy in the final energy consumption, as well as to apply a circular economy for eco-innovation with "zero waste" principle, where residues are used as raw materials for the production of new products (European Union, 2009).

In this context, grape stem is a residue that present currently a low commercial value, being mainly used as soil amendments or as a valuable foodstuff for livestock feed, although their contents in antinutrients, such as phytic acid, or condensed-tannins have not been sufficiently evaluated. Moreover, the pursue of innovative uses of these materials has allowed to envisage other applications like extraction of polyphenols or dietary fiber (Mateo & Maicas, 2015; Teixeira et al., 2014). There is just a few information related to the real applicability of grape stems polyphenols extracts on the cosmetic, pharmaceutic, and food industry, although many authors mention that it can be applied in all these sectors.

As previously proven, grape stems are very rich in phenolic compounds with remarkable antioxidant activity, which makes them valuable candidates to be used by several

industrial sectors (Teixeira et al., 2014). The integration of these materials would allow to replace synthetic antioxidants currently used, and so to develop cost-effective solutions for the current constraints enclosed to food and cosmetic industries, concerning the inclusion of new ingredients. Respecting the application of these materials, their extracts can be directly used as antioxidants, or in the production of coatings (Sharma & Rao, 2015), protecting not only the polymer itself, but also the packed foodstuff, from the oxidation processes, whilst allows the obtaining of marketable products fitting the consumers expectative. Further, these compounds can also be used by the food industry in the production of food additives, or for the enrichment of regular foodstuffs.

Grape stems have already showed potential to be used directly, minimally processed, in the production of spirits, like demonstrated by Barros et al. (2016), leading to an industrial alternative to the traditional distilled spirits produced (Barros et al., 2016). Effectively, the authors formulated and developed a liquor based beverage by the incorporation of grape stems, which displayed a valuable phenolic composition and antioxidant activity, reaching their maximum at 90 days of maceration, proving that this first approach allowed to obtain an added value product fitting a current market demand.

More recently, Vásquez-Armanta et al. (2017) demonstrated the potential of grape stem phenolic extracts to reduce or eliminate the adhesion of pathogenic bacteria (*L. monocytogenes*) in food contact surfaces, such as stainless steel and polypropylene surfaces (Vazquez-Armenta et al., 2017a), and to control the presence of human pathogenic bacteria (*L. monocytogenes*, *Staphylococcus aureus*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium, and *Escherichia coli* O157: H7 in fresh leafy vegetables, namely lettuce and spinach, acting as a disinfectant (Vázquez-Armenta et al., 2017b).

Concerning the cosmetic sector, this kind of compounds is highly demanded for the production of creams or sunscreen lotions. The feasibility of the extraction of these bioactive components have to be explored, in order to assess the economical viability of supplying these compounds for this sector. Until now, there are no studies that really apply the bioactive phenolic compounds isolated from grape stems on this industry, maybe due to the fact that, in this sector, it is mandatory to make several testes over many years until they are marketable.

Due to the strong antioxidant properties of some phenolic compounds, there are also promising applications in the pharmaceutical sector as a substitute to synthetic substances commonly used in this field. As previously discussed, preliminary studies have been already developed regarding the effect of grape stems' phenolics on redox unbalance in human

keratinocytes, demonstrating the potential of individual phenolics to be used in this sector (Domínguez-Perles et al., 2016). Furthermore, these individual phenolics isolated from grape stems combined with vitamins, allow to obtain higher biological capacities, thus preventing the deleterious effects associated with redox unbalance in cells (Queiroz et al., 2017).

1.1.4.2. Non-phenolic applications of grape stems

Regarding the non-phenolic composition, and as previously described, grape stems present also high quantities of cellulose, hemi-cellulose, and lignins in their constitution, making it a good candidate to represent an economically viable alternative to the use of casks, as a substitute of wood, in the process of wine ageing, which maybe a constraint linked to modern industrial procedures in agreement with the European regulations on food safety. Since these residues present a visible quantity of tannins, related to the so-called astringency, a negative sensorial characteristic in wine, grape stems should be tested for this purpose either fresh, or dry, and with distinct deposition times, which can ultimately lead to the degradation of tannins (Bhat, Singh, & Sharma, 1998), making grape stems a suitable material, and inexpensive alternative for the process of wine ageing. Grape stems can also be used as a source of astringent compounds, mainly represented by proanthocyanidins (Llobera & Cañellas, 2007).

Another approach is the bioconversion based in the use of winery by-products to growth microorganisms, once they are considered to be environmentally friendly, reliable and, in most cases, cost effective (Mateo & Maicas, 2015).

However, in order to extend the applicability of these extracts in food, cosmetic or even pharmaceutical industry, their purification have to be explored. Louli et al. (2004) have already studied this question, where supercritical fluid extraction (SFE) was employed. This application demonstrated that the use of pure CO_2 at a pressure higher or equal to 150 bar at 45 °C is sufficient for the significant improvement of the properties of the initial product, namely a higher antioxidant activity, once SC CO_2 removed compounds with none or low antioxidant activity (Louli et al., 2004). Furthermore, the organoleptic properties of the product are improved, meaning that the final product does not have the intense and unpleasant odor, becoming more suitable as a food additive or cosmetic ingredient.

1.1.5. The utilization of winery by-products as animal feed

Winery by-products have been mainly used in the animal feed industry as possible livestock feeds for ruminants and rabbits' diets. The inclusion on these raw matters is basically due to the fact that they are considered to be a potential cheap feed resource as limited processing is required. Although various wine wastes can be included in animal diets, such as the stems, the pomace, the seeds, the skins and pulps, the vine shoots, the stalks and the lees, studies have mainly been conducted in relation to grape pomace and grape seeds, having no available studies regarding the use of grape stems in this way. Furthermore, according to Nicodemus et al. (2007), only a residual amount of 3% is currently used in animal feeding (Nicodemus, García, Carabaño, & De Blas, 2007). In this sense, a little review will be presented related to the use of winery by-products as animal feed, where grape stems could be also comprised.

In a review study, Bekhit et al. (2016) have reported that one of the main constraints of these by-products is their variable chemical composition and nutritive value (Bekhit, El-Din, Cheng, Harrison, Ye, Bekhit, Ng, Kong, 2016). In fact, this is a general problem when studying the inclusion of alternative new feed sources in animals' diets. These authors refer that these variations occur between red and white grapes pomaces, and tend to be higher when applying different fermentation and pressing conditions. Furthermore, the agro-climatic effects should also be taken into account, making it necessary to evaluate the nutritive value of grape pomaces on a case-by-case basis. The same constraints were reported by Ruberto et al. (2008) in relation to the method of wine production and type of grape (Ruberto, Renda, Amico, & Tringali, 2008), and Baumgartel et al. (2007) and Basalan et al. (2011) have enhanced these differences according to the relative proportions of seeds, skin, pulp and stalks in the case of grape pomaces (Basalan, Gungor, Owens, & Yalcinkaya, 2011; Baumgärtel, Kluth, Epperlein, & Rodehutscord, 2007). The second drawback is the relatively low nutritive value of these feedstuffs, traditionally considered to be fibrous raw matters due to its high contents in fibre and lignin, and the presence of polyphenols, such as anthocyanins, that can have a potential negative effect on rumen fermentation (Spanghero, Salem, & Robinson, 2009).

Early results of grape seed inclusion on rabbit's diets (Alicata, Bonanno, Giaccone, 1988) have pointed out good performance results up to a level of 15% in the feeds. More recently other authors have also shown that the same level of inclusion led to normal performance

rates of animals with improvements on the digestible energy intake and average daily gains (García, Nicodemus, Carabaño, & De Blass, 2002), and no negative effects on growth performances or lactation parameters (Nicodemus et al., 2007). For ruminants, grape seed flour has been included up to 20% in the diets of lambs without compromising growth performance parameters and meat quality characteristics (Ragni, Vicenti, Melodia, & Marsico, 2014). In a recent study, Gessner et al. (2015) have pointed the beneficial effects of including a mixture of grape seed and grape pomace in an inclusion rate of 1% on milk production of dairy cows, with animals showing and increase in milk yield (Gessner et al., 2015). Similar results were obtained by Mokni et al. (2017) when evaluating the effects of grape seed in ewes' milk production, reporting substantial higher yields of milk production and higher levels of calcium and iron in the milk chemical composition (Mokni, Amri, Limam, & Aouani, 2017).

Data on grape pomace utilization in animal feeding is scarce and although it can also be included in its dried form in the diets of rabbits up to 5% without adverse effects on growth or health of animals (Guemour, Bannelier, Dellal, & Gidenne, 2010), this feedstuff is more prone to be utilized in ruminant feeding as long as low levels of inclusion are used. In fact high levels of inclusion lead to a decrease in the diets digestibility (Abarghuei, Rouzbehan, & Alipour, 2010), as well as in animal performances. Molina-Alcaide et al. (2008) and Mirzaei-Aghsaghali et al. (2011) conducting *in vitro* studies have evidenced the potential of using grape pomace and other winery by-products as feedstuffs for ruminants (Mirzaei-Aghsaghali, Maheri-sis, Mansouri, & Ebrahim, 2011; Molina-Alcaide, Moumen, & Martín-García, 2008). However, for lambs, Bahrami et al (2010) have verified that a maximum inclusion level of 10% can be used without negative effects on performance parameters (Bahrami et al., 2010).

Given the above mentioned limitations the development of methods that can upgrade the nutritive value of winery residues is one of the areas that should be explored in the animal feed industry. The treatment of these products through solid-state fermentation using fungi and yeast can increase the nutritive value of feedstuffs and develop value-added products. Results have shown that yeast (*Saccharomyces boulardii*) treated grape pomace included in the diets of pigs at a level of 3% improved growth performances, the digestibility of the diets, the meat quality parameters and at the same time promoted alterations in the fatty acid profile of the subcutaneous (Yan & Kim, 2011). Hu et al. (2014) have shown that yellow wine lees could be converted into a high-protein feedstuff using yeast culture by solid state fermentation (Hu et al., 2014). Zepf and Jin (2013) have demonstrated that fungi treated grape mark can

reach levels of crude protein increase up to 280%, from 7% to 27% (Zepf & Jin, 2013). More recently, Jin et al. (2016) in a study performed with thirteen fungi verified that solid state fermentation of grape pomaces and wine lees increase the *in vitro* digestibility (up to 50%) and the protein content of the feedstuffs (Jin, Zepf, Bai, Gao, & Zhu, 2016).

Other possible alternatives for the utilization of winery by-products in animal feeding do not directly relate to its nutritive value and animal performances but to other properties, attributable to concentration and chemical composition of the polyphenolic fraction. For ruminants, grape marc has been mostly studied as a possible ingredient in diets that promote a decrease in methane production (Moate et al., 2014; Russo et al., 2017) due to their content in polyphenols and fat (Spanghero et al., 2009). In fact, reduction of enteric methane emissions from ruminants is one possible major role that winery residues, namely grape pomace, may have in the industry of animal production.

Another viable utilization is related to the potential of these by-products as functional feed ingredients. In a recent review, Brenes et al. (2016) has highlighted the biological activities attributed to the polyphenolic fraction on winery by-products in monogastric animals (Brenes, Viveros, Chamorro, & Arija, 2016). In this work the natural antioxidant properties on meat and meat products and the antimicrobial activity and modulation of gut microbiota are thoroughly revised enhancing the beneficial effects derive from the bioactivities of polyphenols in chicken, pigs and rabbits. In ruminants, the utilization of winery sediments and winery grape pomace at inclusion levels of 7.5 and 16.6% can act as antioxidants in wethers (Ishida, Kishi, Oishi, Hirooka, & Kumagai, 2015). Kafantaris et al. (2016) have also shown that grape pomace silage has the potential to decrease the oxidative stress-induced damage to lipids and proteins and enhanced the growth of facultative probiotic bacteria and inhibited the growth of pathogen populations in lambs fed diets with an inclusion ratio of 45% (Kafantaris et al., 2016).

Although all of the referred possible utilizations of winery by-products show positive effects, it should be noted that that there are also cases being reported where no effect or negative effects have been reported. Thus, the inclusion of these by-products in animal feeding should be carefully planned so that the threshold levels are well defined according to the animal species, the inclusion rate, product variability and objectives in terms of animal performance. Therefore, commercial exploitation of these by-products by the animal feed industry requires a more detailed knowledge of their effects. Nevertheless, it is also clear that one possible way for the use of winery wastes is its inclusion in animal diets. In this respect,

the objective is to develop value-added products in order to reduce the winery waste generation and disposal, providing additional alternatives to decrease the environmental impact of winery activity and at the same time generating a valuable source of animal feed.

Conclusions

The winery by-products represent a negative environmental impact in several regions in the world, despite recent improvement of waste management strategies. The principal objective is to use wastes from one sector as raw material for other sectors. Grape stems reveal to contain a rich source of valuable compounds, namely phenolics, showing potential to be used directly, minimally processed, in distinct applications, besides composting, production of spirits or power production. Effectively, although grape stems constitute a less valorized residue from grapes, this natural source of bioactive compounds allows to go against opportunities to identify innovative uses for this material. Furthermore, the high biological activities demonstrated by several authors concerning the phenolic compounds present in grape stems can be an useful tool to recover and use these bioactive compounds as nutraceutical, natural food additives/ingredients, therefore showing potential to be used in pharmaceutical, cosmetics and food industries. Therefore, multidisciplinary collaborations relative to phytochemical evaluations and biological activities will contribute to found a promising utilization of these by-products towards added value products, contributing for the environmental protection of the wine production zones.

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II – Objectives

2.1. General objective

The main objective of this task is the evaluation of the winery industry by-products (grape stems), respecting their contents in phytochemicals, antioxidant and antibacterial activities, as well as the variation of these compositions/activities over time in order to design and develop optimal valorisation procedures in a future work.

2.2. Specific objectives

- Quantification of (poly)phenols present in grape stems by spectrophotometric and chromatographic screening methods;
- Determination of biological activities of grape stems (antioxidant and antibacterial);
- Assessment of the changes in the identified and quantified compounds during storage, as well as on the biological activities.
- Correlation between the phenolic profile and biological properties namely antioxidant and antibacterial activities.

III – Monitoring the antioxidant and antimicrobial power of grape (*Vitis vinifera* L.) stems over long-term storage

3.1. Monitoring the antioxidant and antimicrobial power of grape (*Vitis vinifera* L.) stems phenolics over long-term storage

Adapted from:

Gouvinhas I, Santos RA, Queiroz M, Leal C, Saavedra MJ, Domínguez-Perles R, Rodrigues M, Barros AIRNA. Monitoring the antioxidant and antimicrobial power of grape (*Vitis vinifera* L.) stems phenolics over long-term storage. *Accepted in Industrial Crops & Products.*

Abstract

The wine industry involves the production of large quantities of by-products, characterized by a valuable composition in phytochemicals with putative health-promoting qualities. Additionally, in light of recently revealed multidrug-resistant bacteria framework, the search for natural antimicrobial compounds has focused its attention on these compounds as promising alternatives. In this study, grape stems were assessed on their phytochemical composition and antimicrobial activity throughout storage (64 days). Upon this characterization, stems were noticed as a valuable source of total phenols, *ortho*-diphenols and flavonoids (42.04-96.29 and 45.52-81.11 mg GA g^{-1}, and 29.46-76.20 mg CAT g^{-1}, respectively), and ABTS and DPPH radical scavenging capacity (4.28-8.56 and 0.46-1.00 mmol Trolox g^{-1}, respectively), which remained stable during storage. In addition, all polyphenolic extracts were competent in inhibiting the bacterial growth of selected Gram-positive and Gram-negative bacteria strains (% of relative inhibition zone diameter ≥47), being quite interesting to the food-pharma industries as functional ingredients.

Keywords: Grape stems; Storage; Polyphenols; Chromatography; Radical scavenging capacity; Antibacterial activity.

3.1.1. Introduction

The grape (*Vitis vinifera* L.) is one of the most important and traditional fruit crops in the world, with productions higher than 74 million metric tons, most of which (80.0%) is allocated to wine production. Nowadays, this agro-food industry encloses valuable socio-economic impact on the local production areas (FAOSTAT, 2014). However, during winemaking, large amounts of by-products are generated, including liquid (wastewater) and solid wastes, such as grape stems, grape pomaces, lees, and trimmed vine shoots. These materials have serious economic and ecological drawbacks in the local production areas. The main by-products consist essentially of grape stems, pomace, and seeds, as well as wine lees which amount for more than 30% (w/w) of the total material transformed (Arvanitoyannis, Ladas, & Mavromatis, 2006).

Valuing agro-food by-products has a history of 40 years of research, the main application of winery residues being focused on bio-fuel production, obtaining food ingredients and animal feed, as well as soil amendments (Mateo & Maicas, 2015). Within this scope, the scarceness of raw foodstuffs, in conjunction with a growing social requirement to lower the pressure on ecosystems, have boosted the interest in exploiting these residues (Dominguez-Perles, Moreno, & Garcia-Viguera, 2018). Indeed, finding appropriate applications for such underexploited materials is, to a significant extent, based on their phytochemical compounds content , such as phenolics, with valuable biological activity.

Among the agro-food by-products evaluated as sources of bioactive compounds, winery by-products have attracted remarkable interest in recent years as an inexpensive source of polyphenols, which can be exploited as active ingredients in the pharmaceutical and cosmetic industries, or as food supplements, contributing to the sustainability of the agro-food system. Consequently, recent studies have provided updated information on the significant amounts of bioactive phenolics, present in grape fruits and their by-products (stems, pomaces, and seeds), responsible for beneficial effects on human health by reducing the incidence, prevalence, and severity of a number of pathophysiological processes, as demonstrated in diverse *in vitro* and *in vivo* studies (Domínguez-Perles et al., 2016; Teixeira et al., 2014; Vazquez-Armenta et al., 2017a). Concerning grape stems, which are discarded before the vinification process thus contributing to preserve almost all phytochemical compounds, there is a body of literature on their antioxidant, antimicrobial, and anti-inflammatory effects (Barros et al., 2014; Dias et al.,

2015; Domínguez-Perles et al., 2014; Domínguez-Perles et al., 2016; Queiroz et al., 2017; Xia et al., 2010). However, to date, incomplete information available on the polyphenolic composition of grape stems and its dependency on variety, sun exposure, and/or processing conditions, as the most relevant factors, must be updated and enhanced to properly design rational valorization procedures for this seasonal material. Indeed, seasonality is one of the major constraints to the design of valorization processes, as long-term storage could compromise the value of these materials as foodstuff (Dominguez-Perles et al., 2018).

Hoping to obtain more information about the potential of grape stems, concerning sample varieties cultivated in the highest production region of Portugal (Douro), this study was aimed at generating data on the phenolic composition of stems during storage, as well as their radical scavenging activity *in vitro*. Correspondingly, information about the antimicrobial potential of polyphenolic extracts present in grape stems against several multidrug resistant Gram-positive and Gram-negative bacteria was also explored to identify promising new uses for this waste.

3.1.2. Material and methods

3.1.2.1. Chemicals

Folin-Ciocalteu's reagent, 3,4,5-trihydroxybenzoic acid (gallic acid), acetic acid, both extra pure (>99%), potassium hydroxide, and sodium hydroxide were purchased from Panreac (Panreac Química S.L.U., Barcelona, Spain). Sodium nitrate, aluminum chloride, and sodium carbonate, all extra pure (>99%), saline water (0.9% NaCl), and methanol were acquired from Merck (Merck, Darmstadt, Germany). Sodium molybdate (99.5%) was purchased from Chem-Lab (Chem-Lab N.V., Zedelgem, Belgium). The compounds 2,2-diphenyl-1-picrylhidrazyl radical ($DPPH^{\bullet}$), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)diammonium salt ($ABTS^{\bullet+}$), potassium phosphate, and dimethyl sulfoxide were obtained from Sigma-Aldrich (Steinheim, Germany), as well as the standards compounds caftaric acid, resveratrol, quercetin, kaempferol, and malvidin 3-*O*-rutinoside for the chromatographic separation. Additionally, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Fluka Chemika (Neu-Ulm, Switzerland). All culture media and antibiotics were acquired from Oxoid (Oxoid Limited, Thermo Fisher Scientific Inc.). Ultrapure water was obtained using a Millipore water purification system.

3.1.2.2. Plant material

The present study was performed on grape stems from three red varieties of *Vitis vinifera* L. ('Tinta Barroca', 'Sousão', and 'Syrah') which are traditionally cultivated in the *Região Demarcada do Douro*, in northern Portugal. Plant material was collected from Quinta do Bonfim (GPS: 41.1872, -7.5841) vineyard, located in Cima Corgo sub-region. Grape stem samples were collected during the 2016 harvest. Once collected, after the grape destemming process, plant material was washed to remove grape berry residues and stored at room temperature for 64 days. Every 4 days, including control (Day 0), samples were collected and lyophilized, grounded to a fine powder, and stored, protected from light, at room temperature until analysis, as showed in **Figure III.1.**

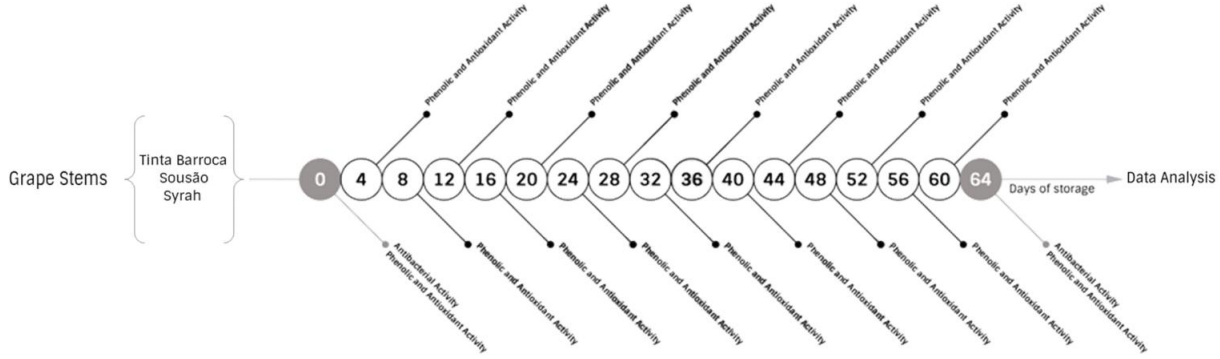

Figure III.1 - Scheme on the sampling schedule and the analytical determinations undertaken on the samples.

3.1.2.3. Preparation of extracts for analysis

For the extraction of polar phenolic compounds, samples (40 mg) were mixed with 1.5 mL of methanol/distilled water (70:30, v/v), vortexed, and finally extracted by agitation at room temperature (RT) for 30 min. Then, the mixture was centrifuged for 15 min at 10000 rpm and 4 ºC, to separate supernatant from the solid residue. The supernatants were reserved in a 5 mL volumetric flask. This extraction was repeated three times and supernatants from successive extractions were collected together. Final volume was made up to 5 mL with the above mentioned solvent. The methanolic extracts were then filtered through 0.45-µm PVDF filters (Millex HV13, Millipore, Bedford, MA, USA) and stored at 4 ºC until spectrophotometric and chromatographic analyses.

3.1.2.4. Phenolic composition

The content in total phenols, flavonoids, and *ortho*-diphenols was determined according to spectrophotometric methodologies previously reported by Machado & Domínguez-Perles (2017), with minor modifications.

The content of total phenolics in grape stems extracts was evaluated by the Folin-Ciocalteu spectrophotometric method, using gallic acid as standard. Briefly, 20 µL of sample appropriately diluted and 100 µL of Folin–Ciocalteu reagent were mixed and vortexed. Then, 80 µL of Na_2CO_3 (7.5%) were added and the mixture was vortexed once again. The reaction was incubated in an oven at 40-45 ºC, for 30 min, protected from light. Absorbance was recorded at 750 nm. Results were expressed in mg of gallic acid per gram of dry weight (mg GA g^{-1} DW).

The content of *ortho*-diphenols in grape stems was determined by adding 40 µL of Na_2MoO_4 (50 g L^{-1}) to 160 µL of the samples appropriately diluted. Mixtures were vortexed and allowed to rest at room temperature, protected from light, for 15 min. The absorbance was recorded at 375 nm and quantified using gallic acid as standard. Results were expressed in mg GA g^{-1} DW.

For the assessment of flavonoid content in grape stems, 24 µL of the sample properly diluted were mixed with 28 µL of $NaNO_2$ (50 g L^{-1}). After 5 min precisely, 28 µL $AlCl_3$ (100 g L^{-1}) were added and the mixture was allowed to react for 6 min. Subsequently, 120 µL of NaOH (1 M) were added to the mixture. The absorbance was immediately recorded at 510 nm, and the flavonoid content quantified using catechin as standard. Results were expressed in mg of catechin per gram of dry weight (mg CAT g^{-1} DW).

The assays were accomplished using 96-well micro plates (Nunc, Roskilde, Denmark) and an Infinite M200 microplate reader (Tecan, Grödig, Austria). For all analyses, three replicates (n=3) of each sample were assessed.

3.1.2.5. In vitro antioxidant activity

The free radical scavenging activity was determined by DPPH and ABTS spectrophotometric methods adapted to a microscale, according to the procedure described by Queiroz et al. (2017), by measuring the variation in absorbance at 520 nm after 15 min of reaction of the phenolic compounds with $DPPH^{\bullet}$, adding 190 µL of the DPPH solution (8.87 mM) to 10 µL the sample, and at 734 nm after 30 min for $ABTS^{\bullet+}$ (20.00 mM), mixing 188 µL of ABTS and 12 µL of the sample (Queiroz et al., 2017). The assays were performed

using 96-well micro plates (Nunc, Roskilde, Denmark) and an Infinite M200 microplate reader (Tecan, Grödig, Austria). The results were expressed in mmol Trolox to each gram of dried grape stem sample (mmol Trolox g^{-1} DW). All analyses were done in triplicate (n=3) for each variety.

3.1.2.6. Chromatographic determination of phenolic compounds

The polyphenolic profile of the grape stem samples was assessed by Reverse Phase - High Performance Liquid Chromatography - Diode Array Detector (RP-HPLC-DAD), in accordance with the method previously described (Queiroz et al., 2017). Chromatographic analyses were carried out on a C18 column (250 x 4.6 mm, 5 µm particle size; ACE, Aberdeen, Scotland). Chromatographic separation was performed using distilled water/formic acid (99.9:0.1, v/v) (Solvent A) and acetonitrile/formic acid (99.9:0.1, v/v) (Solvent B) in the linear gradient scheme (t in min; %B): (0; 0%), (5; 0%), (20; 20%), (35; 50%), (40; 100%), (45; 0%), and (65, 0%). The flow rate and the injection volume were 1.0 mL min^{-1} and 20 µL, respectively. Chromatograms were recorded in the 200-600 nm range and analyzed at 330 and 520 nm. The equipment consisted of a LC pump (SRVYR-LPUMP), an auto-sampler (SRVYR-AS), and a photodiode array detector (SRVYR-PDA5) in succession. Flavonoids, cinnamic acids, and stilbenes were quantified as quercetin/kaempferol, caftaric acid, and resveratrol, respectively, at 330 nm, and anthocyanins as malvidin-3-*O*-glucoside at 520 nm. Concentrations were expressed in mg kg^{-1} of dry weight (mg kg^{-1} DW).

3.1.2.7. Bacterial isolates

Gram positive and Gram negative bacterial isolates, recognized by the WHO (World Health Organization) as human health threatening pathogens, were collected from human patients and supplied by the Hospital Center of Trás-os-Montes and Alto Douro (CHTMAD), in northern Portugal, according to a research collaboration protocol established in 2004 with the University of Trás-os-Montes and Alto Douro. These strains belong to MJS collection and are located at the Microbiology Laboratory of the Veterinarian Science Department at UTAD.

The antimicrobial activity of grape stems' phenolic extracts was evaluated against multidrug resistant bacteria isolated from the gastrointestinal tract of humans, namely *Staphylococcus aureus* (MJS241), *Enterococcus faecalis* (MJS257), *Escherichia coli* (MJS60), and *Klebsiella pneumoniae* (MJS281). Isolates identification was performed by standard biochemical characterization techniques using API 20E, API 20NE, API Staphy, and

API Strep (BioMerieux), followed by genetic identification through 16S rRNA sequencing. Once identified, all strains were stored at -70 °C in aliquots of BHI (Brain Heart Infusion) medium with 15% (v/v) glycerol. *Pseudomonas aeruginosa* and *Listeria monocytogenes* strains were obtained from American Type Culture Collection (ATCC) (**Table III.1**).

Table III.1
Bacterial strains tested in the antibacterial bioassays.

Bacterial isolates	Origin	Class
Listeria monocytogenes ATCC 15313	American Type Culture Collection	Gram +
Staphylococcus aureus MJS241	Clinical-human gastrointestinal segment	Gram +
Enterococcus faecalis MJS257	Clinical-human gastrointestinal segment	Gram +
Pseudomonas aeruginosa ATCC 10145	American Type Culture Collection	Gram -
Escherichia coli MJS60	Clinical-human gastrointestinal segment	Gram -
Klebsiella pneumoniae MJS281	Clinical-human gastrointestinal segment	Gram -

3.1.2.8. Antimicrobial activity

The antimicrobial activity of the phenolic extracts, obtained from the grape stem samples (Day 0 and Day 64 of storage), was assessed using the disc diffusion method described by Bauer et al. (1966) with some modifications (Bauer, Kirby, Sherris, & Turck, 1966). In brief, bacterial inoculum, obtained from inoculating an isolated colony from pure strains at 0.9% NaCl with the turbidity adjusted to 0.5 McFarland standard units, were spread with a sterile cotton swab onto Petri dishes (90 mm of diameter) containing 20 ml of Mueller-Hinton agar. Six-millimeter diameter sterile paper discs were distributed on the seeded agar plates and imprinted with 10 µL of 195 mg mL^{-1} polyphenolic extract (in Dimethyl Sulfoxide (DMSO) 10%). Plates were incubated overnight at 37 ºC, followed by diameter measurement (in millimeters) of the clear inhibitory zones around the discs imprinted with the polyphenolic extracts. In all experiments, a negative control (10 µL DMSO) and 3 positive controls (standard commercial antibiotics: ciprofloxacin (10 µg), gentamicin (10 µg), and gentamicin (30 µg)) were included. All experiments were performed in triplicate.

The antibacterial activity was assessed by the application of the equation '*% RIZD = ((IZD sample – IZD negative control) / IZD antibiotic standard) x 100%*', in which RIZD represents the percentage of relative inhibition zone diameter in mm and IZD is the inhibition zone diameter in mm (Freitas, Aires, Rosa, & Saavedra, 2013; Saavedra et al., 2012). This equation compensates eventual inhibitory effects of any solvents distinct from

water on the inhibitory zone. Additionally, the antibacterial effects of the polyphenol extracts assessed were classified according to the following activity score: 0 - Without effect; 0-100 - Less effective than an antibiotic; >100 - More effective than an antibiotic; Δ - Extract effective and antibiotic without effect.

3.1.2.9. Statistical analysis

The results are presented as mean (n = 3) with the determination of the Least Significant Difference (LSD) for a *p* value <0.05. The data obtained were subjected to variance analysis (ANOVA) and a multiple range test (Tukey's test), using IBM SPSS statistics 21.0 software (SPSS Inc., Chicago, IL, USA). Pearson correlation analysis was performed to corroborate relationships between selected parameters.

3.1.3. Results and discussion

3.1.3.1. Total phenolic, ortho-diphenol, and flavonoid content

The total phenolic content was found at its lowest level at the beginning of storage (Day 0) in 'Sousão' cv. (58.21 mg GA g^{-1} DW), followed by 'Syrah' (76.60 mg GA g^{-1} DW), and 'Tinta Barroca' (82.58 mg GA g^{-1} DW) (**Table III.2**). As to the evolution of the total phenolic content during storage (64 days), 'Sousão' exhibited a decrease of its content by 20.7%, unlike 'Tinta Barroca' and 'Syrah' which did not reveal significant differences over time. The same trend was observed regarding the content of *ortho*-diphenols and flavonoids of 'Sousão' stem samples, as the starting material from this variety, again presenting the lowest content for these phenolic classes on Day 0. Additionally, during storage, the content of *ortho*-diphenols and flavonoids of 'Sousão' stems decreased by 14.3% and 2.0%, respectively. When evaluating 'Tinta Barroca' stems, no significant differences between the first and the last days of storage concerning any of the phenolic classes monitored were noticed. Contrary to the content of total phenols that did not vary in 'Syrah' stems during storage, *ortho*-diphenols and flavonoids experienced a decrease, i.e. from 62.84 to 48.26 mg GA g^{-1} DW and from 69.46 to 47.26 mg CAT g^{-1} DW, respectively, on day 64, relatively to the values recorded in starting materials (**Table III.2**).

Through this first approach, it was possible to verify that, despite the slight decrease in polyphenol content in grape stems during storage, essentially due to the intrinsic characteristics of the varieties studied, this by-product is still a rich source of bioactive compounds. Indeed, this concurs with previous studies, such as the work developed by Anastasiadi et al. (2012) who obtained similar values of total phenols (from 367 to 587 mg GA g^{-1}), although in this work the initial content of phenolics in grape stems was 7.5 times greater than the concentration recorded in the present study (Anastasiadi et al., 2012). Other authors reported concentration of phenolics in 'Syrah' cv. stems (50 mg GA g^{-1}) lower than those found in this study (Alonso, Guillén, Barroso, Puertas, & García, 2002). Similarly, Llobera et al. (2007) also demonstrated lower values of total polyphenols in 'Manto Negro' grape stems (11.6 mg GA g^{-1}) in relation to the levels found in the present study in 'Sousão', 'Tinta Barroca', and 'Syrah' grape stems (Llobera & Cañellas, 2007). However, in 2008, the same authors found in the 'Roditis' variety grape stems similar levels of these compounds relatively to those described in our work in 'Tinta Barroca' stems (87.3 mg GA g^{-1}), despite Roditis being a white variety (Llobera & Cañellas, 2008).

Table III.2. Total phenols (mg GA g^{-1} DW), *ortho*-diphenols (mg GA g^{-1} DW), and flavonoids (mg CAT g^{-1} DW) content of grape (*Vitis vinifera* L.) stems of the varieties 'Sousão', 'Tinta Barroca', and 'Syrah' during storage (64 days).

Days of storage	Total phenols			*Ortho*-diphenols			Flavonoids		
	'Sousão'	'Tinta Barroca'	'Syrah'	'Sousão'	'Tinta Barroca'	'Syrah'	'Sousão'	'Tinta Barroca'	'Syrah'
0	[Z] 58.21^{d}	82.58^{abc}	76.60^{ab}	61.35^{f}	72.87^{abcde}	62.84^{cde}	45.31^{i}	65.52^{abcd}	69.46^{ef}
4	53.69^{bcd}	70.97^{a}	86.77^{bc}	52.99^{abcde}	67.36^{abc}	62.02^{cde}	41.15^{fghi}	58.19^{a}	64.02^{cde}
8	46.02^{ab}	83.72^{abc}	93.49^{c}	52.18^{abcd}	73.11^{abcde}	64.67^{e}	32.85^{abc}	65.90^{abc}	64.68^{cdef}
12	46.12^{abc}	76.56^{ab}	73.58^{a}	51.35^{abcd}	67.97^{abc}	48.05^{a}	34.69^{abcd}	60.54^{abc}	48.01^{a}
16	49.59^{abcd}	80.37^{abc}	72.89^{a}	52.72^{def}	73.20^{abcde}	48.67^{a}	35.49^{bcdef}	66.67^{bcdefg}	47.38^{a}
20	47.70^{abc}	74.20^{ab}	85.98^{abc}	54.73^{bcdef}	65.85^{ab}	55.97^{bc}	37.89^{bcdefgh}	63.76^{abc}	61.90^{cde}
24	54.68^{cd}	90.71^{bc}	78.48^{ab}	56.92^{cdef}	77.81^{cde}	57.82^{bcde}	42.87^{hi}	75.73^{fg}	60.43^{cd}
28	44.00^{a}	89.68^{bc}	88.19^{bc}	50.69^{abcd}	76.69^{bcde}	61.93^{cde}	34.34^{abcd}	68.93^{cdefg}	68.51^{def}
32	49.50^{abc}	84.57^{abc}	83.85^{abc}	57.16^{def}	74.15^{abcde}	60.16^{bcde}	40.89^{efghi}	63.91^{abc}	71.89^{f}
36	47.43^{abc}	85.59^{abc}	73.37^{a}	51.07^{abcd}	77.19^{abcde}	53.80^{ab}	39.57^{defghi}	66.53^{bcdef}	58.54^{bc}
40	53.46^{bcd}	81.58^{abc}	80.30^{ab}	52.01^{abcd}	72.02^{abcde}	57.63^{bcde}	35.78^{bcdef}	67.50^{bcdefg}	64.43^{cdef}
44	42.65^{a}	71.23^{a}	84.27^{abc}	45.52^{a}	66.38^{a}	64.03^{de}	32.20^{ab}	58.69^{ab}	61.55^{cde}
48	42.04^{a}	93.34^{bc}	87.05^{bc}	47.23^{ab}	79.37^{de}	61.70^{cde}	29.46^{a}	76.20^{g}	64.98^{cdef}
52	44.50^{a}	92.73^{bc}	77.73^{ab}	48.33^{abc}	79.01^{de}	56.92^{bcd}	36.65^{bcdefg}	74.59^{defg}	51.34^{ab}
56	48.42^{abc}	96.29^{c}	87.39^{bc}	49.98^{abcd}	81.11^{e}	61.54^{cde}	38.34^{bcdefgh}	75.48^{efg}	57.84^{bc}
60	43.86^{a}	83.15^{bc}	76.09^{ab}	49.35^{abcd}	70.86^{abcd}	53.77^{ab}	35.08^{abcde}	66.53^{bcdefg}	47.54^{a}
64	46.15^{abc}	81.21^{abc}	72.25^{a}	52.68^{abcde}	69.85^{abcd}	48.26^{a}	44.41^{i}	65.88^{bcde}	47.26^{a}
LSD (p<0.05)	13.71	22.45	18.75	11.46	13.56	15.24	12.79	17.42	23.88

[Z] Means (n=3) in the same column followed by different superscript lowercase letters are significantly different at p<0.001, according to Tukey's test.

Effectively, in this regard, some authors have referred higher values of phenolic compounds in red grape stem varieties (Püssa, Floren, Kuldkepp, & Raal, 2006), while others have reported higher values in stem samples obtained from white varieties (Pinelo et al., 2005). Sahpazidou et al. (2014) and Apostolou et al. (2013) also revealed similar total polyphenol content of white (Assyrtiko, Vilana, Robola, and Athiri,) and red (Mavrotragano, Voidomato, Mandilaria, Moschato, Ksinomavro, and Vinsanto) varieties, varying from 318 to 584 mg GAE g^{-1}, which are significantly higher than those presented in this work (Apostolou et al., 2013; Sahpazidou et al., 2014). This inconsistent preponderance of white and red varieties as sources of phenolic compounds suggests that the genetic background and physiological features are not the only factors responsible for the occurrence of such compounds, as environment and agroclimatic conditions seem to be particularly relevant to this.

In order to obtain a closer insight into the extract's composition, eleven individual phenolics were identified and quantified resorting to RP-HPLC-DAD (**Figure III.2**). The tentative identification of the individual compounds found in the polyphenolic extracts of grape stems was performed by comparison with authentic standards on DAD spectra and retention time. Hence, the most abundant phenolics found in stems of the studied varieties were consistent with the presence of hydroxycinnamic acids (caftaric acid and an unidentified hydroxycinnamic acid), flavonols (isorhamentin-3-*O*-(6-*O*-feruloyl)-glucoside, quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucuronide, kaempferol-3-*O*-rutinoside, and kaempferol-3-*O*-glucoside), flavones (unidentified flavone), stilbenes (Ɛ-viniferin), and anthocyanins (malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside and malvidin-3-*O*-rutinoside) (**Figure III.2**).

Apart from profiling the range of phenolic compounds present in this material, this study also analyzed the evolution in concentration of such individual phenolics over 64 days of storage. This analysis revealed that 'Tinta Barroca' presented the highest concentration of most phenolic compounds identified, followed by 'Syrah', while 'Sousão' was, in general, the variety with the lowest concentration of individual phenolics (**Figure III.3**). Furthermore, during storage, a significant decrease in the concentration of some individual phenolics was observed, which was especially significant for caftaric acid, the unidentified flavone, and malvidin-3-*O*-rutinoside. This reduction was more perceptible during the first 10 days of storage, remaining at practically constant levels afterwards (**Figure III.3**).

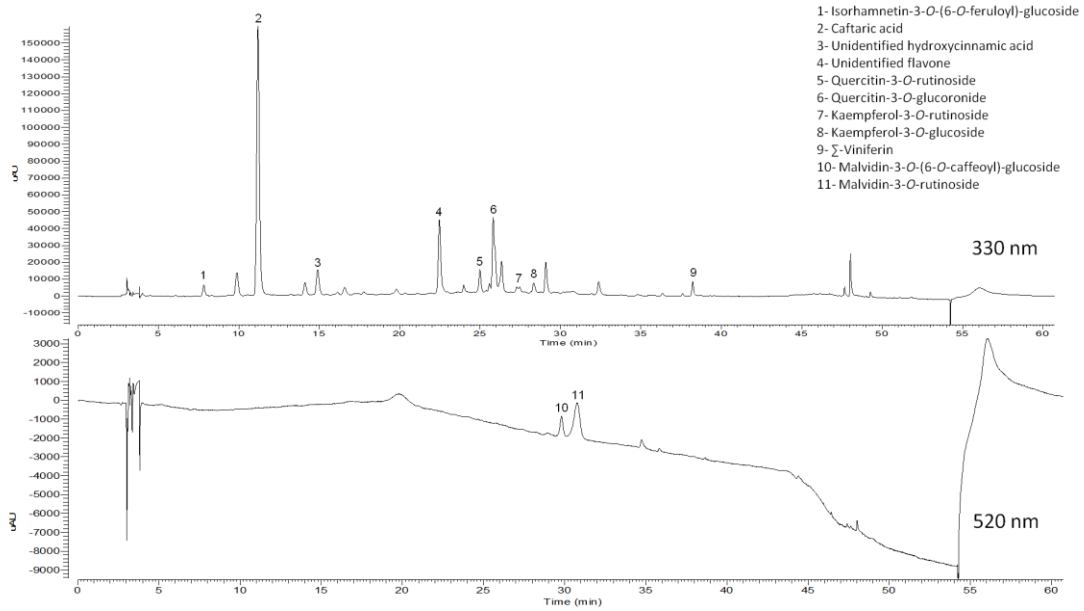

Figure III.2 - Representative RP-HPLC–DAD chromatogram of grape (*Vitis vinifera* L.) stems at 330 (phenolic acids and flavonoids) and 520 nm (anthocyanins) of Tinta Barroca variety at Day 16. (1) isorhamnetin-3-*O*-(6-*O*-feruloyl)-glucoside; (2) caftaric acid; (3) unidentified hydroxycinnamic acid; (4) unidentified flavone; (5) quercetin-3-*O*-rutinoside; (6) quercetin-3-*O*-glucuronide; (7) kaempferol-3-*O*-rutinoside; (8) kaempferol-3-*O*-glucoside; (9) Ɛ-viniferin; (10) malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside; (11) malvidin-3-*O*-rutinoside.

The 'Syrah' cv. presented a decrease in almost all individual identified phenolics, including anthocyanins, with a minimum loss of 25.0%. Concerning 'Tinta Barroca', during the entire storage period (64 days), a decrease in the concentration of isorhamnetin-3-*O*-(6-*O*-feruloyl)-glucoside of about 11%, 36% for caftaric acid, 6% for the unidentified hydroxycinnamic acid, 57% for the unidentified flavone, and a decrease of 42% and 14% for the anthocyanins malvidin-3-*O*-rutinoside and malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside were found. Interestingly, 'the concentration of additional phenolics Tinta Barroca'grape stems, augmented from 4.2 to 60.5%, on average, which can explain the changes found concerning the content of total phenolics which did not vary during storage for this variety.

To date, a number of studies have revealed the presence of several phenolic compounds in grape stems, namely stilbenes such as *trans*-resveratrol and its dimmer Ɛ-viniferin, which have been found in high concentrations, ranging from 0.09 to 0.27 mg g^{-1} DW and from 0.12 to 5.82 mg g^{-1} DW, respectively (Anastasiadi et al., 2012).

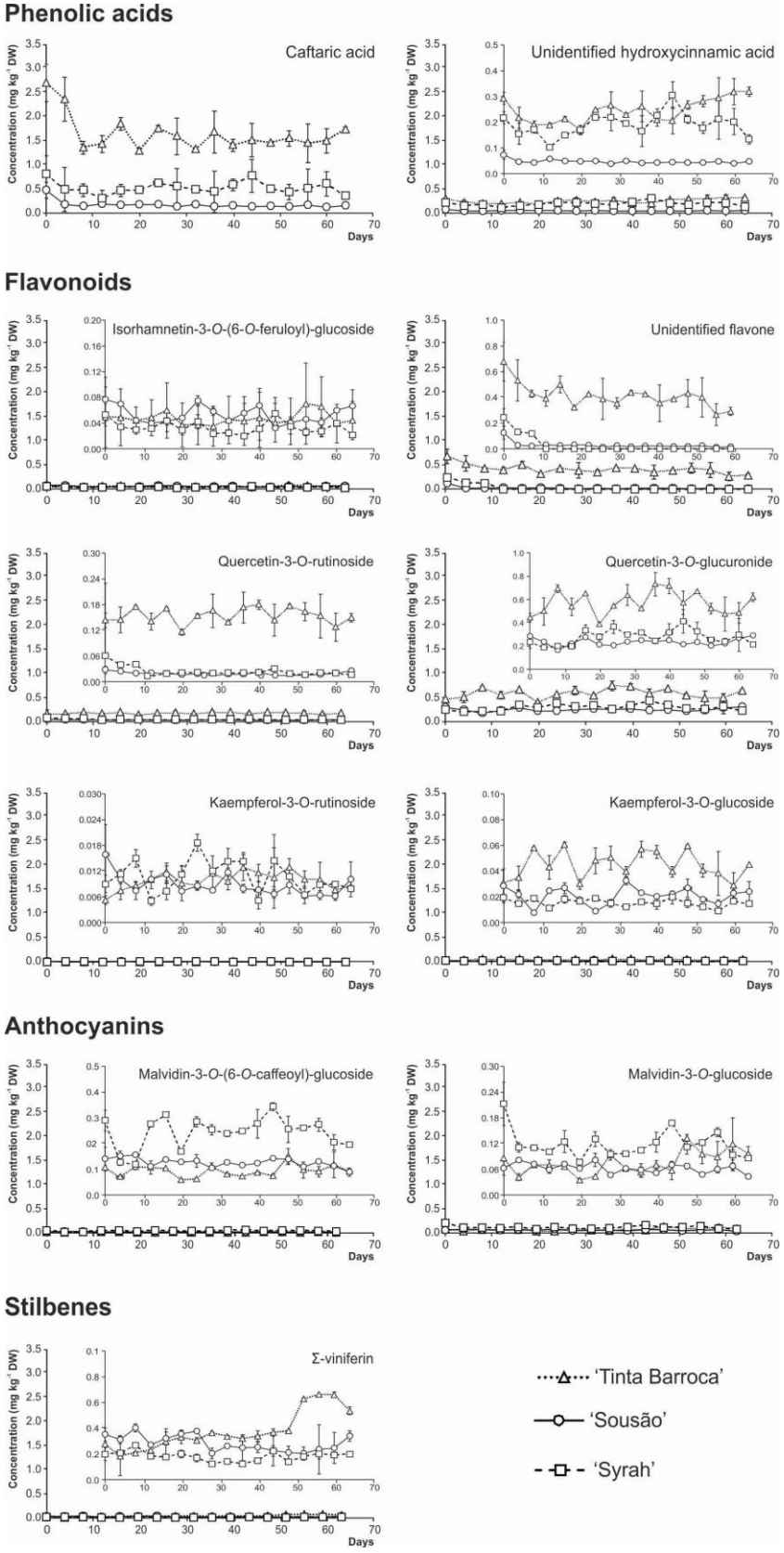

Figure III.3 - Content of individual phenolics (mg kg^{-1} dw) of grape (*Vitis vinifera* L.) stems during 64 days of storage. Statistical treatment notes: data were subjected to analysis of variance (ANOVA) and multiple range test (Tukey's test) with a significance of $p<0.05$.

In this regard, Sun et al. (2006) also found *trans*-piceid and *cis*-piceid, in low concentrations (61.43 and 143.85 mg kg^{-1} DW, respectively) in stems of the red grape varieties (Sun et al., 2006). Furthermore, Piceatannol and vitisin-B are additional phenolics described in grape stems which were firstly identified by Piñeiro et al. (2013), in concentrations equal or lower than 21.1 and 61.1 mg kg^{-1} DW, respectively, in white and red grape varieties (Piñeiro et al., 2013).

Effectively, given the results obtained in the present work and the current knowledge on this issue taken into consideration, one can state that grape stems are a rich source of phenolic compounds, and even after storage these samples still contain a significant content in phenolic compounds. This is especially relevant because of the seasonal production of this material, and the requirement of its storage until its usage in the context of dedicated productive processes aimed at taking advantage of the intrinsic polyphenolic content.

3.1.3.2. Radical scavenging capacity in vitro

The antioxidant activity of the methanolic extracts of grape stems obtained of the 'Sousão', 'Tinta Barroca', and 'Syrah' was determined by ABTS and DPPH methods (**Table III.3**). As expected, due to the above-mentioned description of total and individual phenolic compounds' content , 'Sousão' was the variety which showed the lowermost activity at the beginning of the study (5.41 and 0.53 mmol Trolox g^{-1} DW regarding ABTS and DPPH, respectively), but increased its $DPPH^{\bullet}$ scavenging activity after 64 days of storage (0.65 mmol Trolox g^{-1} DW) relatively to Day 0 (control), whereas no statistically significant differences between day 0 and 64 were found concerning the $ABTS^{\bullet+}$ (5.41 and 5.30 mmol Trolox g^{-1} DW, respectively). Besides 'Sousão', 'Tinta Barroca' and 'Syrah' grape stems demonstrated similar radical scavenging capacity on Day 0 (7.57 and 0.87 mmol Trolox g^{-1} DW, on average, for $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging capacity, respectively). However, while 'Tinta Barroca' stems did not show any differences between the first and the last day (Day 64) of storage, 'Syrah' methanolic extracts exhibited a significant decrease in $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging activity by 26.7% and 22.0%, respectively. Our results are in agreement with additional studies in literature which highlight grape stems with antioxidant activities, establishing appropriate correlation with their radical scavenging power (Barros et al., 2015b; Teixeira et al., 2014). In fact, the presence of specific compounds in grape stems is associated with high antioxidant activity, for example quercetin, *trans*-resveratrol, (–)-epicatechin, (+)-

catechin, gallic acid, and rutin, which have been identified as components with high scavenging potential (Goutzourelas et al., 2015).

Table III.3. ABTS and DPPH radical scavenging activity (mmol Trolox g^{-1} DW) of grape (*Vitis vinifera* L.) stems of the varieties 'Sousão', 'Tinta Barroca', and 'Syrah' during storage (64 days).

Days of storage	ABTS			DPPH		
	'Sousão'	'Tinta Barroca'	'Syrah'	'Sousão'	'Tinta Barroca'	'Syrah'
0	[Z] 5.41^{d}	7.96^{abcd}	7.18^{ef}	0.53^{abc}	0.83^{abcd}	0.91^{d}
4	5.21^{bcd}	7.24^{ab}	6.71^{bcdef}	0.51^{ab}	0.79^{a}	0.88^{bcd}
8	4.61^{ab}	7.61^{abcd}	7.05^{ef}	0.46^{a}	0.86^{abcd}	0.91^{d}
12	4.77^{abcd}	6.92^{a}	5.30^{a}	0.49^{ab}	0.82^{ab}	0.77^{abc}
16	4.58^{ab}	7.29^{ab}	5.46^{a}	0.57^{bcd}	0.92^{bcd}	0.80^{abcd}
20	4.70^{abc}	7.20^{ab}	6.55^{bcde}	0.57^{abcd}	0.86^{abcd}	0.84^{abcd}
24	5.20^{bcd}	8.15^{bcd}	6.58^{bcde}	0.62^{cd}	1.00^{d}	0.85^{abcd}
28	4.40^{a}	8.28^{bcd}	7.53^{ef}	0.51^{abc}	0.96^{cd}	0.91^{d}
32	4.91^{abcd}	7.94^{abcd}	7.66^{f}	0.56^{abcd}	0.95^{cd}	0.86^{abcd}
36	4.58^{ab}	8.03^{abcd}	6.01^{abcd}	0.54^{abc}	0.92^{abcd}	0.76^{a}
40	4.81^{abcd}	7.74^{abcd}	6.57^{bcde}	0.57^{bcd}	0.90^{abcd}	0.83^{abcd}
44	4.28^{a}	7.00^{a}	6.87^{def}	0.51^{ab}	0.84^{abc}	0.86^{abcd}
48	4.39^{a}	8.50^{cd}	6.77^{cdef}	0.51^{ab}	0.96^{cd}	0.85^{abcd}
52	4.69^{abc}	8.50^{cd}	5.80^{abc}	0.52^{abc}	0.91^{abcd}	0.81^{abcd}
56	4.59^{ab}	8.56^{d}	7.03^{ef}	0.55^{abcd}	0.98^{d}	0.89^{cd}
60	4.62^{abc}	7.60^{abcd}	5.72^{ab}	0.55^{abcd}	0.91^{abcd}	0.76^{ab}
64	5.30^{cd}	7.40^{abc}	5.26^{a}	0.65^{d}	0.93^{bcd}	0.71^{a}
LSD (P<0.05)	1.00	1.62	2.07	0.13	0.17	0.12

[Z] Means (n=3) in the same column followed by different superscript lowercase letters are significantly different at p<0.001, according to Tukey's test.

3.1.3.3. Antimicrobial activity

The antimicrobial activity of grape stem phenolic extracts was tested by disc diffusion method against three Gram-positive (*Listeria monocytogenes*, *Staphylococcus aureus*, and *Enterococcus faecalis*,) and three Gram-negative (*Pseudomonas aeruginosa*, *Escherichia coli*, and *Klebsiella pneumoniae*) bacteria, using solvent DMSO (10%) as negative control which was ineffective in the suppression of bacterial growth against all strains tested. In this method, the positive antibiotic controls (Gentamycin 10µg $Disc^{-1}$, Gentamycin 30µg $Disc^{-1}$, and Ciprofloxacin 10µg $Disc^{-1}$) were effective, varying according to the antibiotic used and the sensitivity of the bacteria studied (**Table III.4**). Indeed, the antimicrobial properties of samples determined by the disc diffusion method revealed that all tested stem extracts were not affected by 64 days of storage, once they inhibited the bacterial growth of the three Gram-

positive bacteria and one Gram-negative bacteria (except *E. coli* and *K. pneumoniae*). The resistance of these two Gram-negative bacteria can be explained by the presence of a complex lipopolyssaccharide layer cell membrane, representing a major barrier for phenolics to get into the cytoplasm. On the other hand, Gram-positive bacteria present a simple layer membrane, thus being more susceptible to lipophilic compounds input (Corrales et al., 2009).

When comparing the relative antimicrobial capacity of polyphenolic extracts from the three varieties included in the current study, it was found that 'Sousão' featured samples with lower efficacy than 'Tinta Barroca' and 'Syrah' against *L. monocytogenes* and *P. aeruginosa*. This might be due to its lower content in phenolic compounds. Nevertheless, 'Sousão' still presented a valuable antimicrobial activity (% RIZD between 0-100) (**Table III.4**).

Regarding 'Syrah' cv., stem phenolic extracts exhibited a decreasing efficiency against *P. aeruginosa* during the 64 days storage. However, at the end of the storage period, this variety continued to display a high efficacy (% RIZD=91). It is also important to highlight that in some cases 'Tinta Barroca', 'Sousão', and 'Syrah' grape stems showed higher efficiency than the control antibiotics included in the study regarding the inhibition of *S. aureus* and *E. faecalis* growth. Thus, the fact that the samples did not lose their antimicrobial activity after 64 days of storage demonstrated the opportunity of recovering grape stems at any time of the year, despite being a seasonal product.

Previous studies have reported the antimicrobial activity of grape stems with possible applications in the food industry, such as Vásquez-Armenta et al. (2017), who demonstrated the potential use of these extracts concerning the control of human pathogenic bacteria in fresh leafy vegetables, as well as to inhibit *L. monocytogenes* motility and adhesion to stainless steel and polypropylene surfaces. This has been explained by the possible interaction (synergistic, additive or antagonistic) between individual phenolic compounds present in this plant material (Vazquez-Armenta et al., 2017a; Vázquez-Armenta et al., 2017b). In fact, phenolic compounds are known for their antibacterial activities over a wide range of bacteria due to their structural configuration, the content of hydroxyl groups and polymerization degree being the keys for their antimicrobial activities (Daglia, 2012).

Table III.4. Antibacterial activity of grape (*Vitis vinifera* L.) stems of the varieties 'Sousão', 'Tinta Barroca', and 'Syrah' during storage (64 days).

Samples	Antibiotics														
	Gentamycin 10 µg Disc^{-1}					Gentamycin 30 µg Disc^{-1}					Ciprofloxacin 10 µg Disc^{-1}				
	'Sousão'	'Tinta Barroca'	'Syrah'	LSD (p<0.05)	p-value	'Sousão'	'Tinta Barroca'	'Syrah'	LSD (p<0.05)	P-value	'Sousão'	'Tinta Barroca'	'Syrah'	LSD (p<0.05)	p-value
Storage: 0 days															
L. monocytogenes ATCC 15313	[Z] 53[a]	58[a]	58[a]	11.25	[Y] N.s.	47[a]	52[a]	52[a]	10.92	N.s.	49[a]	55[a]	54[a]	13.79	N.s.
S. *aureus* MJS_{241}	Δ	Δ	Δ		N.s.	Δ	Δ	Δ		N.s.	Δ	Δ	Δ		N.s.
E. faecalis MJS_{257}	Δ	Δ	Δ		N.s.	Δ	Δ	Δ		N.s.	Δ	Δ	Δ		N.s.
P. aeruginosa ATCC 10145	97[a]	>100[b]	>100[b]	12.43	*	76[a]	83[a]	85[a]	10.54	N.s.	50[a]	55[a]	56[a]	12.11	N.s.
E. coli MJS_{260}	0	0	0		N.s.	0	0	0		N.s.	0	0	0		N.s.
K. pneumoniae MJS_{281}	0	0	0		N.s.	0	0	0		N.s.	0	0	0		N.s.
Storage: 64 days															
L. monocytogenes ATCC 15313	49[a]	55[b]	46[ab]	18.38	*	43[a]	48[b]	40[ab]	9.20	**	43[a]	48[b]	40[ab]	8.95	*
S. aureus MJS_{241}	Δ	Δ	Δ		N.s.	Δ	Δ	Δ		N.s.	Δ	Δ	Δ		N.s.
E. faecalis MJS_{257}	Δ	Δ	Δ		N.s.	Δ	Δ	Δ		N.s.	Δ	Δ	Δ		N.s.
P. aeruginosa ATCC 10145	94[a]	>100[b]	91[a]	11.08	*	73[a]	82[b]	71[a]	12.56	**	48[a]	54[b]	47[a]	10.60	**
E. coli MJS_{260}	0	0	0		N.s.	0	0	0		N.s.	0	0	0		N.s.
K. pneumoniae MJS_{281}	0	0	0		N.s.	0	0	0		N.s.	0	0	0		N.s.

[Z] Means (n=3) in the same row for each clinical antibiotic followed by different superscript lowercase letters are significantly different at p<0.05. [Y] N.s., No significant (p>0.05); *significant at p<0.05; **significant at p<0.01;***significant at p<0.001. Δ - Extract effective and antibiotic without effect.

Besides the differences found in the concentration of individual phenolics of stem extracts, they presented similar efficacy in antimicrobial activity against the bacteria studied. In fact, phenolic compounds may act on the microbial cell membrane which could explain the effect of the phenolic extracts of grape stems against pathogens. In this regard, phenolics accumulate in the lipid bilayer causing derangement in membrane function and structure and penetrate into the bacterial cell exerting inhibitory activity in the cell's cytoplasm, leading to lysis and release of intracellular ATP (Daglia, 2012). They can also cause loss of cell constituents, increasing the cytoplasmic membrane permeability. However, the antimicrobial activity revealed by polyphenols depends on their structural conformations, for example, non-flavonoids have demonstrated a weaker capacity to inhibit bacterial growth in comparison with flavonoids. Hence, within the latter group, flavonols, due to their hydrophobic properties, are capable to penetrate cell phospholipids membranes, being responsible for antibacterial activity by modulating molecular pathways inside cells (Van Dijk, Driessen, & Recourt, 2000).

With this approach, the high potential revealed by stem extracts to inhibit the growth of important human clinical pathogens, even after months of storage, and, in some cases, higher potential than clinical antibiotics, highlighted promising applications for this winery waste, as a source of bioactive phytochemicals, namely polyphenols. This would constitute an interesting strategy to identify new antimicrobial agents against multidrug resistant pathogenic bacteria, to be applied alone or in synergetic effect with antibiotics already in clinical use, to increase their efficacy and, therefore, reduce antibiotic resistance.

3.1.3.4. Correlation analysis

In order to establish the correlation between phenolics and antibacterial activity shown in **Table III.5**, only specific values from **Table III.4** were further considered, namely those of *L. monocytogenes* (Gentamicin (10 µg $Disc^{-1}$), Gentamicin (30 µg $Disc^{-1}$), and Ciprofloxacin (10 µg $Disc^{-1}$)) and *P. aeruginosa* (Gentamicin (30 µg $Disc^{-1}$) and Ciprofloxacin (10 µg $Disc^{-1}$)).

Table III.5. Pearson correlation of total and individual grape (*Vitis vinifera* L.) stems phenolics with $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging capacity, and antimicrobial activity.

Compounds	ABTS	DPPH	*Listeria monocytogenes*			*Pseudomonas aeruginosa*	
			Gentamicin (10 µg $Disc^{-1}$)	Gentamicin (30 µg $Disc^{-1}$)	Ciprofloxacin (10 µg $Disc^{-1}$)	Gentamicin (30 µg $Disc^{-1}$)	Ciprofloxacin (10 µg $Disc^{-1}$)
(1) Isorhamnetin-3-*O*-(6-*O*-feruloyl)-glucoside	[Z] 0.026 N.s.	-0.148 N.s.	-0.420 N.s.	-0.442 N.s.	-0.442 N.s.	-0.299 N.s.	-0.296 N.s.
(2) Caftaric acid	**0.866***	0.663 N.s.	**0.954****	**0.940****	**0.940****	0.789 N.s.	0.821 N.s.
(3) Unidentified hydroxycinnamic acid	**0.849***	**0.823***	**0.964****	**0.974****	**0.974****	**0.902***	**0.910***
(4) Unidentified flavone	**0.874***	0.628 N.s.	**0.898***	**0.876***	**0.876***	0.684 N.s.	0.719 N.s.
(5) Quercetin-3-*O*-rutinoside	**0.874***	0.790 N.s.	**0.857***	**0.858***	**0.858***	**0.934****	**0.953****
(6) Quercetin-3-*O*-glucuronide	0.651 N.s.	0.615 N.s.	0.578 N.s.	0.587 N.s.	0.587 N.s.	**0.823***	**0.837***
(7) Kaempferol-3-*O*-rutinoside	-0.282 N.s.	-0.512 N.s.	-0.356 N.s.	-0.360 N.s.	-0.360 N.s.	-0.417 N.s.	-0.416 N.s.
(8) Kaempferol-3-*O*-glucoside	0.673 N.s.	0.537 N.s.	0.453 N.s.	0.460 N.s.	0.460 N.s.	0.697 N.s.	0.706 N.s.
(9) Ɛ-viniferin	0.295 N.s.	0.221 N.s.	0.113 N.s.	0.126 N.s.	0.126 N.s.	0.444 N.s.	0.449 N.s.
(10) Malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside	-0.095 N.s.	0.069 N.s.	-0.041 N.s.	-0.013 N.s.	-0.013 N.s.	-0.123 N.s.	-0.164 N.s.
(11) Malvidin-3-*O*-rutinoside	0.453 N.s.	0.607 N.s.	0.427 N.s.	0.458 N.s.	0.458 N.s.	0.439 N.s.	0.404 N.s.
Total *ortho*-diphenols	**0.937****	0.751 N.s.	0.761 N.s.	0.757 N.s.	0.757 N.s.	0.809 N.s.	**0.824***
Total flavonoids	**0.954****	**0.960****	0.795 N.s.	0.809 N.s.	0.809 N.s.	**0.895***	**0.888***
Total phenols	0.692 N.s.	0.657 N.s.	**0.939****	**0.955****	**0.955****	0.805 N.s.	0.811 N.s.

[Z] N.s., no significant (p>0.05); *significant at p<0.05; **significant at p<0.01; ***significant at p<0.001.

The analysis of the correlation between phenolic concentration with antioxidant power and antimicrobial activities demonstrated a positive connection of the caftaric acid, unidentified hydroxycinnamic acid, unidentified flavone, and quercetin-3-*O*-rutinsoside contents with $ABTS^{\bullet+}$ scavenging capacity (r=0.866, r=0.849, r=0.874, and r=0.874, respectively) and antimicrobial activity (against *L. monocytogenes*) (r=0.945, r=0.971, r=0.883, and r=0.858, respectively), these phenolics being the main compounds responsible for the activities found (**Table III.5**). On the other hand, the radical scavenging activity based on the DPPH assay was correlated only with the concentration recorded for the unidentified hydroxycinnamic acid (r=0.823). Quercetin-3-*O*-rutinoside and quercetin-3-*O*-glucuronide also revealed to be the main compounds responsible for the high inhibition demonstrated against *P. aeruginosa*, in comparison with Gentamicin (30 µg $Disc^{-1}$) and Ciprofloxacin (10 µg $Disc^{-1}$) (**Table III.5**).

However, no significant correlation of additional individual phenolics with antioxidant and antimicrobial activities was found. Additionally, in some cases, negative correlations were found which can be explained by the existence of synergistic/antagonistic work between compounds.

The colorimetric determinations (*ortho*-diphenols, flavonoids, and total phenols) also showed correlations with the antioxidant and antimicrobial activities, even more significant statistically, which demonstrated the relevance of these bioactive compounds on the biological activities of grape stem extracts.

Therefore, the data reported in the current study reinforces polyphenols as a valuable tool against pathogenic bacteria which can be used as a natural alternative to chemicals or in combination with antibiotics to increase their efficacy, and, consequently, reduce antibiotic resistance of multidrug resistant strains.

Conclusions

This work reports an accurate identification of the phenolic composition and *in vitro* antiradical and antimicrobial activities of grape (*V. vinifera* L.) stems throughout 64 days of storage. In some cases, a decrease in the phenolic content was observed during storage; however, this content, as well as the antioxidant and antimicrobial activities, remain high after more than two months of storage. Furthermore, this study proved that stem extracts are effective to inhibit human clinical pathogens and, in some cases, more so than commercial antibiotics. It is expected that these values remain constant for months of storage, leading to the possibility to access this by-product all year-round, and keeping the biological activities that these wastes present. In this sense, grape stems can be used as a substrate to produce a high-added value product in several industries (cosmetic, food, and/or pharmaceutic), giving rise to sustainable agricultural production.

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IV – Conclusions

The winery by-products are a cheap source of polyphenols with potential biological activities, such as antioxidant and antibacterial properties. Since the constitution of these materials changes rapidly in the timeline during disposal or silage, depending on the environmental conditions, these variations need to be addressed. In this way, the main goal of this study was to demonstrate the effect of long-term storage on the phenolic composition and antioxidant and antimicrobial activities of grape stems.

The results demonstrated that, in a general way, the storage of this by-product presented no significant variations concerning some of the minor components identified, as well as respecting their biological activities. Furthermore, strong correlations were observed between the presence of phenolic compounds and all the studied bioactivities. In this sense, it is extremely important to add value to this type of by-products in order to increment their use in the extraction of biomolecules for applications in food, pharmaceutical and cosmetic industries, which is essential for the present and future of the wine industry and derived socio-economical activities in the Northern of Portugal.

Furthermore, it is essential to take in account that the structure-activity relationship (SAR) of the separate phenolics of grape stems turns into valuable even those compounds present in low concentrations, which are responsible for instance of valuable biological activities. Thus, the conservation upon storage of those phenolic compounds identified in grape stems in the present work, responsible for the functional (antiradical and antimicrobial) characteristics, or even on other activities of interest for the cosmetic industry, is of high relevance for the design of further valorization procedures for this material (specially taking into consideration its seasonal features), and to inform on the processing time that will be required for this material in order to take the highest advantage possible from its bioactive composition.

Further studies are needed in order to complete this first work, namely in terms of the determination of more biological activities, such as anti-proliferative and anti-inflammatory. Furthermore, prior to a proper and safe use, this by-product should be completely characterized respecting the potential presence of bioactive pesticides and toxins, in order to be applied in these several industrial sectors.