Universidade de Trás-os-Montes e Alto Douro

Evaluation of grape stems extracts from cultivar ‘Sousão’ as a source of compounds inhibitors of oxidative stress *in vitro* using keratinocytes

Dissertação de Mestrado em Bioquímica

Marcelo Flávio Jesus Queiroz

Orientador: Professora Doutora Amélia Maria Lopes Dias da Silva
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Professora Doutora Amélia Maria Lopes Dias da Silva

Data: 22 de Fevereiro de 2017

Classificação: 19 (dezanove) valores

Vila Real 2017

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

_____

(Marcelo Flávio Jesus Queiroz)

Vila Real, Março 2017

Agradecimentos

Em primeiro lugar quero agradecer à Universidade de Trás.-os-Montes e Alto Douro, por fornecer as condições necessárias para que fosse possível a realização desta tese de mestrado, agradecendo também aos laboratórios do CITAB onde efetuei todo o trabalho prático desta dissertação.

Quero agradecer à Professora Amélia Silva e ao Doutor Raúl Domínguez-Perles os meus orientadores que sempre foram incansáveis comigo e mostraram toda a disponibilidade para me orientar em todas as situações. A eles desejo o maior dos sucessos e felicidades quer a nível pessoal quer profissional.

Aos meus pais, irmã e família mais chegada quero agradecer por todo o apoio e paciência e apoio que sempre tiveram comigo eles que são os principais responsáveis por eu poder seguir os meus objetivos e sonhos e sem os quais tudo seria impossível de alcançar.

Quero também agradecer à Professora Ana Barros, que sempre se mostrou disponível para me ajudar em todas as situações para além de por todos os recursos do laboratório à minha disposição para que o meu trabalho corresse sempre na perfeição, a quem desejo a maior das sortes e do sucesso a todos os níveis. Aos meus companheiros de laboratório Ana Abraão, Carla Sobreiro, Irene Gouvinhas, André Lemos, Davide Oppozler e Nelson Machado que depressa se tornaram amigos quero agradecer por toda a ajuda, conselhos e companheirismo que sempre demonstraram para comigo e desejar-lhes as maiores felicidades e todo o sucesso do mundo.

Aos meus amigos de sempre "Mambos" e aos meus companheiros desta jornada universitária Carlos, Siopa, Tiago, Luís e Jorge quero agradecer por todos os momentos e por todo o apoio e quero desejar-lhes todo o sucesso do mundo e que todos cumpram com o brilhantismo de sempre os seus objetivos.

Por fim, mas não menos importante quero agradecer à Carla por toda a paciência apoio e força que me deu durante esta jornada e desejar todo o sucesso do mundo para alcançar todos os seus objetivos.

A todos, um muito obrigado

Results in this Master's dissertation were published/submitted or presented in meetings:

Research article:

Queiroz, Marcelo; Oppolzer, David; Gouvinhas, Irene; Silva, Amélia; Barros, Ana; Dominguez-Perles, Raul (2017). Vitamins C and E boost grape stems' phenolics against oxidative stress by modulating intracellular ROS, GSH, and Lipid peroxidation. *Journal of Natural Products* (submitted Manuscript ID: np-2017-000263).

Oral and poster communications:

[1] Queiroz M., Barros A. I.R.N.A, Silva A.M, Domínguez-Perles R. **Engaço de Uva como fonte de compostos fenólicos bioativos**, 2016, em Livro de Resumos de 10ª Edição Jornadas de Biologia, Universidade de Trás-os-Montes e Alto Douro (pp. 22) (Oral communication).

[2] Queiroz M., Barros A. I.R.N.A., Silva A.M., Domínguez-Perles R**. Relevance of Grape Stems as a source of purified phenolics with valuable radical scavenging capacity**, 2016, em Livro de resumos do Congresso Internacional de Vitivinicultura Unforgettable Wines 5th infowine forum (pp. 41-42) (Poster).

[3] Queiroz M., Barros A. I.R.N.A, Silva A.M, Domínguez-Perles R. **Relevance of Grape stems as a source of purified Phenolics with valuable radical scavenging capacity**, 2016, em Livro de resumos de 9ª Edição das Jornadas de Bioquímica, Universidade de Trás-os-Montes e Alto Douro (pp. 31) (Poster).

Resumo

A vinicultura é uma das mais importantes atividades sócio-económicas em Portugal, bem como em toda a bacia do Mediterrâneo. De todos os resíduos resultantes do processo de vinificação o engaço, que é descartado antes do início da produção do vinho, está entre os resíduos que aparecem em maiores quantidades.

O potencial antioxidante dos extratos de engaço de uva tem sido muito falado na ultima década, no entanto existe uma falta de informação sobre o potencial dos seus compostos fenólicos individuais e o estabelecimento de combinações desses compostos individuais com outros compostos antioxidantes. Neste trabalho, extratos polifenólicos de engaço de uvas da casta 'Sousão' (*Vitis vinifera* L.) foram analisados por HPLC-UV-*Vis* semi-preparativo obtendo-se compostos fenólicos isolados e purificados (isorhamnetina-3-*O*-(6-*O*-feruil)-glicosídeo, ácido caftárico, malvidina-3-*O*-glucosídeo, quercitina-3-*O*-rutinosídeo, quercitina-3-*O*-glucoronídeo, kaempferol-3-*O*-rutinosídeo, malvidina-3-*O*-(6-*O*-cafeoíl)-glucosídeo, malvidina-3-*O*-rutinosídeo e Σ-viniferina) que foram caracterizados por HPLC-PDA-ESi-MSn. Os compostos isolados e purificados foram testados para a sua capacidade anti-radicalar (DPPH e ABTS) para estabelecer o valor de metade da sua concentração máxima inibitória (IC_{50}). De acordo com o potencial anti-radicalar destes compostos onde foi calculado o IC_{50} (malvidina-3-*O*-glucosídeo (0,41 µmol/L), quercetina-3-*O*-glucuronídeo (1,25 µmol/L), e malvidina-3-*O*-(6-*O*-cafeoíl)-glucosídeo (0,43 µmol/L)) foi testado o seu efeito na viabilidade celular, atividade anti-inflamatória, capacidade de modulação dos marcadores de stresse oxidativo e na supressão do mesmo e a capacidade de diminuir os processos apoptóticos usando os modelos celulares (*in vitro*) HaCaT (linha celular não tumoral de queratinócitos humanos) e RAW 264.7 (linha celular não tumoral de macrófagos murinos) sob condições basais e em ambiente oxidativo. Os resultados obtidos durante estas determinações permitiram identificar as combinações malvidina-3-*O*-glucosídeo + Vitamina E e quercetina-3-*O*-glucuronide + Vitamina C como as mais valiosas e efetivas no que diz respeito à atividade anti-inflamatória (inibiram cerca de 75 % da produção de NO nas células RAW 264.7 quando estimuladas por lipopolissacarídeo) e à atividade anti-radicalar (ex. mostrou os menores valores de ROS medidos pela técnica da citometria de fluxo). A quercitina-3-*O*-glucoronídeo e a malvidina-3-*O*-(6-*O*-cafeoil)-glucosídeo mostraram o maior potencial anti-apoptótico como comprovado pela diminuição em caspase-3 clivada que é um marcador pró-apoptótico. Estes resultados permitiram

melhorar o conhecimento das atividades biológicas exercidas pelos extratos completos e pelos compostos individuais, indicando que estes exercem uma proteção celular benéfica contra o stresse oxidativo e que podem ser potenciais candidatos a ser usados no desenvolvimento de novos ingredientes funcionais para comidas, bebidas, cosméticos e também nutracêuticos.

Palavras-chave: Bioquímica; *Vitis-vinifera* L.*;* Stresse oxidativo; Propriedades bioativas; Apoptose; Engaço; Compostos fenólicos.

Abstract

Viticulture represents one of the most important socio-economic activities in Portugal, as well as in the whole area of the Mediterranean Basin. From all the residues resulting from the production of wine, grape stems, which are discarded at the beginning of the vinification process, are among the residues that appear in higher quantities.

The antioxidant potential of phenolic extracts from grape stems have been widely reported in the last decade, whilst there is a lack of information on the potential of their individual compounds and the establishment of improved combinations with other antioxidant compounds. In this work, polyphenolic extract of grape stems (*Vitis vinifera* L.), from the cultivar 'Sousão' was processed resorting to semi-preparative HPLC-UV-*Vis*, allowing to obtain purified phenolics (isorhamnetin-3-*O*-(6-*O*-feruloyl)-glucoside, caftaric acid, malvidin-3-*O*-glucoside, quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucuronide, kaempferol-3-*O*-rutinoside, kaempferol-3-*O*-glucoside, malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside, malvidin-3-*O*-rutinoside, and Σ-viniferin), which were fully characterized by HPLC-PDA-ESi-MSn. Isolated and purified compounds were featured on their radical scavenging capacity (DPPH and ABTS) in order to establish the half maximum inhibitory concentration (IC_{50}). Those compounds on which could be established the IC_{50} according to their radical scavenging power (malvidin-3-*O*-glucoside (0.41 µmol/L), quercetin-3-*O*-glucuronide (1.25 µmol/L), and malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside (0.43 µmol/L)) were further tested on cell viability, anti-inflammatory activity, capacity to modulate oxidative stress markers and overall oxidative stress and also on their capacity to decrease the apoptotic process in biological (*in vitro*) models HaCaT cells (human keratinocytes) and RAW 264.7 (murine macrophages from blood) under basal and under oxidative conditions. The results obtained through the plethora of determinations developed allowed to identify the combinations malvidin-3-*O*-glucoside + Vitamin E and quercetin-3-*O*-glucuronide + vitamin C as the most valuable and most effective regarding the anti-inflammatory (inhibited about 75 % the NO produced by RAW 264.7 cells, stimulated with lipopolysaccharide) and anti-radicalar activities (e.g. showed the lower values of ROS as measured by flow cytometry). Quercetin-3-*O*-glucoronide and malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside showed the higher anti-apoptotic potential, as evaluated by the decrease in cleaved caspase-3 a pro-apoptotic marker. These results allowed to improve the knowledge on biological activities exerted by complete extracts, or their individual

compounds, indicating beneficial cellular protection against oxidative stress and as potential candidates to be used in the development of new functional ingredients to foods, beverages, cosmetics and nutraceuticals.

Keywords: Biochemistry, *Vitis vinifera* L. ; Oxidative stress; Bioactive properties; Apoptosis; Grape stems; Phenolic compounds.

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Abbreviations

AB	Alamar Blue®
ABTS	2,2-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt
AMPS	Ammonium Persulfate
Annexin-V-FITC	Annexin-V conjugated with FITC
CE	Catechin equivalents
DCFDA	2',7'-Dichlorofuorescein diacetate
DHPE-FITC	(N-(Fluorescein-5-Thiocarbamoyl)-1.2-Dihexadecanoyl-*sn*-Glycero-3-Phosphoethanolamine) conjugated with FITC
DMEM	Dulbeco's modified Eagle's medium
DNA	Deoxyribonucleic Acid
DPPH	2,2-Diphenyl-1-picrylhidracyl radical
dw	dry weight
FAO	Food and Agriculture Organization
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GAE	Gallic acid equivalents
GSH	Glutathione
GST	Glutathione *S*-transferase
HBSS	Hank's balanced salt solution
HPLC	High performance liquid chromatograph.
HPLC-PDA-ESi-MSn	High Performance Liquid Chromatography-photodiode Array-Electrospray-mass Spectrometry
IL-6	Interleukin 6
LDL	Low density lipoproteins
LP	Lipid peroxidation
LPS	Lipopolysaccharide
Mv-3-*O*-(6-*O*-caff) -glc	Malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside
Mv-3-*O*-glc	Malvidin-3-*O*-glucoside
NO	Nitric oxide
PGE_2	Prostaglandin E_2
PI	Propidium iodide

Preparative-HPLC-UV-*Vis*	Preparative High Performance Liquid Chromatography-Ultraviolet Ultraviolet-Visible
PVDF	Hybond®-Polyvinylidene difluoride
Q-3-*O*-glc	Quercitin-3-*O*-glucoronide
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RPM	Revolutions per minute
RT	Room temperature
SDS	Sodium dodecyl sulfate
SPSS	Statistical package for the social science
TBS-T	Tris-buffered saline-tween
TE	Trolox equivalents
TEMED	Tetramethylethylenediamine
TPTZ	2,4,6-Tris(2-pyridyl)-*s*-triazine
Tris/HCl	Tris(hydroxymethyl) aminomethane hydrochloride
Trolox	6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
Trypsin-EDTA	Trypsin with EDTA (ethylenediaminetetraacetic acid)
UV	Ultraviolet
Vit C	Vitamin C
Vit E	Vitamin E
WB	Western Blot
WE	Whole Extract

Chapter I

Introduction and Objectives

Introduction

1. Introduction

1.1 State-of-the-art

Winemaking industry represents one of the most important socio-economic activities in the Mediterranean Basin, and particularly in Portugal. The latest data collected by FAO (Food and Agriculture Organization) showed that in the year 2012 the grape (*Vitis vinifera* L.) production was about 67 Mtons (FAO, http://faostat.fao.org/, 2015), with about 15 % of this grape production being intended for the wine industry (IXth General Assembly of the International Vine and Wine Organization, 2011). This industrial use of grapes entails the production of large amounts of solid wastes, mainly constituted by pomace (seeds, stems, and pulp), stems, lees, and leaves (Ana Barros *et al.,* 2015) (**Figure 1.1**).

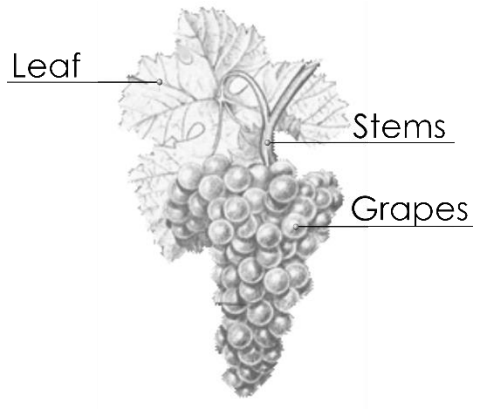


Figure 1.1 Illustration of grape cluster and its constituents (adapted form http://como-fazer-vinho.blogspot.pt, 2016)

These residues can make up to 1.5 Mtons (González-Centeno *et al.,* 2012) and have a direct impact in local environments, as well as on the sustainability of these industries that have to dispend innumerous human and economic resources to process and/or disposal these materials according to the European and national regulations (Barros *et al.*, 2015).

Although solid materials from wine industries, in their unprocessed form, represent a serious constraint linked to these agro-food companies, these by-products are also a potential source of sustainable functional compounds still underexplored, which once assessed on their concentration and biological activity could provide an opportunity for the development of new added value products with a great economic potential. Nonetheless, the final valorization of these materials needs to be supported by a body of scientific data on the actual composition, the possibility of isolate bioactive compounds, and the chance regarding their use in the development of new formulations featured by improved biological activities.

1.2 Chemistry of grape (*Vitis vinifera* L.) stems

Grape stems are one of the major by-products of the wine industry, which removal occurs before vinification processes, to avoid the transfer of compounds responsible for negative effects on the organoleptic characteristics (excessive astringency) of wines (Teixeira *et al.,* 2014). This plant material represents about 25% of the plant material processed by the wine industry and, given its features as unprocessed materials, is characterized by a high preservation ratio of chemical and phytochemical compounds (Piñeiro *et al.,* 2013).

Even though grape stems have a valuable phytochemical content, the knowledge on this issue has been mainly generated in the last decade, whilst during this period, the lack of effective transfer of information to the added value chain has not allowed to design proper valorization processes. Instead, currently grape stems remain mostly addressed to the production of spirits and as a source of dietary fiber as well as concentrates of plant protein for ruminants feeding (Arvanitoyannis, 2006; González-Centeno *et al.,* 2012). Nonetheless, these uses of grape stems are strongly limited by the high content of complex carbohydrates and other anti-nutritional compounds (hydrolysable and condensed tannins) making part of their chemical composition, which chelate nutrients reducing the nutritional value of livestock feeds (Teixeira *et al.,* 2014). Another use of grape stems is the production of fertilizers, but this process requires the prior extraction of the polyphenols due to its phyto-toxicity and antimicrobial activity, which could jeopardize the efficiency of the composting process (González-Centeno *et al.,* 2012).

As a brief description of the chemical composition of grape stems, it should be stated that the nowadays developed evaluation of the physico-chemical features of grape stems have noticed that, on average, the percentage of humidity and soluble alcohols are 67.5 and 71.0 %, respectively (Llobera *et al.,* 2007).

Complex carbohydrates are the major components of grape stems, which conditions their valorization, ranging from 60.0 to 90.0 % of dry weight (w/w), and are comprised mainly by cellulose (40.0 to 49.0 %) and lignin (22.5 %) (González-Centeno & Rosselló, 2010; Barros *et al.,* 2015). The content in proteins and soluble sugars of grape stems is 7.0 and 1.7 %, respectively (Llobera & Cañellas, 2007).

1.3 Grape stems as a source of phenolic compounds

In addition to the basic chemical composition, recent studies have shown that grape stems are rich in bioactive polyphenols, which together represent about 6 % of ºdry weight (Katalinić *et al.,* 2010; Teixeira *et al.,* 2014). In this regard, the studies developed on this issue have noticed that the majority of the polyphenols present in this vegetable matrix are flavan-3-ols, hydroxycinnamic acids, flavonols, and stilbens (Katalinić *et al.,* 2010; Teixeira *et al.,* 2014). These bioactive compounds are the most important secondary metabolites produced by plants, developing critical functions, essential for plants' development, reproduction, and general physiology (Dzialo *et al.,* 2016). Their production enhances when plants are exposed to stressing conditions by two major biosynthetic pathways, shikimate and acetate pathways (Barros *et al.,* 2015), being associated also to interesting functions in diverse biological systems (mammalians, for instance) (Domínguez-Perles *et al.,* 2016). Indeed, data available concerning the phytochemical profile of grape stems suggest their potential use as a source of compounds with high biological interest.

The biological properties of the phenolic compounds are related to their chemical structure, wherein they are mostly formed by at least one phenolic ring in which a hydrogen atom is replaced by a hydroxyl group. The different variability in biological properties of phenolics derives from the various compounds that can replace hydrogen bonding and how this bind to the phenolic ring. Usually, in plants, these phenolic compounds appear with more than one phenolic ring, so called polyphenols (Dzialo *et al.,* 2016).

Regarding the individual phenolic compounds, the ones more frequently found in grape stems are catechin, procyanidin B, quercetin-3-*O*-glucuronide, quercetin-3-*O*-rutinoside, caftaric, ferulic, gallic, and syringic acids, and Σ-viniferine. According to the data available in literature agro-climatic conditions (genetic background and environmental conditions) drastically influence the final concentrations of these compounds found in the stems (Teixeira *et al.,* 2014).

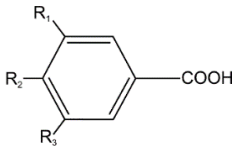
Apart from these compounds it has also been verified the presence of micronutrients such as Vitamin C and Vitamin E, which could contribute to the biological activity described in the extracts of the plant material (Barros *et al.,* 2015), though to date there is a lack of proper demonstration of this fact.

1.3.1 Phenolic Acids

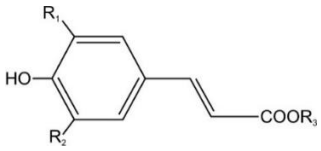
Within this class of phenolic compounds, hydroxybenzoic acids appears in greater abundance in stems and other by-products of winemaking (Teixeira *et al.,* 2014). Besides, among the phenolic acids present in the pulp, it has been described the occurrence of benzoic, caftaric, and cinnamic acids, as well as their derivative compounds (**Table 1.1**).

Table 1.1 Chemical structure of phenolic acids**.**

Benzoic Acid



Cinnamic Derivatives



	R_1	**R_2**	**R_3**		**R_1**	**R_2**	**R_3**
Benzoic acid	H	H	H	Caffeic Acid	H	OH	H
Gallic acid	OH	OH	OH	*p*-Coumaric Acid	H	H	H
p-Hydroxybenzoic acid	H	OH	H	Caftaric Acid	OH	H	$C_4H_6O_6$
p-Chlorobenzoic acid	H	Cl	H				
Syringic acid	CH_3O	OH	CH_3O				
Vanillic acid	CH_3O	OH	H				

Regarding the hydroxybenzoic acids, gallic acid is the most commonly found in grape stems at the highest concentrations, followed by the syringic acid. Thus, the former develops a very important role, mainly because of is activity as a precursor of hydrolysable tannins (Apostolou *et al.,* 2013; Teixeira *et al.,* 2014), which have been associated with several technological and biological functions (Barros *et al.,* 2015).

It can also be found in grape stems, either in white and red cultivars, caftaric acid that constitutes the most abundant hydroxycinnamic acid. The *trans* isomers are always in higher concentrations than the *cis*, because it is believed that the *trans* configuration is the natural conformation, whereas the *cis* configuration represents an isomerization product induced by UV radiation during the extraction processes (Singleton, 1978; Teixeira *et al.,* 2014).

1.3.2 Flavonoids

In general flavonoids are widely distributed in foods and beverages of plant origin such as fruits, vegetables, tea, and wine. These compounds are featured by low molecular weight and

are structurally composed of 15 carbon atoms (C_6-C_3-C_6). To date, the flavonoids identified in the different by-products derived of the vinification process belong to the subclasses flavonols, flavanols, flavones, and anthocyanins (Teixeira *et al.,* 2014) (**Table 1.2**).

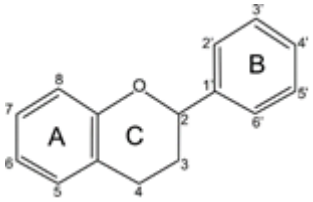
The flavanols or flavan-3-ols are flavonoids accounting with a hydroxyl group at the C3 position and do not have a carbonyl at the C4 carbon. This type of flavonoid has been described from the point of view of physiological functions in plants. In this respect, the flavan-3-ols serve as attractants to pollinators such as coloring source and also contribute to the organoleptic properties of plant foods. They are also responsible for the astringency and bitterness of wines (Teixeira *et al.,* 2014). When comparing the concentrations of different types of flavan-3-ols in grape stems, it was found that catechin is the major compound in either red and white varieties, whilst its concentration is far higher in stems than in skins and/or seeds. Despite being described both in red grapes and white, the highest concentration of catechin is found in red cultivar grapes (Barros *et al.,* 2015; Teixeira *et al.,* 2014). Other flavan-3-ols present in grape stems but in smaller concentrations are epicatechin gallate, epicatechin, and procyanidins B1, B2, and B3 (Teixeira *et al.,* 2014).

Flavonols are flavonoids with the presence of a double bond between the C2 and C3 carbons and a hydroxyl group in the C3 carbon. There are different sugars that can bind to flavonols originating glycosides, glucuronides, galactosides, and diglycosides (Castillo-Muñoz *et al.,* 2007; Jeffery *et al.,* 2008; Teixeira *et al.,* 2014). Flavonols can also react with anthocyanins, forming more stable pigments, and with phenolic acids in order to contribute in the copigmentation phenomena (Shrikhande 2000).

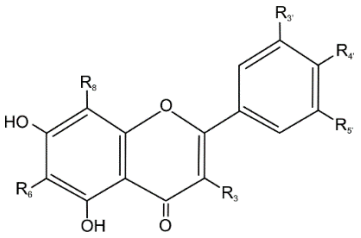
Flavonols can be found widely in the plant kingdom with the exception of fungi and algae. Flavonols are also present in the various wastes from the vinification process, appearing at different concentrations. Besides, diverse types of flavonols in the distinct wastes (stems, seeds, pomace and leaves) have been described. The most common flavonols are kaempferol, quercetin, isohamnetin, and myricetin, whilst they are majorly found as glycosides (Del Rio *et al.,* 2013; Teixeira *et al.,* 2014). Within flavonols, quercetin is the compound most frequently found in foods and foodstuffs (Ross & Kasum 2002).

Table 1.2 Chemical structure of flavonoids present in the grape stems

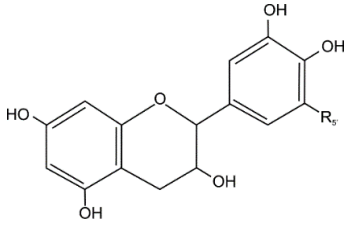
Flavonoids



Flavonols (1)



Flavanoids (2)



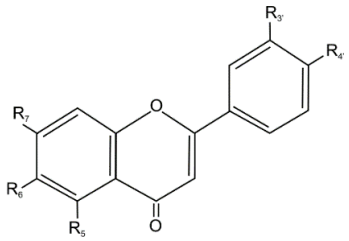
\+ 2*R* 3*S* + 2*S* 3*S*

Catechin [epi]catechin

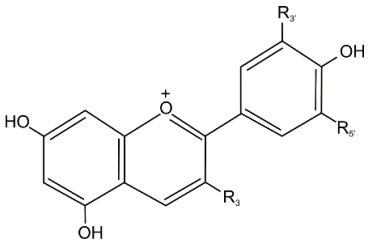
\- 2*S* 3*R* - 2*R* 3*R*

Catechin [epi]catechin

Flavones (3)



Anthocyanins (4)



	R_3	R_7	R_8	$R_{3'}$	$R_{5'}$
Flavonoids (1)					
Kaempferol (K)	OH	OH	H	H	H
K-3-*O*-Glc[Z]	*O*-Glc	OH	H	H	H
K-3-*O*-Gluc	*O*-Gluc	OH	H	H	H
K-3-*O*-Rut	*O*-Glc-Rha	OH	H	H	H
Quercetin (Q)	OH	OH	H	OH	H
Q-3-*O*-Gal	*O*-Gal	OH	H	OH	H
Q-3-*O*-Glc	*O*-Glc	OH	H	OH	H
Q-3-*O*-Gluc	*O*-Gluc	OH	H	OH	H
Q-3-*O*-Rut	*O*-Glc-Rha	OH	H	OH	H

Flavanoids (2)							
Catechin [Epi]catechin	/	OH	OH	H	H		OH
Flavones (3)							
Luteolin		H	H	OH	H	OH	
Anthocyanins (4)							
Mv-3-*O*-Glc		*O*-Glc	OH	H	OCH_3		OCH_3
Mv-3-*O*-(6-*O*-caf) -Glc		*O*-(Caf)-Glc	OH	H	OCH_3	OCH_3	
Mv-3-*O*-Rut		*O*-Glc-Rha	OH	H	OCH_3	OCH_3	

[Z] Caf, caffeoyl; Gluc, glucuronide; Gal, galactoside; Glc, glucoside; K, kaempferol; Mv, malvidin; Q, quercetin; Rut, rutinoside.

Anthocyanins are highly soluble flavonoids and are directly responsible for the red color of grapes and wines. These compounds consist of anthocyanidin aglycone (cyanidin, delphinidin, malvidin, petunidin, and peonidin), chemically featured by an aromatic ring attached to a heterocyclic ring containing an oxygen, which is also connected to a third aromatic ring by a carbon-carbon bond. This last aromatic ring forms conjugates with sugars and organic acids in order to create a multitude of different anthocyanins of various color intensities (Del Rio *et al.*, 2013). Anthocyanins are relatively unstable and readily undergo oxidation processes. They are also very sensitive to many factors that can affect its color and its stability such as temperature, pH, and ultraviolet radiation (Pascual-Teresa *et al.*, 2010; Teixeira *et al.*, 2014). Regarding the anthocyanins currently described as being present in grape stems, the limited information available has noticed the presence of malvidin-3-*O*-glucoside, malvidin-3-*O*-(-3-*O*-caffeoyl)-glucoside, and malvidin-3-*O*-rutinoside (Barros *et al.*, 2014).

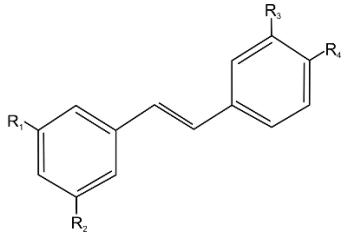
Flavones are flavonoids with a double bond between the C2 and C3 carbon, and differ from the flavonols because the lack of the hydroxyl group in C3 carbon. Despite being uncommon flavonoids in winery by-products, as a representing of this flavonoid subclass, luteolin has been found in very low concentrations in the stems of either red and white varieties of grape stems (Çetin *et al.*, 2011; Teixeira *et al.*, 2014).

1.3.3 Stilbenes

Stilbenes are phenolic compounds chemically consisting of two aromatic ring linked by an ethylene bridge (Garrido & Borges, 2013). In relation to by-products, the stilbenes are mostly found in grapes' skin, but they are also present in smaller amounts in the stems. Actually, in this plant material different stilbenes have been identified *trans*-piceid, *trans*-resveratrol, *cis*-resveratrol-3-*O*-glucoside,*trans*-reveratrol-3-*O*-glucoside, and ε-viniferin (Schoedl *et al.*, 2012; Teixeira *et al.*, 2014; Barros *et al.*, 2014; Dias *et al.*, 2015) (**Table 1.3**).

Table 1.3 Chemical structure of stilbenes present in the stem.

Stilbenes



	R_1	R_2	R_3	R_4
Resveratrol	OH	OH	H	OH
Resveratrol-3-*O*-glc[Z]	*O*-Glc	OH	H	OH

[Z] Glc, glucoside.

Grapes produce stilbenes as a response to physiological stressing factors like the levels of ozone and UV-C radiation. These factors lead to increasing levels of stilbenes hundred-folds. This fact indicates that it might be possible to modify the stilbene content of grape residues by initial industrial processes after harvest, allowing an augment of the value of winery by-products as sources of bioactive phytochemicals (Teixeira *et al.*, 2014).

Different studies on the phytochemical features of all winemaking by-products have shown that, in red grapes, stilbenes are found in stem, seeds, leafs, and pomace, whilst regarding white grapes they have been only found in the grape's stems and skins (Schoedl *et al.*, 2012; Teixeira *et al.*, 2014).

1.4 Biological Properties and Metabolic effects of Phenolic Compounds

Phenolic compounds have been deeply studied in relation to their biological properties. Several studies have provided information that proved that flavonoids have a wide variety of biological activities *in vitro* and also *in vivo*. These phenolic compounds have shown to exert antimicrobial, antiviral, antioxidant, anti-inflammatory, anti-hypertensive, antineoplastic, mutagenic, and antiplatelet properties (Guardia *et al.*, 2001; Ross & Kasum, 2002) (**Figure 1.2**).

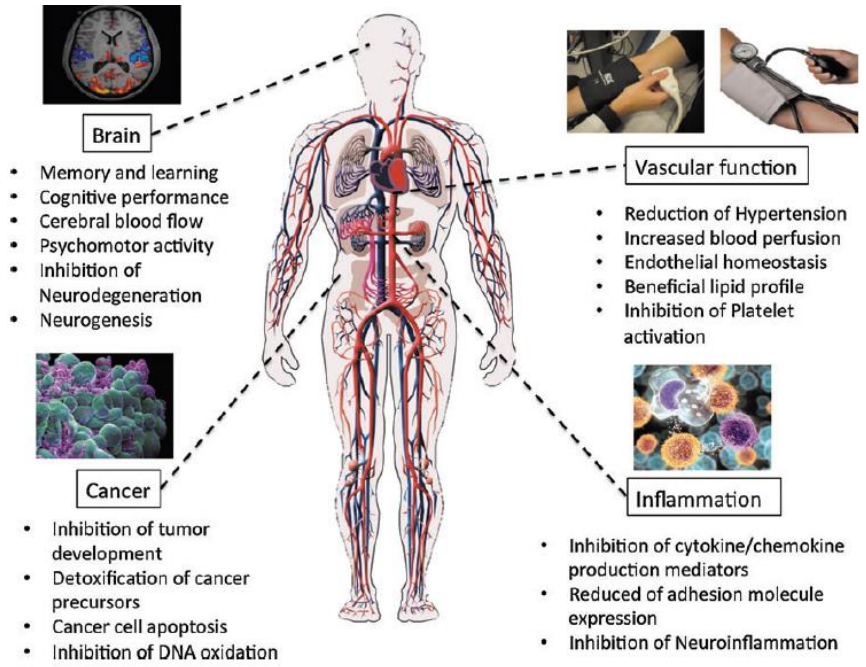


Figure 1.2 Potential benefices of phenolic compounds in humans (adapted from Del Rio et. al 2013).

1.4.1 Antioxidant Activity

Oxygen reactive species (ROS) (**Figure 1.3**), produced in cells as consequence of the respiration chain of oxidative phosphorylation, can attack different biological macromolecules such as deoxyribonucleic acid (DNA), proteins, and lipids. When the natural defense mechanisms of the organisms, either enzymatic or non-enzymatic, are overwhelmed, oxidative stress occurs because of an excessive accumulation of ROS species. The accumulation of damages caused by ROS can result in the appearance of several diseases, such as tumors, arteriosclerosis, diabetes, and chronic inflammation (Ross & Kasum, 2002; Apak *et al.*, 2004).

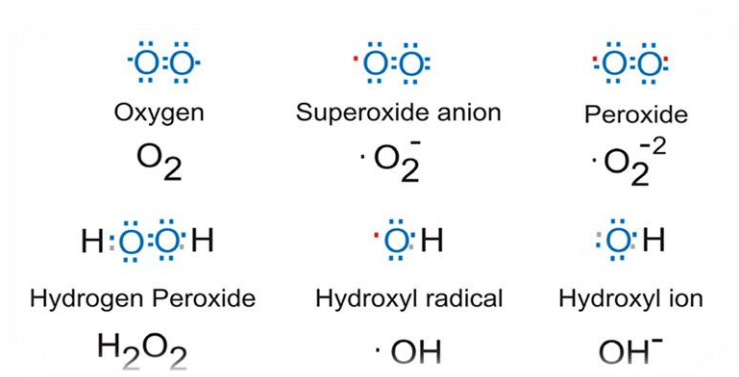


Figure 1.3 Different types of Reactive Species of Oxygen (adapted from: http://www.biotek.com)

Antioxidants can delay and prevent oxidative processes caused by free radicals and ROS. In this connection, the phenolic compounds present in various plant materials have shown a wide range of biological activities, being one of the most widely reported the ability to provide defensive tools against oxidative stress from the endogenous ROS. This ability is commonly known as antioxidant activity (Heo *et al.,* 2007; Dzialo *et al.,* 2016). The total radical scavenging power exerted by the phytochemical composition of such materials is the final consequence of three major different types of interactions between their phenolic compounds like the synergic effect, the antagonism, and additive effect.

Derived from the specific polyphenolic content, it has been reported that grape stems' extracts are featured by a high radical scavenging activity, which provides a protective capacity against damage to the DNA induced by ROS and reactive nitrogen species (RNS) (Apostolou *et al.,* 2013).

The antioxidant activity of the phenolic compounds is associated with their chemical properties, the presence of conjugated double bonds, and the presence of functional groups bonded to the phenolic ring. The anti-oxidant capacity of phenolic compounds occurs throughout diverse mechanisms of action, such as the inhibition of ROS formation and decreasing the levels of ROS complexation, the extinction of the oxygen singlet, reduction of chelated metal ions responsible for catalyzing reactions leading to the formation of ROS, interrupting the cascade of free radical in lipid peroxidation, and the protection of cell components that already have antioxidant capacity (Dzialo *et al.,* 2016).

The flavonoids when joined with other antioxidants like vitamins C and E can inhibit the lipid peroxidation at the phospholipid bilayer. Unlike what occurs with vitamins C and E that are respectively concentrated in the aqueous phase and phospholipid bilayer, flavonoids are more likely to be located between the two places because of their amphipathic capacity (Ross

& Kasum, 2002). This differential distribution allows to envisage a complementary work between these diverse groups of bioactive molecules, suggesting that their combination in the design of novel functional formulations could contribute to arise additional benefits.

Numerous flavonoids have been tested to relieve oxidative stress by glutathione-*S*-transferase (GST) induction, which is an enzyme that protects cells against damage caused by free radicals, showed an increased resistance to oxidative stress caused by hydrogen peroxide. Some, including quercetin, have been promoted as significantly increasing the specific activity of this enzyme. Indeed, GST plays a protective role against cancer by detoxifying xenobiotics with mutagenic potential, therefore, compounds which upregulate GST can also ease oxidative stress and assist in detoxification of xenobiotic mutagens (Fiander & Schneider 2000; Ross & Kasum 2002), which turn into even more interesting their collaborative work with flavonoids.

Quercetin as previously said have an *in vitro* antioxidant activity, however several investigations have demonstrated that *in vivo* there is no detectable free quercetin in human plasma after consumption of a meal rich in quercetin. This happens because of the metabolic activity of the human small intestine and liver that makes difficult the presence of quercetin. glicosides to in human plasma (Janisch *et al.,* 2004).

Flavonoids have also shown the ability to inhibit the low density lipoproteins (LDL) oxidation, in special quercetin. The protective capacities from flavonoids in contradiction of LDL oxidation are derived from binding to copper receptor of the Apo-B protein of the LDL particle, scavenging of lipid peroxyl radicals, regeneration of the endogenous antioxidants of the LDL or chelating copper(III) ions (Morand *et al.,* 1998; Janisch *et al.,* 2004).

Flavonoids can interfere with endogenous antioxidants by increasing their functions and also interfere with three different types of systems responsible for the production of free radicals like the direct radical scavenging, the nitric oxide, and xanthine oxidase (Nijveldt *et al.,* 2001).

The antioxidant capacity of grape stems extracts was related to the ability to prevent the oxidation of low density lipoproteins (LDL) in the presence of small concentrations of (poly)phenols, as well as the reduction of intracellular levels of ROS (Teixeira *et al.,* 2014).

Given the importance of intracellular oxidation reactions in the development of degenerative processes and associated pathologies, the biological activity described for phenolic compounds present in the stems suggests its potential application in the development of functional products focused on prevention of diseases like Alzheimer, skin diseases (skin cancer, *Psoriasis, Rosacea*) and also in the combat against the effects of aging (Dzialo *et al.*, 2016).

1.4.2 Anti-inflammatory capacity

Every day our body is exposed to external factors that can cause different types of damage, irritation or allergies, boosting or activating the immune system to normalize the internal environment. The natural defenses of our organism respond against those negative factors by two main mechanisms: inflammation and anti-viral response. Inflammation is not a defense system, but it's a response to tissue injury which involves five components tumor, redness, heat, pain, and lack of functionality (*functio laesa*) (Vane & Botting, 1987; Nathan, 2002). These hemo-dynamic-based modifications in affected tissues are addressed to recruit cell components responsible for removing etiological factors (bacteria, toxins, etc.) and damaged cells. Neutrophils and monocytes are the main leukocytes recruited, the second ones became macrophages. The main proteins involved are released by affected cells and by leukocytes, including cytokines such as interleukins and chemokines (Abbas et al. 2014). During the inflammatory process, activated macrophages and neutrophils convert molecular oxygen into reactive oxygen species (ROS), increasing the release of free radicals at damaged site. Macrophages, in addition to ROS, also produce reactive nitrogen species (RNS) mainly constituted by nitric oxide (NO). The formation of these radicals is responsible for the activation of other enzymes and or transcriptional factors, such as 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 and activation. Given the feature of skin as the outmost cell layer, this tissue is exposed to innumerous pro-inflammatory aggressions, like for example the solar radiation (Dzialo *et al.,* 2016).

Cyclooxygenase also plays an important role as inflammatory mediators. Phospholipase C and A2 are involved in the liberation of arachidonic acid that is one of the starting points for the inflammatory process, as a precursor of eicosanoids such as pro-inflammatory prostaglandins (Williams *et al.,* 1999). These are involved in various immunologic responses and are the final products of the cyclooxygenase and lipoxygenase metabolic pathways (Formica & Regelson, 1995; Nijveldt *et al.,* 2001). Certain phenolic compounds can inhibit both mediator's pathways, reducing that way the release of arachidonic acid. Nonetheless, the exact mechanisms in which the phenolics inhibit the enzymes involved in this process are poorly understood. Quercetin is believed to be able to inhibit both enzymes activity, being responsible for the declining in the formation of the inflammatory metabolites (Nijveldt *et al.,* 2001)

An additional anti-inflammatory property of the flavonoids is their assumed capacity to inhibit neutrophil degranulation. This is important because that is a direct way to diminish the release of arachidonic acid by neutrophils and other immune cells (Tordera *et al.,* 1994).

That way, the crucial function of the polyphenols regarding the inflammation process is to inhibit the formation of pro-inflammatory factors, neutralizing free radicals and also inhibiting lipid peroxidation (Dzialo *et al.,* 2016).

According to Almeida *et al.,* (2015), photoaging can be mitigated by polyphenols. Incubating HaCaT cells with *C sativa* leaf extract has reduced UV-induced damage in DNA, suggesting a protective mechanism based on direct antioxidant action (involving O_2). However, as this process can occur with the intermediation of the nuclear factor Nrf2 (nuclear factor E2-related factor 2) activation, which is a transcription factor sensitive to the reduction, and is known to be stimulated by various phytochemical compounds. The quantity of DNA damaged by UV radiation was also reduced after the treatment with polyphenols (especially anthocyanins) from blackberry berries (*Rubus adenotrichos* L.), honeyberry (*Lonicera caerulea* L.), and blueberry (*Vaccinium myrtillus* L.) (Calvo-Castro *et al.,* 2013; Hu *et al.,* 2015; Dzialo *et al.,* 2016).

In addition to DNA, UV radiation can also disrupt, through enhancing oxidative stress, other components of the cell. Hence, intracellular lipids are highly susceptible to peroxidation caused by the formation of ROS. A study showed that production of ROS induced by UV-A radiation was suppressed by phenolic compound extracted from *Prunella vulgaris* L. leading to a decrease in lipid peroxidation (Dzialo *et al.,* 2016).

Many natural plant polyphenols have been considered effective as sunscreen agents. Phenolic compounds are able to absorb radiation of the UV-B region due to their suitable chemical structure (Dzialo *et al.,* 2016).

An important aspect of the induced photo-oxidative stress is the inflammatory reaction. There are many reports on the attenuation of inflammatory mediators due to the action of phenolic compounds. Often mentioned, are the signaling molecules such as interleukin 6 (IL-6) and prostaglandin E2 (PGE2). The decrease expression of inflammatory mediators was evident after treatment of cells with veratric acid, phloretin dihydrochalcone, luteolin, and afzelin (Dzialo *et al.,* 2016).

Objectives

2. Objectives

Sustainability is one of the major concerns of the current world, so it is important to find new ways to reutilize materials at our disposal, and also find potential beneficial uses for some of the by-products that are less studied and cause environmental problems, our contributing to a circular, more sustainable and competitive, economic model. Following that line of thought, this work intends to find new and biological relevant uses for grape stems.

Therefore, the general objective of the present work is to evaluate the potential of grape stems to obtain isolated and purified bioactive phenolics, and to design dedicated combination with other antioxidant compounds that contribute to gain more efficient molecular tools against oxidative stress.

To achieve this general objective, it was pursued the consecution of the following specific goals:

- To identify and isolate the major phenolic compounds present in grape stems;
- Assess the isolated phenolics on the radical scavenging capacity in order to establish inhibitory concentrations compatible with *in vivo* administration;
- Test the cytotoxic effect and anti-inflammatory properties of the isolated phenolic compounds in biological systems (mouse macrophages and human keratinocytes);
- Determine the scope of combinations of purified phenolics with Vitamins E and C, concerning the capacity to prevent oxidative stress;

Evaluate the effects of the combinations of the purified phenolics with the Vitamins E and C on the apoptotic process.

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Chapter II

Evaluation of the phytochemical features of grape stems polyphenolic extracts from cultivar ‘Sousão’

Evaluation of the phytochemical features of grape stems polyphenolic extracts from cultivar 'Sousão'[†]

ABSTRACT

Viticulture represents one of the most important socio-economic activities in Portugal, as well as in the whole area of the Mediterranean Basin. From all the residues resulting from the production of wine, grape stems, which are discarded at the beginning of the vinification process, are among the residues that appear in higher quantities. This plant material represents about 25 % of the vegetal material processed by the wine industry and has the characteristic of being able to preserve all its chemical and phytochemical content. Although the grape stem is a material with a valuable phytochemical content, it continues to be one of the less studied and valued by-products. The objective of this work was to evaluate the general characteristics of grape stems (*Vitis vinifera* L.) as a source of bioactive phytochemicals, resorting to spectrophotometric determinations of the content of total phenolic compounds, *ortho*-diphenols, and flavonoids of hydromethanolic extracts from grape stems of the cultivar 'Sousão'. The results of this work show that, this cultivar represents a good choice to further studies like the isolation of the different phenolic compounds to determinate their biological effects.

Keywords: *Grape stems, phenolic compounds; spectrophotometric methods; ABTS, DPPH, 'Sousão'*

[†] ***Abbreviations:*** *ABTS, 2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt; DPPH, 2,2-diphenyl-1-picrylhidracyl radical; MO, Missouri; PVDF, Hybond®-Polyvinylidene difluoride; Dw, dry weight; GAE, gallic acid equivalents; CAT; Catechin equivalents; TE, Trolox equivalents; HPLC, High performance liquid chromatography.*

1. Introduction

The use of grapes by the winery industry represents the major utilization, reaching more than 200 Mtons per year around the world (Food and Agriculture Organization (FAO), 2010; Instituto Nacional de Estatística (INE), 2008/2009). Though the evident socio-economic advantages linked to this industrial activity, the vinification process causes a concentration of residues within a very short timeframe, from September to November, annually (Barros *et al.,* 2014). In this concern, finding new valorization procedures for these materials (grape seeds, peels, pomace, and stems) merits to be considered, towards an enhanced sustainability of the wine-making activity.

The analysis of the information available in the literature on the composition and uses of these plant materials reveals that grape stems is one of the less studied and less valorized, whilst it is mainly referred to the polyphenolic content (Souquet *et al.,* 2000). This material is removed before the vinification process to avoid an excessive astringency of the wine or a negative effect on their organoleptic characteristics (Teixeira *et al.,* 2014), and therefore keeps almost all bioactive compounds unaltered.

The evaluation of the physical and chemical characteristics of grape stems has noticed that on average the moisture percentage is between 55.0 and 80.0 %, depending of the cultivar analyzed and agro-climatic conditions (Llobera & Cañellas, 2007), informing this fact on a plant material with a high potential to yield high concentrations of bioactive phenolics.

As mentioned before, the previous characterization regarding the phytochemical composition of grape stems have pointed out their content in phenolic compounds that are mainly represented by flavan-3-ols, flavonols, stilbens, and hydroxycinnamic acids, which can reach up to 5.8 % of the dry weight (dw) (Katalinić *et al.,* 2010; Barros *et al.,* 2015). These compounds are secondary metabolites produced by plants under stressing conditions, and are synthetized by two major biosynthetic pathways, the shikimate and the acetate pathways (Herrmann, 1995; Ross & Kasum, 2002). This phenolic compounds present are responsible for a plethora of biological activities, namely anti-inflammatory, antioxidant, and anti-apoptosis activities, among others (Domínguez-Perles *et al.,* 2016).

The present chapter is focused on the preliminary evaluation of the general features of grape stems as a source of bioactive phytochemicals, resorting to spectrophotometric determinations of the content of total phenolic compounds, *ortho*-diphenols, and flavonoids of hydromethanolic extracts from grape stems of the cultivar ‘Sousão’. This

cultivar was selected, because of previous descriptions on the phenolic composition of grape stems by authors discriminating plant material from diverse cultivars traditionally grown in northern Portugal (Barros *et al.*, 2014). Besides the polyphenolic composition, its polyphenolic extracts obtained were also assessed on the radical scavenging power by using the ABTS and DPPH methods.

2. Materials and methods

2.1 Chemicals

The reagents Folin-Ciocalteau, ABTS (2,2-azino-bis(3-ethylbenzothiazoline)-6 sulphonic acid), DPPH (2,2-diphenyl-1-picrylhidracyl radical), Trolox (6-hydroxy-2,5,7,8-tetremethychroman-2-carboxylic acid), as well as the standards gallic acid and catechin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Food quality ethanol was from Panreac (Castelar del Valles, Barcelona, Spain). Inorganic membrane filters of 0.22 mm were from (ANOTOP 10 plus, Whatman, maidstone, UK). All the chemicals used were of analytical grade. Methanol was obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Plant material and extraction

Grape stems from the cultivar 'Sousão' (*Vitis vinifera* L.) were obtained from Quinta da Cavadinha (Pinhão, Portugal) in the Spring–Autumn season (2015). For analytical purposes plant material was washed in tap water and chopped into small pieces. Then, samples were dried in oven (Memert, Schwabach, Germany), at 40 ºC, for 72-hours, grounded to a fine powder and stored, protected from light and humidity, for further extraction of phenolic compounds.

Each sample (100 mg) was mixed with 1.5 mL of methanol/distilled water/formic acid (70:29:1, v/v/v). Then, samples were vortexed and phenolic compounds were extracted by sonication at room temperature (RT) for 60 min, afterwards were centrifuged at 15000 *rpm* for 5 min, at 4 ºC (Sigma, Steinheim, Germany), and the supernatant was collected. Supernatants were filtered through a 0.45-μm PVDF filter (Millex HV13, Millipore, Bedford, MA, USA) and stored at 4 ºC for spectrophotometric determinations.

2.3 Colorimetric Methods

2.3.1 Total phenolic content

The total phenolic content in the grape stems' hydromethanolic extracts was evaluated by a spectrophotometric method resorting to the Folin-Ciocalteau reagent, following the methodology previously described by Curvelo-García (1998) adapted to microplate scale (Domínguez-Perles *et al.,* 2016). Total phenolics were expressed in mg of gallic acid equivalents per g of dry weight of plant material (mg GAE/g dw).

2.3.2 Flavonoids

The flavonoids' concentration in the polyphenolic extracts was determined by the spectrophotometrical method described by Zhishem *et al.,* (1999), which is based in the complexation reaction of aluminum chloride ($AlCl_3$) with the flavonoids present in the sample. The reaction is read at 510 nm, being the absorbance directly proportional to the flavonoid concentration. For this determination 24 µL of hydromethanolic extract were added to each well, followed by addition of 28 µL of sodium nitrite (50 g L^{-1}). This mixture was leaving to react for 5 minutes and then 28 µL of aluminum chloride were added. Again, the mixture was incubated for 6 minutes at room temperature and then, 120 µL of sodium hydroxide (NaOH) (1 M) were added. The microplate was shacked for 30 seconds in the plate reader (Thermo Fisher Scientific, Lisbon, Portugal) and the absorbance was measured at 510 nm. Flavonoids were quantified resorting to catechin standard curve freshly prepared and expressed as catechin equivalents per gram of dry weight (mg CE/g dw).

2.3.3 *Ortho*-diphenols

The content in *ortho*-diphenols was determinate colorimetrically using the method adapted by Gouvinhas *et al.,* (2015). Shortly, the content in *ortho*-diphenols was evaluated, at the microplate scale, by the addition of 40 µL of sodium molybdate (Na_2MoO_4) (50 g L^{-1}) to 160 µL of the sample or standard dilution. The reaction was incubated at room temperature, protected from light, for 15 min. After the incubation, the plate was read at 375 nm (Thermo Fisher Scientific, Lisbon, Portugal). The concentration in *ortho*-diphenols was expressed in mg GAE/g dw.

2.4 Anti-radicalar activity

In order to obtain consistent information on the anti-radicalar activity of the grape stem extracts two methodologies were applied, the DPPH (determination of the neutralizing capacity of the radical DPPH$^{\bullet}$) and the ABTS (determination of the neutralizing capacity of the radical ABTS$^{\bullet+}$). The anti-radicalar capacity was quantified resorting to freshly prepared Trolox calibration curves according to Ozgen *et al*. (2006) and expressed as Trolox equivalents (*Trolox Equivalentet Antioxidant Capacity* – TEAC) per gram of dry weight (TE/g dw).

Regarding the DPPH assay, to evaluate the antioxidant capacity of the samples, the grape stem's extracts were left reacting with the stable radical DPPH$^{\bullet}$. The DPPH$^{\bullet}$ solution was featured by purple color, which disappears in the presence of an antioxidant caused by the reduction of the radical to diphenyl-picryl hydrazine. This reaction was measured at 520 nm (**Figure 2.1**).

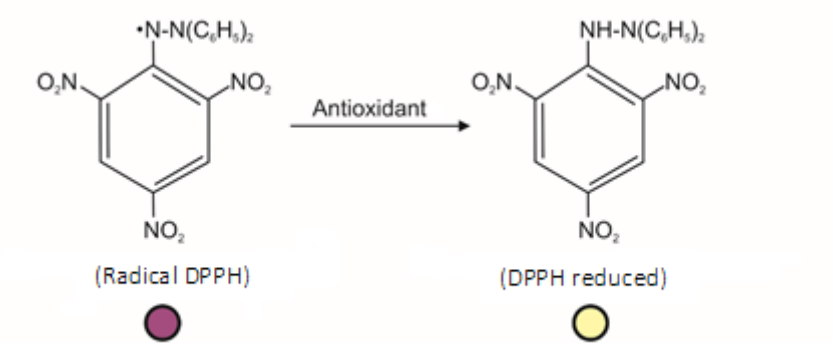

Figure 2.1. Chemical reaction of DPPH reduction.

The working solution of DPPH$^{\bullet}$ consists in the dissolution of DPPH in methanol (8.87 mM). To evaluate the antioxidant activity, an aliquot from the working solution was diluted in MeOH/H_2O (70:30, v/v) until presenting an absorbance of almost 1.000 at 520 nm wavelength. Once obtained the proper DPPH$^{\bullet}$ solution, 196 µL were mixed with 4 µL of sample. The reaction was incubated at room temperature, protected from light, for 30 min. Afterwards the absorbance was measured at 520 nm.

Concerning the ABTS method, this is based in the capacity of potassium persulfate to oxidize the radical to the cation ABTS$^{\bullet+}$, featured by a dark green color. When adding an antioxidant extract the cation ABTS$^{\bullet+}$ is reduced to $ABTS^{2-}$, which entails the discoloration of the starting solution. This reaction can be monitored spectrophometrically at 735 nm (**Figure 2.2**).

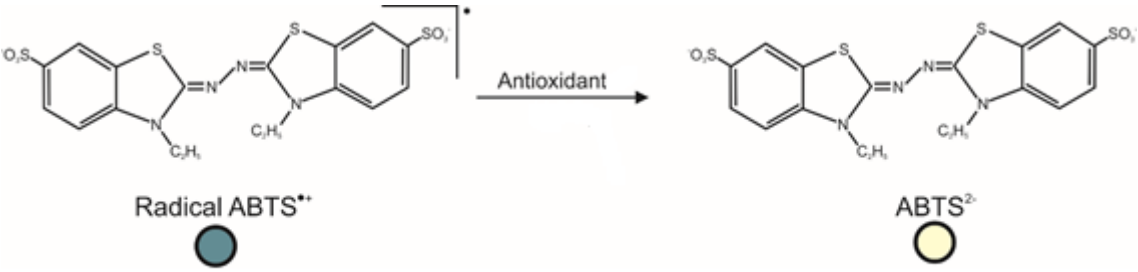

Figure 2.2. Chemical reaction of ABTS reduction by the antioxidant agent.

Based on these chemical properties, to measure the antioxidant activity using the ABTS method, it was prepared an $ABTS^{\bullet+}$ solution by adding 88 µL of a solution of 148 mM sodium persulfate (in water) to 5 mL of 7 mM $ABTS^{\bullet+}$ (in water). This mixture was stirred and hold at room temperature, protected from light, for 12-16 hours to obtain an $ABTS^{\bullet+}$ concentrated solution in a stable oxidative state. The stable $ABTS^{\bullet+}$ solution was then diluted with a 100 mM solution of sodium acetate buffer (pH 4.5) until displaying a stable absorbance of 0.70 ± 0.02 at 734 nm (working solution). The reaction was then developed by adding 12 µL of sample or standard dilution to 188 µL of the working solution. The plate was then incubated for 30 min, protected from light, at RT and finally, the absorbance was measured at 734 nm.

2.5 Statistical analysis

All measurements were performed in triplicate (n = 3) and values were expressed as media ± standard deviation. The results were subjected to an analysis of variance (ANOVA) and a multiple range test (Tukey's test) using SPSS statistic 21.0 software package (SPSS Inc., Chicago, USA).

3. Results and discussion

The polyphenolic composition of grape stems has been partially studied in the last couple of years regarding their content in total phenols, flavonoids, and *ortho*-diphenols, as well as on radical scavenging either by biochemical techniques, enzymatic models, like the activity of catalase and superoxide dismutase, or using biological models (Teixeira et al. 2014). In that way, the study of the biological activity of the whole extracts did not allow the identification of the bioactive potential of different phytochemical compounds

presents in this vegetal matrix. Furthermore, the presence of certain compounds might difficult the understanding of the true bioactive potential of the identified compounds.

To identify the potential of the stem as a source of individual phenolic compounds, using HPLC (High Performance Liquid Chromatography) in order to isolate the compounds, it is necessary to study varieties with high amounts of total phenols, flavonoids and *ortho*-diphenols. With this objective, and considering the information available regarding it phenolic composition and biological activity previously published (Barros *et al.,* 2014; Barros *et al.,* 2015) the cultivar 'Sousão' was chosen to be evaluated as a potential source of individual phenolic compounds.

3.1 Phenolic composition of the grape stems

3.1.1 Total phenolic content

The Folin reagent constitutes a mixture of phosphomolybdic acid ($H_3PMo_{12}O_{40}$) and phosphotungstic acid ($W_{12}O_{40}H_3P$), which has a yellow color in alkaline environments. This reagent, in the presence of reducing agents like the phenolic compounds, is reduced to oxides of molybdenum (Mo_8O_{23}) and tungsten (W_8O_{23}), which results in the formation of blue complexes that can be monitored spectrophotometrically at the wavelength of 750 nm (Magalhães *et al.,* 2008). At this wavelength, the absorbance is expected to increase with the concentration of the phenolic compounds.

The application of this technique informed on the concentration of total phenolics in hydromethanolic extracts of grape stems of 'Sousão' as being, 56.3 mg GAE/g dw, on average (**Figure 2.3**).

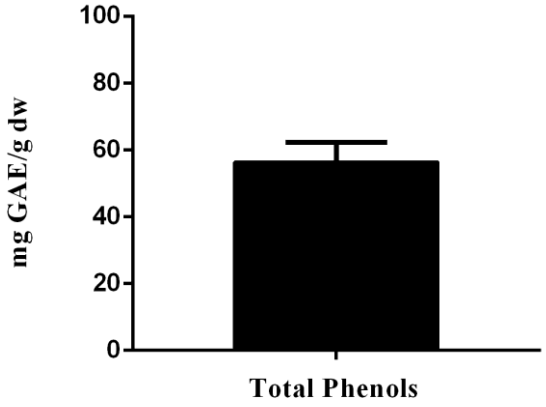

Figure 2.3 Content in total phenols in the grape stem extracts. (n=3)

These results, corresponding to plant material harvested in 2015, agreed with those previously described by Barros *et al.*, (2014) that reported a concentration around 55.0 mg GAE/g dw in grape stems from the same cultivar in the 2013 harvest. The reproduction of concentration in almost equal level inform on the correct development of the optimized technique as well as on the similar level of production phenolics, in the analyzed years. Similar results were found by Makris *et al.*, (2007) using the white grape cultivar Roditis (*Vitis vinifera* sp.), in these study the values of total phenols were 5798 mg GAE per 100g of dry grape stem.

In addition, having into consideration the different climatic conditions in the years 2013 and 2015, the coincidence observed regarding the content of phenolic compounds, suggest a weak impact of fluctuating climatic conditions on the polyphenolic content. Interestingly, this fact also indicates that grape stems may constitute a consistent source of bioactive compounds.

3.1.2 Flavonoids

Regarding the concentration of flavonoids in grape stems' polyphenolic extracts, the analysis performed evidenced an average of 29.5 mg CE/g dw (**Figure 2.4**).

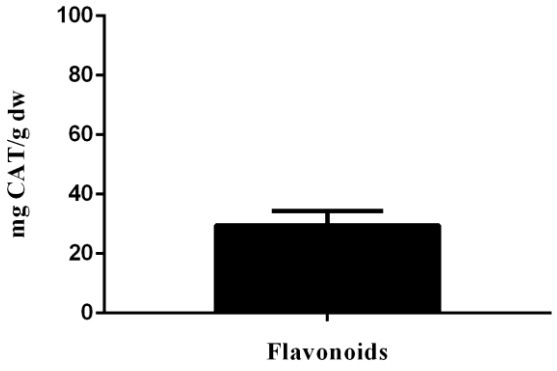

Figure 2.4 Content of flavonoids in the grape stem extracts. (n=3)

As said before, in the total phenols, the results from the flavonoid content in the extracts of grape stems agree with those described by Barros *et al.*, (2014), which indicate once again the correct development of the determination assays, as well as the consistency of the polyphenolic content of grape stems from plants grown under variable climatic conditions. This results also evidence that this cultivar present higher values than other extracts from different types of cultivars, especially stems from grapes of white cultivars.

3.1.3 *Ortho*-diphenols

Concerning the content in *ortho*-diphenols, it was found that the stems of this grape variety had an average of 89.1 mg GAE/g dw (**Figure 2.5).**

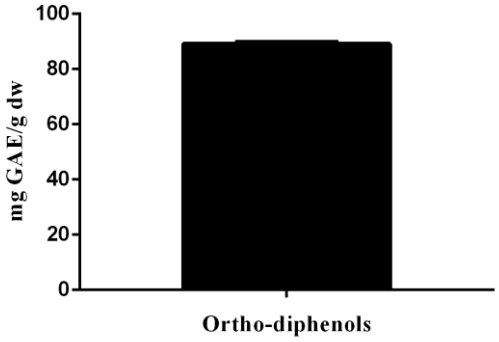

Figure 2.5 Content in *ortho*-diphenols do in the grape stems extracts (n=3)

Once again this result evidenced that the grape stems constitutes a plant material with a high content in phenolic compounds that can have potential biological uses (Barros et al. 2015). The content in *ortho*-diphenols was the highest among the analysis, which was the expected, having in consideration the data available in the literature (Apostolou *et al.*, 2013).

3.2 Anti-radicalar activity of the grape stems' polyphenolic extracts

3.2.1 DPPH• radical scavenging capacity

The evaluation of the anti-radicalar capacity of the grape stems extracts evidenced that they present an anti-radicalar activity of 1.6 micromol Trolox/g dw, on average (**Figure 2.6**).

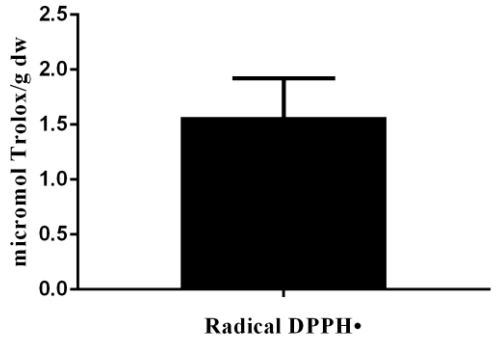

Figure 2.6 DPPH• radical scavenging power of the grape stem extracts. (n=3)

The DPPH based antiradical activity, which has been attributed to the phenolic compounds present in the grape stems, is again in agreement with the range of values described by Barros *et al.,* (2014) referent to the same analysis. This results show that this cultivar shows interesting levels of anti-radicalar capacity when compared with other cultivars especially stems from white varieties, and evidences that this sub-product have an interesting potential as a source of anti-radicalar activities (Llobera *et al.,* 2008; Apostolou *et al.,* 2013).

3.2.2 $ABTS^{\bullet+}$ radical scavenging capacity

In what regards the neutralization capacity of the $ABTS^{\bullet+}$ radical, it was verified that the anti-radicalar activity obtained using this method is 0.6 micromol TE/g dw (**Figure 2.7**).

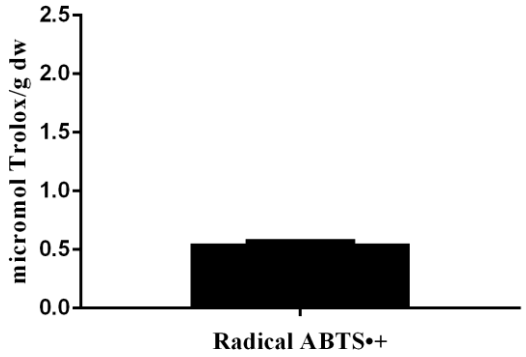

Figure 2.7 $ABTS^{\bullet+}$ radical scavenging power of the grape stem extracts. (n=3)

This once more is a clear consequence of the content in phenolics of the stems extracts. The results of the neutralization capacity of the $ABTS^{\bullet+}$ radical showed values that agree with what is shown by Apostolou *et al.,* (2013) relatively to the grape stems of several cultivars. In that work is shown that for grape stem extracts from V. *vinifera* have a stronger antioxidant potential than extracts from seeds (Apostolou *et al.,* 2013).

4. Conclusions

The results obtained on the polyphenolic composition of grape stems' extracts and the radical scavenging activity of all the analysis have shown that stems from the cultivar 'Sousão' present a higher and constant content in total phenols, flavonoids, and *ortho*-diphenols than other cultivars especially when compared with withe types of grapes. The

measurements obtained for the antiradical activities also shown that these extracts have a potential antioxidant capacity.

Therefore, this cultivar represents a good choice to further studies like the isolation of the different phenolic compounds to determinate their biological effects as well as to find which ones are more responsible for the antioxidant activity shown by the grape stems extracts of this cultivar.

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Chapter III

Vitamins C and E as selected boosters to phenolics against oxidative stress: An effect depending on lowering intracellular ROS, GSH and lipid peroxidation

Vitamins C and E as selected boosters to phenolics against oxidative stress: An effect depending on lowering intracellular ROS, GSH, and lipid peroxidation[†]

ABSTRACT

The antioxidant potential of phenolic extracts from grape (*Vitis vinifera* L.) stems have been widely reported in the last decade, whilst there is a lack of information on the potential of their individual compounds and the establishment of improved combinations with also antioxidant compounds. In this work, polyphenolic extract of grape stems was processed resorting to semi-preparative HPLC-UV-*vis*, allowing to obtain purified phenolics (isorhamnetin-3-*O*-(6-*O*-feruloyl)-glucoside, caftaric acid, malvidin-3-*O*-glucoside, quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucuronide, kaempferol-3-*O*-rutinoside, kaempferol-3-*O*-glucoside, malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside, malvidin-3-*O*-rutinoside, and Σ-viniferin) which were fully characterized by HPLC-PDA-ESi-MSn. Isolated and purified compounds were featured on their radical scavenging capacity (DPPH and ABTS) in order to establish the inhibitory concentration 50 (IC_{50}). Those compounds on which could be established the IC_{50} according to their radical scavenging power (malvidin-3-*O*-glucoside (0.41 µmol/L), quercetin-3-*O*-glucuronide (1.25 µmol/L), and malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside (0.43 µmol/L)) were further tested on cell viability, anti-inflammatory activity, and capacity to modulate

[†] ***Abbreviations:*** *AB, Alamar Blue®; ABTS, 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)diammonium salt; annexin-V-FITC, annexin-V-fluoresceina; ANOVA, analysis of variance; DCFDA, 2',7'-dichlorofuorescein diacetate; DHPE-FITC, (N-(Fluorescein-5-Thiocarbamoyl)-1.2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine); DM, dry matter; DMEM, Dulbeco's modified Eagle's medium; DPPH, 2,2-diphenyl-1-picrylhidracyl radical; FBS, fetal bovine serum; GSH, gluthatione; HBSS, Hank's balanced salt solution; HPLC-PDA-ESi-MSn, high performance liquid chromatography-photodiode array-electrospray-mass spectrometry; LP, lipid peroxidation; LPS, lipopolysaccharide; PI, propidium iodide; preparative-HPLC-UV-vis, preparative high performance liquid chromatography-ultraviolet visible; NO, nitrite; ROS, reactive oxygen species; SPSS, statistical package for the social services; TPTZ, 2,4,6-Tris(2-pyridyl)-s- triazine; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Trypsin-EDTA, trypsin-Ethylenediaminetetracetic acid.*

oxidative stress markers and overall oxidative stress in biological (*in vitro*) model (HaCaT cells – human keratinocytes) in basal and oxidative environments. The results obtained through the plethora of determinations developed allowed to identify the combinations malvidin-3-*O*-glucoside + Vitamin E and quercetin-3-*O*-glucuronide + vitamin C as the most valuable most effective, allowing to improve the biological activities exerted by complete extracts or individual compounds, indicating beneficial cellular protection against oxidative stress and being candidates to be used in the development of new functional products.

Keywords: *Grape stems' phenolics, preparative-HPLC, vitamin E, vitamin C, inflammation, oxidative stress*

1. Introduction

Cells are exposed to diverse deleterious factors affecting the redox balance and thus the aging process in humans. In this connection, skin is the outmost cell layer and its cellular constituents are exposed to the harmful effects of ultraviolet radiation and exogenous chemicals, which cause detrimental skin effects that can result in inflammation, premature skin aging, and malignant processes (Melnikova & Ananthaswamy, 2005; Dzialo *et al.*, 2016). These are initiated by an increased production of reactive oxygen species (ROS) and oxidative stress, which has been highlighted as crucial actors in the initiation of signaling events responsible for the cellular reply to damaging stimuli (Dzialo *et al.*, 2016).

The replacement of drugs by natural compounds is now being re-evaluated resorting to extensive research on different plant materials and their bioactive compounds, especially regarding the occurrence of radical scavenging molecules capable to modulate oxidative stress' severity. In this concern, new antioxidant formulations may be developed, helping to decrease oxidative damage and giving response to current claims for more effective treatments against oxidative stress (Biswas, Haldar, & Ghosh, 2010).

In the last decades, several works have stressed the powerful capacity of phenolic compounds, which are relevant secondary plant metabolites synthesized through the pentose phosphate, shikimate, and phenylpropanoid pathways (Balasundrama *et al.*, 2006), regarding the prevention or attenuation of skin disorder symptoms (Wittenauer *et al.*, 2015; Karim *et al.*, 2014; Danciu *et al.*, 2015). However, the diverse phenolic compounds present in edible plant materials and residues of the agro-food industry display variable biological properties concerning both type of activity and intensity depending on chemical structure (Del Rio *et al.*, 2013).

The use of agro-food by-products as a source of bioactive phenolics would contribute to reduce the competition between distinct economic activities, boosting the competitiveness of the agro food sector and contributing to a more sustainable and circular economic model. In this sense, in northern Portugal the winery industry is one of the most relevant socio-economic activity, which entails the production of a huge amount of solid residues, with an interesting potential as a source of bioactive phytochemicals (Teixeira *et al.*, 2014; Barros, Gironés-Vilaplana, Teixeira, Baenas, & Domínguez-Perles, 2015). From winery by-products, grape (*Vitis vinifera* L.) stems have been exhaustively evaluated by our group over the last years concerning the phenolic composition and biological activity (Domínguez-Perles, Teixeira, Rosa, & Barros, 2014; Barros *et al.*,

2014; Domínguez-Perles, Guedes, Queiroz, Silva, & Barros, 2016). Data obtained throughout these previous works encourage the use of this material as a source of individual compounds with valuable biological activity against oxidative stress. According to the study of whole antioxidant extracts and the establishment of concentration-activity correlations, caftaric acid, isorhamnetin-3-*O*-(6-*O*-feruloyl)-glucoside, quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucuronide, kaempferol-3-*O*-rutinoside, malvidin-3-*O*-gluoside, the coeluted anthocyanins malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside and malvidin-3-*O*-rutinoside, and Σ-viniferin have been emphasized as the most hopeful compounds on this biological function (Domínguez-Perles *et al.*, 2016).

Besides the evaluation of isolated compounds with interesting biological activity, the design and development of new formulations requires the establishment of optimal combinations with well reputed adjuvants on antioxidant activities, competent to provide additional advantages concerning the biological activity foreseen (Shehata & Kamel, 2008).

In order to advance towards formulations suitable to be used by the cosmetic industry in the development of new products capable to protect skin cells against environmental insults, the present work pursues the isolation of the most abundant phenolic compounds by semi-preparative-HPLC. The obtained compounds were assessed on their radical ($ABTS^{\bullet+}$ and $DPPH^{\bullet}$) scavenging capacity and bioactivity against oxidative stress by assessing their capacity to modulate cellular levels of glutathione (GSH), ROS, lipid peroxidation (LP), and general oxidative stress in biological systems under basal conditions and H_2O_2-induced oxidative environments. The adjuvant capacity of vitamins C and E concerning the biological activities under study was also determined.

2. Material and methods

2.1. Chemicals

The compounds 2,2-diphenyl-1-picrylhidracyl radical ($DPPH^{\bullet}$), 2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt ($ABTS^{\bullet+}$), 2,4,6-Tris(2-pyridyl)-*s*- triazine (TPTZ), monobasic sodium phosphate, dibasic sodium phosphate, trizma® hydrochloride, potassium phosphate, annexin V-FITC, Mercury Orange, 2',7'-dichlorofuorescein diacetate (DCFDA), and propidium iodide (PI) were obtained from Sigma-Aldrich (Steinheim, Germany). Meanwhile, 6-hydroxy-2,5,7,8-

tetramethylchroman-2-carboxylic acid (Trolox) and magnesium chloride hexahydrate were purchased from Fluka Chemika (Neu-Ulm, Switzerland). Ultrapure water was produced using a Millipore water purification system (Millipore, Bedford, MA, USA). The Alamar Blue® (AB) reagent was purchased from Invitrogen (Life-Technologies, Oporto, Portugal). Trypsin-EDTA, Hank's balanced salt solution (HBSS), Dulbeco's modified Eagle's medium (DMEM), L-glutamine, fetal bovine serum (FBS), penicillin/streptomycin, glycine, sodium pyruvate, and (*N*-(Fluorescein-5-Thiocarbamoyl)-1.2-Dihexadecanoyl-*sn*-Glycero-3-Phosphoethanolamine) (DHPE-FITC) were purchased from Gibco (Life-Technologies, Oporto, Portugal). The flat bottom 96-well plates were from Corning (New York, USA).

2.2. Extraction and isolation of grape stems' phenolics

Grape (*Vitis vinifera* L.) stems from the variety 'Sousão' were obtained from Quinta da Cavadinha (Pinhão, Portugal) in the Spring–Autumn season (2015). For analytical purposes plant material was washed in tap water and chopped into small pieces. Then, samples were dried in oven (Memert, Schwabach, Germany) at 40 ºC for 72-hours, grounded to a fine powder, and stored protected from light and humidity for further extraction of phenolic compounds. Each sample (100 mg) was mixed with 1.5 mL of methanol/distilled water/formic acid (70:29:1, v/v/v). Then, samples were vortexed and phenolic compounds were extracted by sonication at RT for 60 min, afterwards were centrifuged at 20627*g* for 5 min, at 4ºC (Sigma, Steinheim, Germany), and the supernatant was collected. Supernatants were filtered through a 0.45-µm PVDF filter (Millex HV13, Millipore, Bedford, MA, USA) and stored at 4 ºC until isolation of individual phenolics compounds by preparative HPLC.

From the previously prepared polyphenolic extracts, the major individual phenolic compounds were isolated resorting to a ACE C18 column (250 x 4.6 mm i.d., 5 µm particle size; ACE, Aberdeen, Scotland), using distilled water/TFA (99.9:0.1, v/v) (solvent A) and acetonitrile/TFA (99.0:0.1, v/v) (solvent B) in the linear gradient scheme (t in min; %B): (0; 5 %), (15; 15 %), (30; 30 %), (40; 50 %), (45, 100 %), (55, 100 %), and (58; 5 %). The flow rate and injection volume were 1 mL/min and 200 µL, respectively. Fractionation was performed with a Gilson 322 Controller and pump connected to a Gilson GX-271 Liquid Handler. Chromatograms were recorded with a Gilson 157 UV-*vis* Detector and visualized with the TRILUTION LC 3.0 Service Pack 4

4.0.11.1 software. Fractionation was performed with the Gilson GX-271 Liquid Handler. All Gilson instruments were supplied by Gilson International B.V. (Madrid, Spain).

Fractions were collected when peak front slope reached 87 % till a peak back slope of 60 % into 10-mL crystal tubes. The column eluent was monitored at 320, 370, and 520 nm for phenolic acids, flavonols, and anthocyanins, respectively, and fractionation was performed at RT. A total of 10 x 200 µL injections were fractionated. Isolated compounds with matching retention time and UV-vis spectra were pooled and stored at 4ºC until HPLC-PDA-ESi/MSn analysis and freeze drying.

2.3. HPLC-PAD-ESi-MSn analysis of isolated compounds

Chromatographic analyses for the identification were carried out on a ACE C18 column (250 x 4.6 mm i.d., 5 µm particle size; ACE, Aberdeen, Scotland). Water/formic acid (99:1, v/v) (solvent A) and acetonitrile/formic acid (99:1, v/v) (solvent B) were used for chromatographic separation in an Thermo Finnigan Surveyor HPLC system composed of a photodiode array detector and a mass detector in series (Thermo Fisher Scientific, Inc., Waltham, USA) with the same conditions described by Gironés-Vilaplana, Mena, Moreno, and García-Viguera (2013). Briefly, the flow rate was 1 mL min−1 using the linear gradient scheme (t in min; % B): (0; 5 %), (15; 15 %), (30; 30 %), (40; 50 %), (45, 95 %), and (50; 5 %). The equipment consisted of a Thermo Finnigan Surveyor quaternary LC pump, auto sampler, degasser and a photodiode array detector. The HPLC system was controlled by Xcalibur software (Thermo Electron Corporation, version 1.3). For the quantification, the HPLC–DAD system was used, as previously described (Gironés-Vilaplana *et al.*, 2013), using chromatographic conditions described for identification. Flavonols and flavones were quantified as quercetin-3-*O*-glucoside at 360 nm, hydroxycinnamic acids as 5-*O*-caffeoylquinic acid at 320 nm, anthocyanins as cyanidin-3-*O*-glucoside at 520 nm, and stilbenes as resveratrol at 280 nm.

Once identified and quantified, fractions (2 mL) were frozen at -80 ºC and freeze-dried on a Benchtop Pro with Omnitronics^{TM} System from SP SCIENTIFIC (Ipswich, UK) coupled to a TRIVAC Vane pump (Oerlikon Leybold Vaccum; Cologne, Germany). The freeze-dried fractions were stored at -80 ºC until chromatographic analysis and assessment of the biological (radical scavenging and decrease of cellular oxidative stress activity). For bioassays, fractions were prepared as 1 mg/mL stock solutions in distilled

water, filtered through a 0.22-µm PVDF filter (Millex HV13, Millipore, Bedford, MA, USA), and stored at -80 ºC.

2.4. DPPH• and ABTS•+ scavenging capacity

The free radical scavenging activity was determined by DPPH• and ABTS•+ methods adapted to a micro-scale. The antioxidant activity was evaluated by measuring the variation in absorbance at 515 nm after 50 min of reaction with the radical for DPPH• and at 414 nm after 50 min for ABTS•+, using 96-well microplates and Multiscan FC microplate reader (Thermo-Fisher Scientific, Oporto, Portugal). The results on the radical scavenging capacity were expressed as µmoles TE/mL.

To evaluate the antioxidant capacity of the grape stem samples, they were leaving to react with the radical DPPH• in a methanolic solution. The DPPH•, which is purple, in the presence of an antioxidant is reduced to diphenyl-picryl-hydrazine, which does not present any color (**Figure 3.1**).

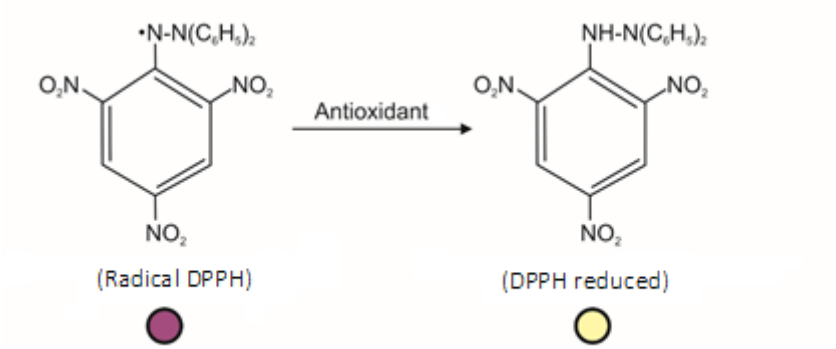

Figure 3.1 Chemical reaction of DPPH reduction.

To have a calibration curve, six concentrations of the Trolox standard (2500, 1250, 625, 313, 156 e 78 µM) allowing to obtain the calibration curve: '*y = 7603.9*x*', with a R^2= 0.9955.

For the development of the anti-radicalar activity relative to DPPH• on the microplate scale, was added in each well 196 µL of the DPPH• solution (8.87 nM with absorbance of 1.000 at 520 nm) and 4 µL of each sample. After that the reaction is leaved for incubation at room temperature and protected from light for 30 minutes. In the end the absorbance readings are made at 520 nm in a microplate spectrophotometer (Thermo Fisher Scientific, Lisbon, Portugal).

The percentage of inhibition for each sample is calculated by the following formula: '*% Inhibition* $_{SAMPLE}$ *= 100 x (*$Abs520_{BLANK}$ *–* $Abs520_{SAMPLE}$*) /*$Abs520_{BLANK}$*)* '. The blank valor is the absorbance of 196 µL of DPPH$^{\bullet}$ solution 8.87 nM with absorbance of 1.000 at 520 nm) and 4 µL of methanol/water (70:30 v/v).

For interpolation of the standard curve was determinate the antioxidant activity. The determination of each sample was made in triplicate (n=3). The results were expressed in nM TE/g dw.

To have the neutralization capacity of the $ABTS^{\bullet+}$ radical, is added potassium persulfate ($K_2S_2O_8$) to the $ABTS^{\bullet+}$ radical, that have a dark green color. This mixture will reduce the $ABTS^{\bullet+}$ cation to $ABTS^{2-}$, which lead to a discoloration of the initial solution (**Figure 3.2**)

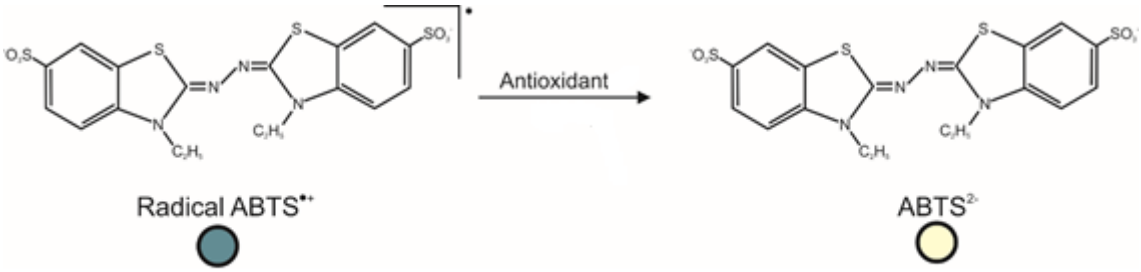

Figure 3.2. Chemical reaction of ABTS reduction by the antioxidant agent.

To create a calibration curve, eight concentrations of Trolox were used (1000, 500, 250, 125, 63, 31, 16 and 8 µM), allowing to have the following curve: '*y=83.719*x*', with a R^2 = 0.9952.

To evaluate the anti-radicalar capacity related to the ABTS radical in microplates, was added to each well 188 µL of the ABTS working solution (7 nM) and 12 µL of each sample and Trolox concentration. In the end the microplate was leaved 30 minutes protected from light. In the end the microplate was read at 734 nm in a microplate spectrophotometric reader (Thermo Scientific, Lisbon, Portugal).

The percentage of inhibition to each standard was calculated by the following formula: '*% Inhibition* $_{SAMPLE}$ *= 100 x (*$Abs734_{BLANK}$ *–* $Abs734_{SAMPLE}$*) /* $Abs734_{SAMPLE}$*'*, where $Abs734_{BLANK}$ corresponds to the absorbance of 188 µL of $ABTS^{\bullet+}$ plus 12 µL of distillated water.

By interpolation of the calibration curve the antioxidant activity of the samples was calculated. The determination of each sample was made in triplicate (n=3). The final results were expressed in mM TE/g dw.

2.5. Cell culture and cell viability measurement

The adherent human keratinocytes cell line, HaCaT, Cell Lines Services (CLS) Eppelheim, Germany) (Boukamp *et al.*, 1988), was cultured in flasks (Orange Scientific, Frilabo, Portugal), by maintaining them in DMEM culture medium, containing 25 mM glucose, supplemented with 10 % FBS (v/v), 2 mM L-glutamine, and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin), at 37 ºC in an atmosphere of 5 % CO_2 in air with controlled humidity. When confluent, cells were detached with Trypsin-EDTA at working concentration. The reaction was stopped with supplemented culture medium, and cells were resuspended at a density of 5.0×10^4 cells/mL and plated in 96-well culture plates (100 µL/well). Cells were allowed to stabilize for 24 h. Afterwards culture media was removed and cells were incubated with fresh medium (control), or fresh medium containing vitamin C (0.200 µmol/L), vitamin E (0.200 µmol/L), whole phenolic extract (2 µmol/L), or the isolated phenolic compounds malvidin-3-*O*-glucoside, quercetin-3-*O*-glucuronide, and malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside (at the half maximum inhibitory concentration (IC_{50}); 0.410, 1.250, and 0.432 µmol/L, respectively).

The cytotoxic effect of the isolated compounds on keratinocytes (HaCaT cell line) was assessed by the Alamar Blue® assay according to the methodology previously reported (Domínguez-Perles *et al.*, 2016).

2.6. Anti-inflammatory activity

The anti-inflammatory power of grape (*Vitis vinifera* L.) stems' complete polyphenolic extract and isolated phenolic compounds was determined on RAW 264.7 macrophage cell line. The cell line (RAW 264.7) was acquired from Cell Lines Service (CLS, Eppelheim, Germany). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum and antibiotics (100 U/mL of penicillin and 100 µg/mL of streptomycin), and maintained at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2.

Cells were detached from the culture flasks, counted, diluted at density of 2.0×10^5 cells/mL, and plated in 96-well culture plates (100 µL/well). Cells were allowed

to stabilize for 24 h. Afterwards culture media was removed and cells were incubated during 24 h with fresh medium (control), or fresh medium supplemented with the antioxidant extract/compounds (whole phenolic extract (2.000 µmol/L), isolated phenolic compounds (malvidin-3-*O*-glucoside, quercetin-3-*O*-glucuronide, and malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside (at the average IC_{50})), vitamin C (0.200 µmol/L), and vitamin E (0.200 µmol/L)) and 1 µg/mL lipopolysaccharide (LPS) as inflammatory stimuli. The nitrite (NO) production was measured by the colorimetric reaction with the Griess reagent, which detects accumulated nitrites in the cells supernatants. Briefly, 50 µL of supernatant (culture medium) of each well was diluted with 50 µL of Griess reagent (0.1% (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride and 1% (w/v) sulfanilamide prepared in 5.0% (w/v) H_3PO_4 (v/v)) and maintained for 30 min, in the dark, before the absorbance reading at 550 nm using a Multiskan EX microplate reader (MTX Labsystems, USA). Percentage of nitrite production by cells cultured exposed to LPS was quantified by freshly prepared nitrite standard curves (0 to 100 µM).

2.7. Flow cytometry analysis of oxidative stress markers

Oxidative stress markers (GSH, intracellular ROS, and lipid peroxidation) were monitored resorting to one-color and two-colors flow cytometry, which were carried out on using a BD Accuri™ C6 cytometer (Becton Dickinson, CA, USA). Five thousand gated events were collected from each sample and analyzed using BD Accuri™ C6 Software (Becton Dickinson, CA, USA).

For cytometric determinations, HaCaT cells ($3.0x10^5$ cells/mL) were cultured in 6-wells culture plates. After 24 hours, culture media was removed and cells were exposed to FBS-free culture media supplemented with grape stems' polyphenolic extracts (2.000 µmol/L), isolated phenolic compounds (IC_{50}), vitamins C (0.200 µmol/L) and E (0.200 µmol/L), and dedicated combinations. Thirty-six hours later, control and exposed cells were harvested using Trypsin-EDTA detachment solution (~10 minutes, 30 ºC).

The induction of oxidative stress was developed by exposing cells to 50 µM H_2O_2. After 1 h, samples were centrifuged at 500 *g* for 5 min, at 4 ºC. Then, supernatants were removed, cells were resuspended in 100 µL PBS and stained of the separate markers of oxidative stress and apoptosis monitored.

The quantification of GSH, ROS, and LP level was developed according to the procedures previously described (Domínguez-Perles *et al.,* 2014).

2.8. Statistical analyses

All measurements were performed in triplicate (n = 3) and values were expressed as media ± standard deviation. The results were subjected to an analysis of variance (ANOVA) and a multiple range test (Tukey's test) using SPSS statistic 21.0 software package (SPSS Inc., Chicago, USA). Significant differences between the biological activity of the compounds and combinations assessed were set at $p < 0.05$.

3. Results and discussion

3.1. Semi-preparative-HPLC isolation of phenolic compounds of grape stems

The phenolic composition of the whole grape stems extract determined by HPLC-PDA-ESi/MSn noticed a profile according to previous descriptions available in the literature (Barros *et al.*, 2014), being the most abundant compounds identified isorhamnetin-3-*O*-(6-*O*-feruloyl)-glucoside, caftaric acid, malvidin-3-*O*-glucoside, quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucuronide, kaempferol-3-*O*-rutinoside, kaempferol-3-*O*-glucoside, mailvidin-3-*O*-(6-*O*-caffeoyl)-glucoside, malvidin-3-*O*-rutinoside, and Σ-viniferin (**Table 3.1**).

All identified compounds were isolated by semi-preparative HPLC, and fractions with matching retention time, and UV-*vis*, and MS spectra were grouped. Afterwards, the isolated compounds were tentatively identified resorting to their UV-*vis* features, mass spectra, and fragmentation patterns in careful comparison with previously reported data (Domínguez-Perles *et al.*, 2014; Hisamoto, Muneda, & Okuda, 2011; Anastasiadi, Pratsinis, Kletsas, Skaltsounis, & Haroutounian, 2012).

Table 3.1
Identification of the individual phenolic compounds in grape (Vitis vinifera L.) stems by HPLC-PDA-ESi/MSn in negative/positive

Peak	*Compound*	*Rt (min)*	*λ UV-vis (nm)*	*$[M-H]^-$ / MS^2 m/z*	*$[M-H]^+$ / MS^2 m/z*
1	Isrhm-3-*O*-(6-*O*-feruloyl)-Glc[Z]	4.1	274, 320	653/315	-
2	Caftaric acid	4.8	300, 330	311/179	-
3	Mv-3-*O*-Glc	24.7	288, 526	-	493/331
4	Q-3-*O*-Rut	26.2	260, 360	609/301	-
5	Q-3-*O*-Gluc	27.5	258, 354	477/301	-
6	K-3-*O*-Rut	27.9	274, 345	593/385	-
7	K-3-*O*-Glc	29.4	282, 345	447/285	-
8	Mv-3-*O*-(6-*O*-caffeoyl)-Glc[Y]	33.5	284, 532	-	655/331
9	Mv-3-*O*-Rut[Y]	33.5	284, 532	-	637/331
10	Σ-viniferin	38.3	310, 325	453/347	-

[Z] *Glc, glucoside; Gluc, glucuronide; Ishrm, isorhamnetin; K, kaempferol; Mv, malvidin; Q, quercetin; Rut, rutinoside.* [Y] *Mv-3-O-(6-O-caffeoyl)-Glc and Mv-3-O-Rut coeluted.*

All compounds isolated with purities higher than 95.0 % according to the integral of the peak areas determined by HPLC-DAD (caftaric acid, malvidin-3-*O*-glucoside, quercetin-3-*O*-glucuronide, malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside, and Σ-viniferin) were chosen out as candidate compounds for the evaluation of the radical scavenging activity. The selected phenolics are indicated in **Figure 3.3**, along with their fragmentation pattern and chemical structure. Although these five phenolics were obtained in a degree of purity equal or higher than 95.0 %, as expected not all were obtained in the same ratio relative to the amount of dry matter processed. Thus, the highest yield corresponded to caftaric acid and quercetin-3-*O*-glucuronide (41.7 and 37.5 μg/g dw, respectively), whilst malvidin-3-*O*-glucoside, malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside, and Σ-viniferin accounted for 9.7 μg/g dw, on average).

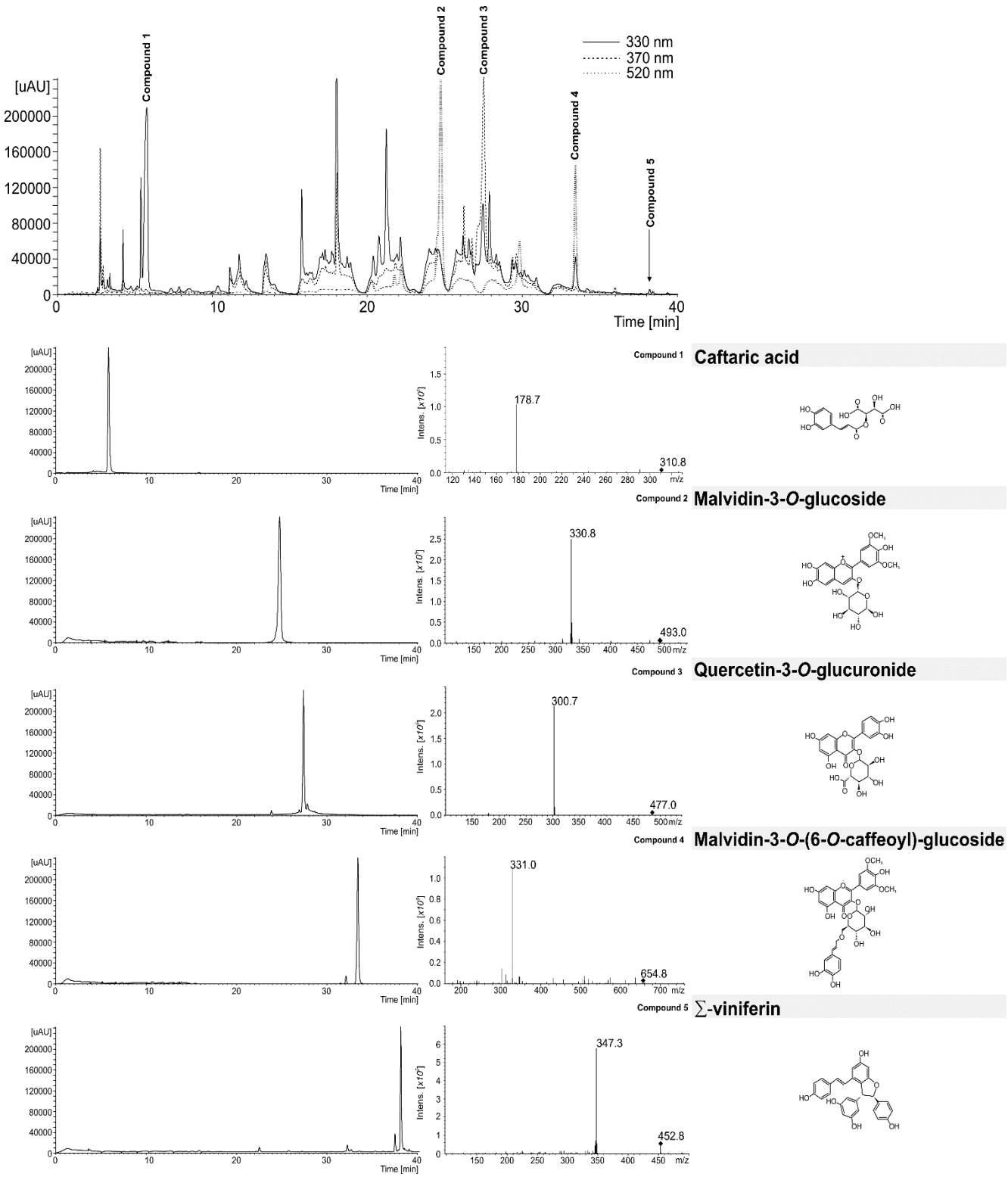

Fig. 3.3. Chromatograms corresponding to whole grape stems extracts (recorded at 330, 270, and 520 nm) and individual compounds isolated by semi-preparative-HPLC: caftaric acid (320 nm), quercetin-3-*O*-glucuronide (370 nm), malvidin-3-*O*-gluoside (520 nm), malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside (520 nm), and Σ-viniferin (320 nm). Mass spectra of the isolated compounds.

3.2. Radical scavenging power of whole extracts, isolated compounds, and vitamins C and E

The radical scavenging capacity of the isolated compounds was evaluated through monitoring their capacity to react with either $DPPH^{\bullet}$ and $ABTS^{\bullet+}$. In addition to the isolated compounds, the whole phenolic extracts and vitamins C and E (envisaged in the present work as feasible boosters of the antioxidant activity to individual phenolics) were also assessed on their scavenging power against $DPPH^{\bullet}$ and $ABTS^{\bullet+}$. Thus, the determination of the capacity to decrease in 50% the initial concentration $DPPH^{\bullet}$ (IC_{50}) of decreasing concentrations (2.00, 1.00, 0.50, 0.25, and 0.13 µM) of the whole extracts (quantified as equivalents of gallic acid) and isolated compounds under evaluation evidenced that three out of the five compounds tested were highly efficient in scavenging $DPPH^{\bullet}$ and $ABTS^{\bullet+}$.

Total phenolic extracts, and vitamins C and E were considered controls that allowed a further understanding on the actual scope of the antioxidant potential of individual phenolics. Hence, the analysis of the differential antioxidant capacity of grape stem extracts and their individual phenolics, as well as of vitamins C and E, provided valuable information to understand the relative contribution of the different constituents of grape stems to this biological function. In addition, the information retrieved from these determinations informed on the suitability to develop proper combinations of compounds with increased antioxidant activity that would be further tested through biological models. In this concern, the reduction of the DPPH and ABTS absorbance indicates the capacity to scavenge free radicals, thus, contributing to the maintenance of a proper redox balance (Domínguez-Perles *et al.,* 2016)

In which concern to DPPH, the colored flavonoids (anthocyanins) malvidin-3-*O*-glucoside and malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside were the most efficient compounds exerting the inhibitory ranges 89.7-21.8 % and 82.7-18.9 %, respectively, which corresponded to IC_{50} of 0.500 and 0.450 µmol/L, respectively (Fig. 4). Quercetin-3-*O*-glucoside also presented a high $DPPH^{\bullet}$ inhibitory power with values ranging from 53.3 to 20.0 %, which by integrating provided an IC_{50} of 1.750 µmol/L (Fig. 4).

The results obtained by analyzing the $ABTS^{\bullet+}$ scavenging capacity agreed with those from the DPPH assay, informing on the ranges of inhibition 93.7-19.5 %, 75.3-5.5 %, and 91.1-6.3 % for malvidin-3-*O*-glucoside, malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside, and quercetin-3-*O*-glucoside, respectively. These inhibition ranges allowed to calculate IC_{50} of 0.320, 0.413, and 0.750 µmol/L, respectively (**Figure 3.4**).

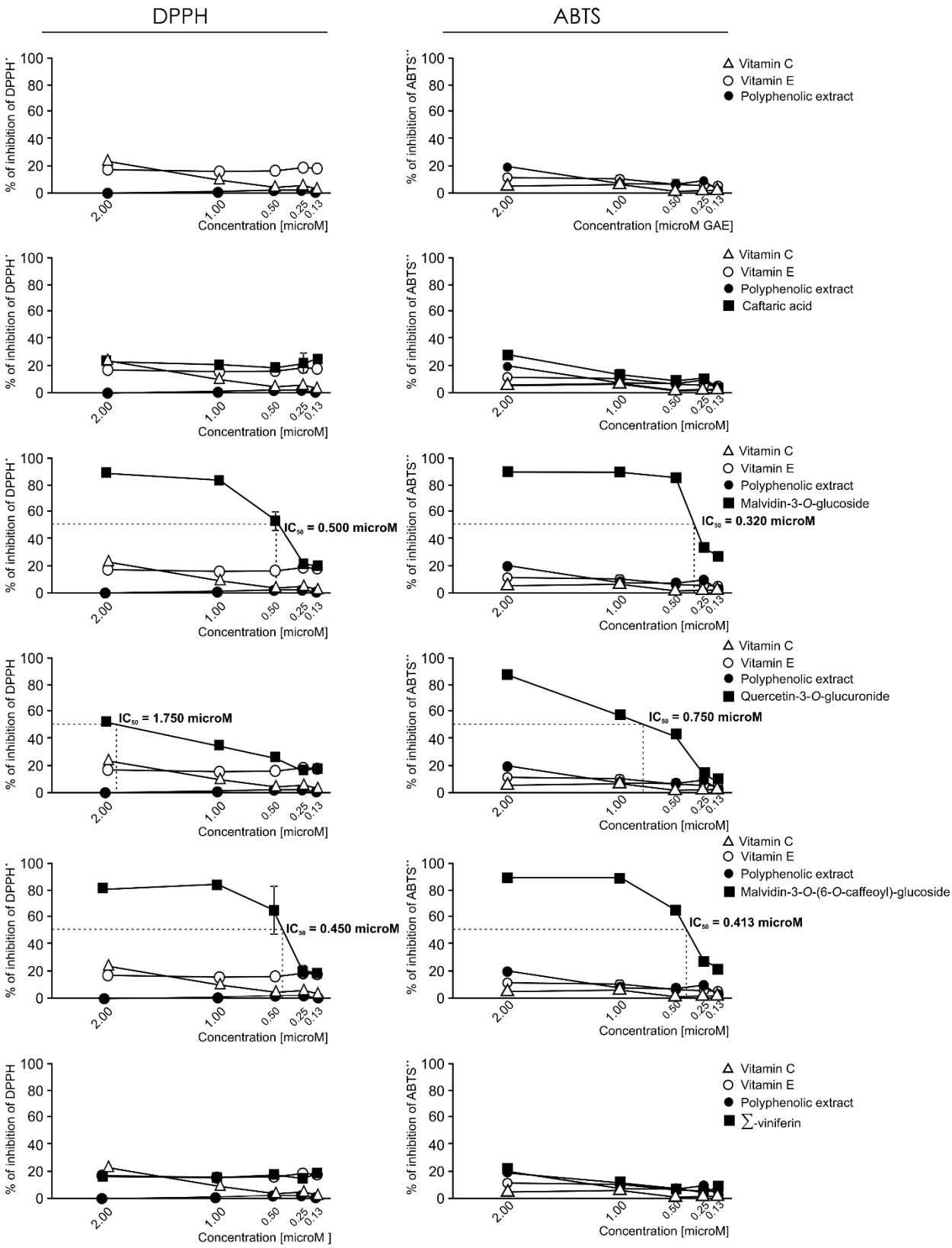

Fig. 3.4. $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging capacity of complete phenolic extract, individual phenolics (caftaric acid, quercetin-3-O-glucuronide, malvidin-3-O-gluoside, malvidin-3-O-(6-O-caffeoyl)-glucoside, and Σ-viniferin), and vitamin C and E at concentrations: 2.00, 1.00, 0.50, 0.25, and 0.13 mol/L.

The differential efficiency observed concerning the scavenging activity against ABTS and DPPH radicals, could be due to the diverse polarity of both, in conjunction with the chemical properties of the bioactive compounds under evaluation. In this concern, ABTS technique allows to study the radical scavenging capacity against hydrophilic and lipophilic solutions, whilst DPPH is based in a system including hydrophobic radicals solved in organic solvents (Kim *et al.,* 2002). In this concern and given that the bioactive compounds tested are addressed to be used in hydrophilic environments, the ABTS assay could be the most proper technique, being this the one that provided the highest efficiency for the three compounds selected.

In contrast to the results obtained for the above-mentioned compounds, the complete phenolic extract and the additional individual compounds evaluated (caftaric acid, Σ-viniferin, and vitamins C and E) in the same range (0.13 - 2.00 µmol/L) did not react with the free radicals in so efficient form, do not reaching inhibitory activities equal or higher than 50.0 %. Hence, the whole phenolic extract displayed a maximum inhibitory concentration of $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ of 16.7 and 5.5 %, respectively, whilst caftaric acid, Σ-viniferin, and vitamins C and E presented maximum inhibition of $DPPH^{\bullet}$ of 24.2, 16.6, 21.2, and 17.7 %, respectively; and of $ABTS^{\bullet+}$ of 26.6, 23.3, 5.1, and 7.2 %, respectively. The much lower capacity of the whole extract regarding the scavenge $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ could be due to the lower concentration of the most active compounds as well as to the existence of antagonistic activity between them, which could be avoided by the purification done, whilst the lower potential of caftaric acid and Σ-viniferin seems to be closely related with their specific chemical properties (Barros *et al.*, 2014; Dominguez-Perles *et al.,* 2016).

To the best of our knowledge, no studies are available on the real scope of grape stems as a source of individual (purified) compounds with antioxidant capacity, which in our view merits to be explored in order to develop rational and sustainable exploitation procedures for this less used material. However, some authors determined the correlation coefficient (*r*) between $DPPH^{\bullet}$ or $ABTS^{\bullet+}$ radical scavenging activity and the concentration of individual plant polyphenols found in complete in grape stems' extracts (Apostolou *et al.,* 2013; Barros *et al.,* 2014; Domínguez-Perles *et al.,* 2016). The application of this statistical tool has supported the virtual identification of the phenolic compounds present in whole grape stems' extract, responsible for the main radical

scavenging observed for phenolic extracts of this plant material, even though no evaluation of the purified individual compounds has been performed to date.

In these works, quercetin-3-*O*-glucuronide, malvidin-3-*O*-glucoside, and malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside presented a significant positive correlation with the decrease of the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ absorbance (Barros *et al.,* 2014; Domínguez-Perles *et al.,* 2016), in agreement with the results observed when analyzed the radical scavenging activity of the isolated compounds, which support the valuable potential of these compounds. However, although other phenolics were identified in previous work as compounds with interesting radical scavenging capacity (caftaric acid) or as compounds with variable activity when tested under diverse extraction conditions (Σ-viniferin) (Domínguez-Perles *et al.,* 2016; Apolostou *et al.,* 2013), their true antiradical power was not confirmed as isolated compounds. This fact could be due to the collaborative work of these phenolics with additional compounds present in the whole extracts that are removed during the purification process, which encourage the development of further evaluations of dedicated combination of the isolated compounds with the highest scavenging capacity with vitamins (C and E) with demonstrated radical scavenging capacity.

Data obtained on the antioxidant capacity of grape stem extracts and their individual phenolics, as well as of vitamin C and E in an individual form, prompted us to design combinations of quercetin-3-*O*-glucuronide, malvidin-3-*O*-glucoside, and malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside (at IC_{50}) with different combinations of vitamins C and E (2.000, 0.200, and 0.020 µmol/L). This approach pursued the identification of dedicated combinations that allowed to increase the radical scavenging capacity based on the synergistic or antagonistic effects that would be further tested *in vitro* by biological models (HaCaT cells) (**Figure 3.5**). The selection of the vitamins concentration was according to previous descriptions available in the literature reporting the range of concentration in which vitamins C and E exert the best antioxidant activity (Thevanayagam, Mohamed, & Chu, 2014). The polyphenolic extract at 2.000 µmol/L was also included as control to understand the effect of supplementing complex polyphenol mixtures with antioxidant vitamins and therefore, the actual interest of using and purified compounds in the development of functional products.

In which respect to the adjuvant activity of vitamin E, it was not found a significant increase of the DPPH radical scavenging capacity of the IC_{50} of quercetin-3-*O*-glucuronide, malvidin-3-*O*-glucoside, and malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside, whilst for malvidin-3-*O*-glucoside and 0.200 µmol/L of vitamin E, decreased

significantly the efficiency of the individual anthocyanin by 37.0 and 19.0 %, respectively. Interestingly, this effect was observed when applying a vitamin E concentration that did not evidence detectable DPPH$^{\bullet}$ scavenging capacity.

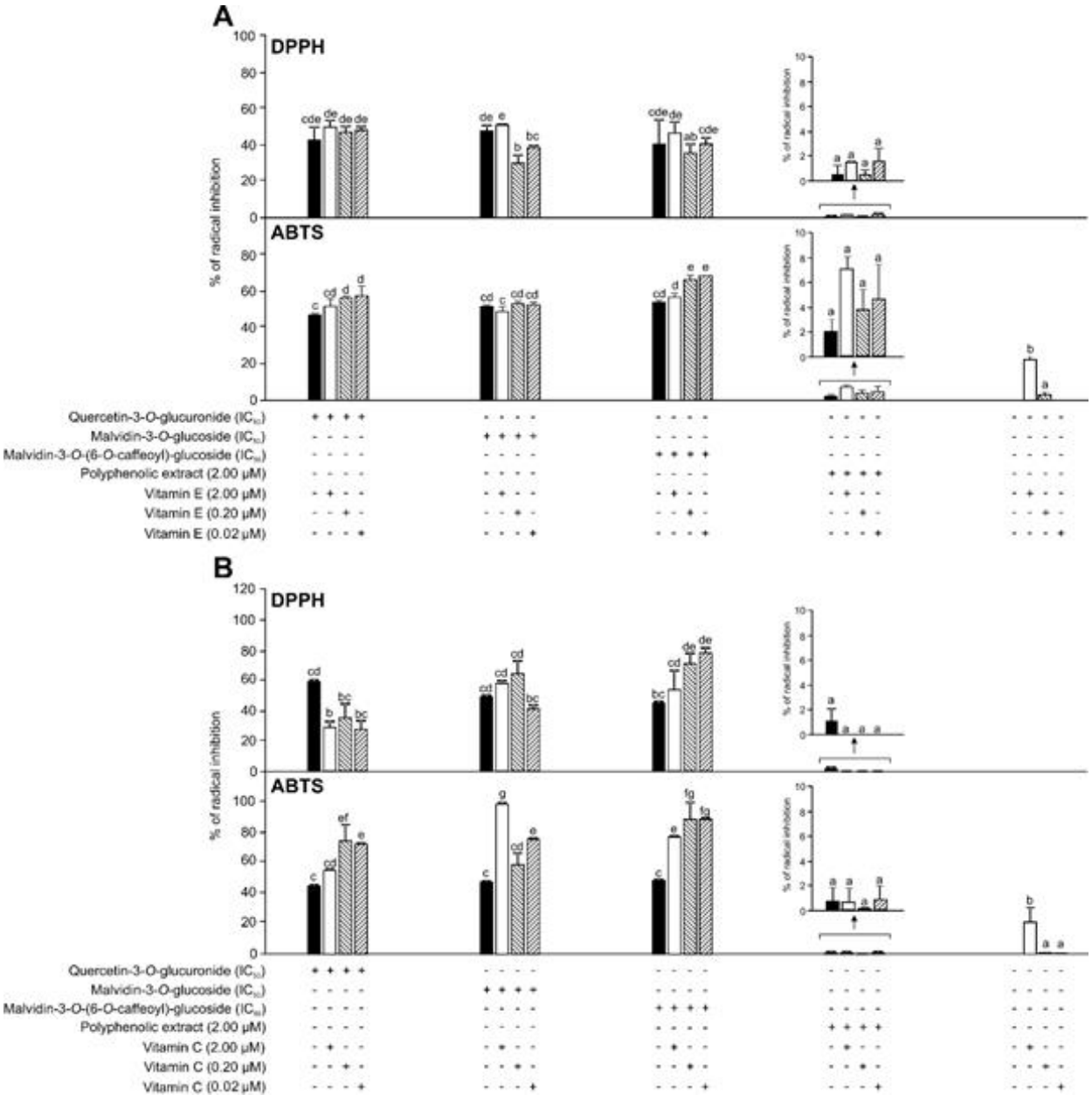

Fig. 3.5. Adjuvant ABTS$^{\bullet+}$ and DPPH$^{\bullet}$ scavenging contribution of vitamin C (0.02 µmol/L) (A) and vitamin E (0.2 µmol/L) (B) to the inhibitory concentration 50 (IC50) of the isolated phenolic compounds. Data are expressed as the mean ± SD (n = 3). Distinct lowercase letter indicates values significantly different at $p < 0.001$

In which respect to ABTS radical scavenging capacity the vitamin E concentrations 0.020 and 0.200 µmol/L were competent to increase the ABTS-based antioxidant activity of quercetin-3-*O*-glucoside (by 20.0 and 22.4 %, respectively) and

malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside (by 22.9 and 27.2 %, respectively). No significant effect of any of the vitamin E concentrations tested was observed regarding the basal antioxidant activity of the polyphenolic extract. Paradoxically, this significant augment was provided by the concentrations of vitamin E displaying the lowest $ABTS^{\bullet+}$ scavenging, that could mean that the synergic effect is not directly related with the bioactive compounds concentration, but related to the chemical properties of the molecules considered. Other reason may be that in the higher concentrations of vitamin E, some interactions may occur that are responsible for the minor antioxidant activity like some sort of competition between both of the whole extract and the vitamin E. This seems to be also an indication of the interactions between the different compounds present in whole extracts one to each other and with the vitamins assayed, that could contribute to the different results obtained from the combination of the isolated phenolic compounds respecting to the whole extract. (Rietjens *et al.*, 2002; Bendary, *et al.*, 2013)

When evaluating the interest of a joint application of individual phenolics and vitamin C for obtaining improved antioxidant activity, it was found that even though no activity was detected for vitamin C alone at the three concentrations evaluated, the two lowest concentrations (0.020 and 0.200 µmol/L) allowed to increase significantly the DPPH radical scavenging capacity of malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside from 37.4 % to 59.1 % and 65.1 %, respectively. This trend was also partially observed when analyzing the ABTS-based antioxidant activity. In this case, 0.020 and 0.200 µmol/L vitamin C improved the $ABTS^{\bullet+}$ scavenging activity of the anthocyanins malvidin-3-*O*-glucoside (by 49.2 and 46.7 %, respectively), whilst 2.000, 0.200, and 0.200 µmol/L vitamin were capable to enhance the ABTS radical scavenging activity of malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside (by 59.3, 83.5, and 83.5 %, respectively). Thus, from the ABTS assay, it was retrieved that the most powerful adjuvant activity was observed from those concentrations exhibiting the lowest antiradical activity when tested alone that support a possible competence with the phenolic compounds when applied together.

These data were in agreement with the previous description of the potential of *alpha*-tocopherol (vitamin E) and/or ascorbic acid (vitamin C) to improve the antioxidant activity of diverse phenolic compounds, namely caffeic acid, *p*-coumaric acid, gallic acid, catechin, epicatechin, myricetin, quercetin, and quercetin-3-*O*-rutinoside (Liao & Yin, 2000; Parker, Miller, Myers, Miguez, & Engeeseth, 2010). This fact is supported by the synergistic or additive activities that seem to emerge between a large variety of compounds or extracts. However, combinations of low concentrations of bioactive

compounds (even no active when tested alone) are the most valuable alternatives. This fact has been already partially addressed with resort to stoichiometric factors, which contribute to differential scavenging potentials concerning mixtures of bioactive compounds (Bendary, Francsis, Ali, & Hady, 2013).

The evaluation of the compounds tested at various concentrations provided also information on the influence of diverse amounts of antioxidants on the pro- and antioxidant activities. This constitutes a relevant issue because nowadays have been demonstrated the relevance of concentrations for the determination of the final activity, especially regarding oxidative effects. Thus, it has been demonstrated that quercetin-3-*O*-rutinoside acts as a significant pro-oxidative at concentrations of higher than 400 μmol/L (Liao *et al.,* 2000). This dependency on concentration is due to the capacity of glycosylated flavonoids to interact one to each other, with iron, and with oxygen at high concentrations, instead to act as a quencher of hydroxyl radicals, giving a net result that is pro-oxidative (Cao, Sofic, & Prior, 1997). A similar mechanism has been proposed for vitamin C, which exhibits the ability to act as a redox reagent. Hence, at concentrations between 0.2 to 400.0 μmol/L, vitamin C contributes to recycle iron from Fe^{+3} to Fe^{+2}, thus significantly increasing the iron species available to react with hydrogen peroxide, whilst at concentrations higher than 400.0 μmol/L, it begins to donate hydrogen atoms to the hydroxyl radicals to a greater degree than it recycles Fe^{+3} (Liao *et al.,* 2000). These complementary mechanisms against free radicals may allow the antagonist, additive or synergic antioxidant activities observed in the experiments developed with combinations of diverse compounds and concentrations. The beneficial effect of combining compounds with complementary mechanisms of action is also supported by the almost lack of a significant increase of the radical scavenging capacity when applying together compounds sharing phenolic structure (Liao *et al.,* 2000). However, measuring these compounds individually in an *in vitro* system does not allow the extrapolation of the actual antiradical activity to biological systems, which is essential to obtain rational evidences on what might be expected to occur *in vivo*, whilst contribute to clarify the properties of the compounds under study and to select those candidates to be analyzed in systems with higher biological relevance.

3.3. Effect of grape stems phenolics and vitamins C and E on viability and LPS-dependent inflammation in HaCaT Cells

The effect of grape stems' whole phenolic extract and individual phenolics, as well as vitamins C and E on the viability of HaCat cells (human keratinocytes) was determined by a colorimetric method, Alamar Blue® (resazurin) assay, which is a sensitive oxidation-reduction indicator that fluoresces and changes coloration after being reduced by the action of mitochondrial enzymes of metabolically active cells (Severino *et al.*, 2014). Cells viability was expressed as percent of the control at 24 and 48 hours after treatments (**Figure 3.6A**). These analyses provided essential information for the study, concerning the suitability of the final harmless concentrations applied for the prevention of redox unbalance in human keratinocytes and allowing to understand the cells behavior in the presence of the bioactive compounds assayed.

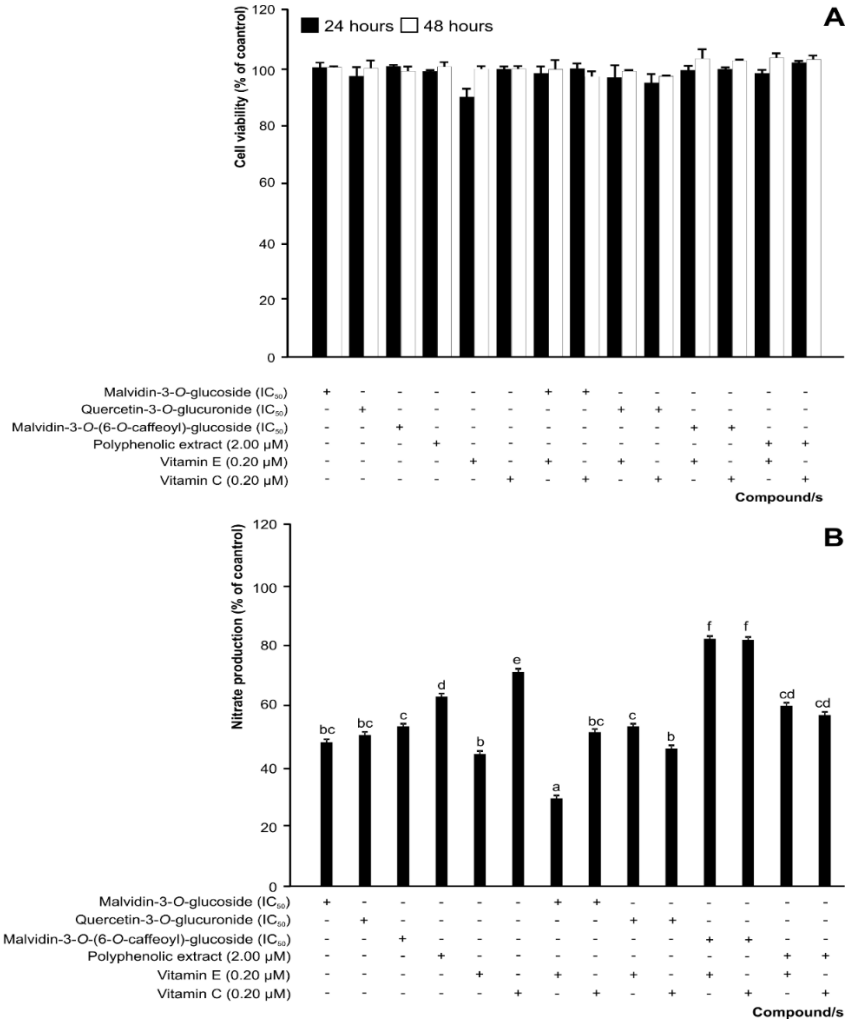

Fig. 3.6. Cytotoxic (A) and anti-inflammatory (B) activities of isolated phenolic compounds at inhibitory concentration 50 (IC_{50}), vitamins C (0.02 µmol/L) and E (0.2 µmol/L), and phenolics-vitamins combinations on RAW 264.7 cells (Murine macrophages from blood) at 24 and 48 hours. Data are expressed as the mean ± SD (n = 3). Distinct lowercase letter indicates values significantly different at $p < 0.001$.

The results obtained indicated that after 24 and 48 h of treatment with complete polyphenolic extract (2.00 µmol/L), malvidin-3-*O*-glucoside (0.41 µmol/L), quercetin-3-*O*-glucuronide (1.25 µmol/L), and malvidin-3-*O*-(caffeoyl)-glucoside (0.43 µmol/L), these did not modify the integrity and viability the HaCaT cells, which remained in values higher than 97.0 % after both 24 and 48 h exposition. Also vitamins C and E (both at 0.200 µmol/L) did not show any deleterious effect compared to the control cultures, allowing a percentage of cells viability between 89.9 and 99.8 % (**Figure 3.6A**).

In order to understand the extent in which the combination of phenolic compounds with antioxidant vitamins (C and E) could display a synergic effect, in the present work it was envisaged the evaluation of their joint effect on oxidative stress in biological systems (HaCaT cells). Nonetheless, to advance towards this objective the deleterious effect of phenolic-vitamin combinations needs also to be monitored. For this, the cytotoxic activity of 0.200 µmol/L of vitamins C and E was tested in combination with 2.00 µmol/L of whole polyphenolic extract of grape stems and the IC_{50} of isolated and pre-selected compounds (malvidin-3-*O*-glucoside, quercetin-3-*O*-glucuronide, and malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside). Thus, when evaluating the foreseen combinations of bioactive compounds, it was stated the harmless effect of their joint application, since HaCaT cells viability remained in values higher than 94.0 and 96.0 % relative to the control at 24 and 48 h, respectively (**Figure 3.6A**). These results indicated that combinations at these concentrations do not have a negative impact in the cells viability and therefore, subsequent experiments were performed using these concentrations.

Next to cell toxicity, whole phenolic extracts, isolated compounds, and vitamins C and E were evaluated on its cytoprotective effects against LPS-induced inflammation in human macrophages (RAW 264.7 cell line) by monitoring their capacity to inhibit NO production (Ghosh *et al.*, 2014; Rodrigues *et al.*, 2015). For this, RAW 264.7 cells were treated with whole polyphenolic extract, malvidin-3-*O*-glucoside, quercetin-3-*O*-glucuronide, malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside, and vitamins C and E at the concentrations previously established, together with an inflammatory stimulus (1 µg/mL LPS) for 24 h (**Figure 3.6B**). All the interventions tested by adding bioactive phenolics and vitamins allowed a significant decrease of the NO production relative to the control. Hence, incubation of human macrophages with the complete extracts or isolated bioactive compounds under evaluation led up to 52.4 % decrease in NO production (indicator of inflammation severity) relative to the untreated control. Therefore, whilst the NO

production by cells treated exposed to LPS and simultaneously treated with whole phenolic extract was of 62.8 %, the isolated compounds decreased the NO production by 52.4 (malvidin-3-*O*-glucoside), 48.9 (quercetin-3-*O*-glucoside), and 47.2 % (malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside). This fact evidenced a significantly ($p < 0.05$) higher capacity of the isolated compounds relative to the complete extract. Regarding antioxidant vitamins, vitamin E provided a reduction of the inflammatory dependent NO production by 56.3 %, whilst vitamin C was the less efficient purified compound with 71.0 % NO production of the control.

In which respect to the combinations of bioactive compounds tested, only the combination of malvidin-3-*O*-glucoside administered concomitantly with 0.200 µmol/L vitamin E provided an improved anti-inflammatory activity (29.0 % NO production of the control) relative to the capacity exerted by the IC_{50} of malvidin-3-*O*-glucoside power (a 47.6 % NO production of the control) (**Figure 3.6B**).

Interestingly, when combining malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside with both vitamins C and E, the anti-inflammatory capacity observed was much lower (18.7 % lower than the control) in comparison with the individual compounds tested on this biological activity (47.2, 56.3, and 29.0 % lower than the control, for malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside, vitamin C and vitamin E, respectively) (**Figure 3.6B**). This difference regarding the synergic activity of antioxidant vitamins with the diverse phenolic compounds under evaluation supports the relevance of the differential chemical features of isolated phenolics to establish efficient collaboration with other molecules for the final biological activity. Finally, in which respect to quercetin-3-*O*-glucuronide, it was found that although it was observed a decrease in the NO production when applied with vitamin E, the differences were not significant. The results obtained with isolated compounds (47.2-52.4 % decrease) were in agreement with the scarce characterization developed so far on the anti-inflammatory activity of wine products and individual phenolics that described a decrease of the NO production by 50.0% (Wang & Mazza, 2002; Rebelo *et al.*, 2014). In general, the percentages of NO reduction of anthocyanins in the study mentioned above were higher than the ones obtained by us, this may result because the extracts use were different and because in this study the anthocyanins isolated were glycosylated, which can decrease the inhibitory activity of the compounds due to the increased hydrophilicity and/or steric hindrance by additional sugar moieties, which may lower the absorption and penetration of glycosylated compounds into cells (Kim, *et al.*, 1999). However, in this concern, it is important to highlight that the concentrations

tested by Wang et al. are in the range of micromolar (16.00 - 500.00 μmoles/L), whilst in the present work these were reduced to nanomolar values (equal or lower than 2.00 μmoles/L), which are close to those obtained after an *in vivo* administration. Interestingly, at these concentrations, the combinations with antioxidant vitamins appeared as a valuable intervention capable to decrease in a higher extent the anti-inflammatory power of these bioactive compounds.

The development of new formulations with improved capacity to decrease NO production is of special relevance because of its participation in a collaborative way with superoxide (O^{2-}) and their reaction product peroxynitrite ($ONOO^-$), involved in some pathogenic pathways by promoting oxidative stress and tissue injury. The proposed mechanisms associated with the reduction in NO production are scavenging of NO radicals, direct inhibition of iNOS enzyme activity, and/or inhibition of iNOS gene expression (Kim *et al.*, 1999; Park *et al.*, 2000; Sheu *et al.*, 2001) that, according to the results obtained in the present work, could be developed in a more efficient extent with phenolics applied with other bioactive compounds, particularly vitamin E.

3.4. Effect of grape stems phenolics and vitamins C and E on basal and H_2O_2-induced ROS, glutathione, and lipid peroxidation in HaCaT Cells

During the last decades it has been demonstrated the close relationship existing between oxidative aggressions and the onset of degenerative diseases and aging, being also related to the severity of these processes (Halliwell, 2005). For this reason, the development of new preventive or therapeutic compounds targeting redox unbalance has been foreseen with resort to the biological activity of newly examined compounds and their combination into new formulations. Involving this situation, the present work dealt with the identification of the most powerful (individual) phenolics from grape stems and their combination with well reputed antioxidant vitamins (C and E), concerning the modulation of oxidative stress markers (ROS, GSH, and LP) in human keratinocytes (HaCaT cells) that will allow to develop new formulations with enhanced biological activities and properties.

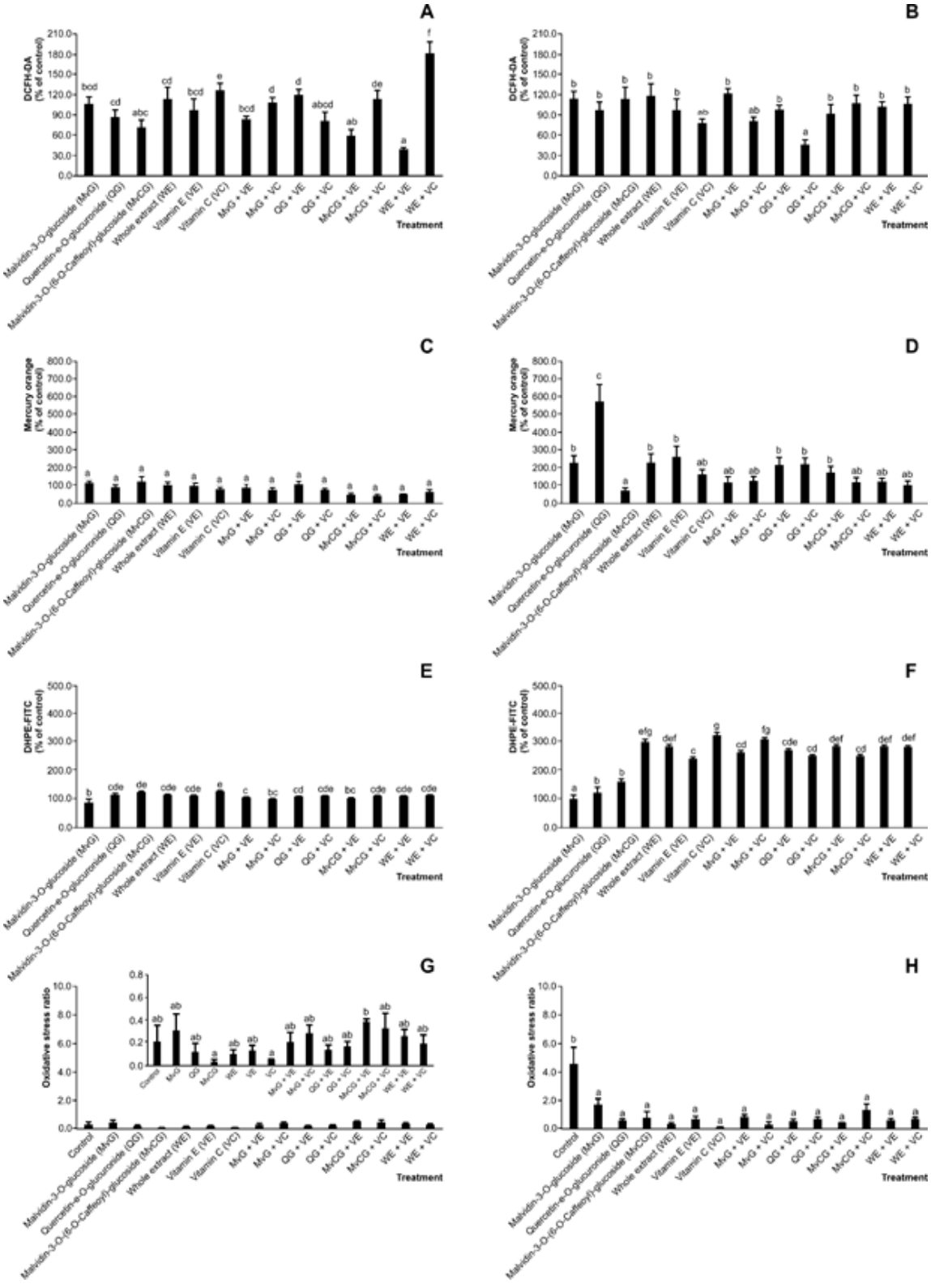

Fig. 3.7. DCF-DA-dependent reactive oxygen species (ROS), Mercury Orange-based glutathione (GSH), DHPE-FITC based lipid peroxidation, and overall oxidative stress in human keratinocytes (HaCaT cells) in basal conditions and exposed to individual phenolics isolated of grape stems (**A**, **C**, **E**, and **G**, respectively) and exposed to individual phenolics isolated of grape stems in and oxidative environment (H_2O_2 50 µM) (**B**, **D**, **G**, and **H**). Data are expressed as the mean ± SD (n = 3). Distinct lowercase letter indicates values significantly different at $p < 0.001$.

With respect to the ROS level in HaCaT cells, under basal conditions it was found no capacity to decrease the levels observed in the control cells, with the exception of cells exposed to the combination of both malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside and complete polyphenolic extracts with vitamin E (**Figure 3.7**), thus, demonstrating the capacity of vitamin E to improve the capacity of antioxidant compounds closely dependent on their chemical features (Kadoma et al. 2006). In addition, these combinations improved the capacity to lower the intracellular ROS level observed in cells treated with malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside and complete polyphenolic extracts by 17.5 and 65.9 %, respectively. Surprisingly, the combination of whole polyphenolic extracts with vitamin C increased the intracellular level of ROS relative to the concentrations recorded for the separate conditions. This situation has been suggested to be due to the combination of the whole extract with vitamin C, which represents a higher concentration of antioxidant compounds in the cell than what its normal, and therefore instead of acting as an antioxidant promote pro-oxidant reactions, being that the reason of the augmented levels of intracellular ROS. Some studies have shown that vitamin C and E and phenolic compounds can also display pro-oxidant activity *in vivo* in specific conditions and concentrations (Bendary *et al.*, 2013). The fact that this situation occurred in the cells not exposed to an oxidative stimulus can be the reason because there is an excessive concentration of anti-oxidant compounds in cells (Rietjens et al 2002). Additional combinations with antioxidant vitamins tested, showed no significant modifications relative to the individual treatments (**Figure 3.7**).

Regarding cells exposed to H_2O_2, as expected presented almost 2-fold higher intracellular ROS concentration relative to basal conditions, whilst when evaluating the capacity of target compounds to modify the higher level of intracellular ROS, it was found that only quercetin-3-*O*-glucoronide, jointly applied with vitamin C, decreased significantly ROS level in comparison with the control and the additional treatments (isolated compounds or combinations) tested (a 65.3 % lower, on average). Under these conditions, quercetin-3-*O*-glucoronide and vitamin C working as individual actors also decreased the ROS level, though this activity was not statistically significant ($p > 0.05$). The combination of this vitamin with malvidin-3-*O*-glucoside was also able to lower ROS below the value of the control group, though this diminution was no statistically significant (**Figure 3.7**).

Glutathione (L-ɣ -glutamyl-L-cysteinylglycine, GSH) is one of the most important component of the cellular antioxidant system (the most abundant thiol into cells), which

reduced form is responsible for scavenging reactive oxygen and nitrogen species, providing a valuable contribution to the redox homeostasis. In this sense, a disulfide bridge is formed between two glutathione molecules to yield the oxidized dimer (GSSG) when ROS donate one electron to the reduced form. By this reaction, GSH system acts as the major redox buffer in most of cells, being responsible for the overall highly reduced environment (Couto *et al.,* 2016). Thus, the evaluation of the contribution of phenolic compounds to the maintenance of the GSH level provides valuable information to understand the extent in which they contribute to the redox balance.

When evaluating target compounds and extracts on the capacity to prevent the decrease of GSH concentrations in both basal and H_2O_2 induced oxidative environments (**Figure 3.7**), it was found that under basal conditions no compound or extract modified significantly the GSH concentration relative to the control. In which concern to the activity of individual compounds on cells exposed to H_2O_2-induced oxidative environment, it was observed 2-fold higher concentration of GSH in cells treated with malvidin-3-*O*-glucoside and vitamin E, whilst the most efficient treatment corresponded to quercetin-3-*O*-glucuronide, which increased the GSH concentration almost 6-folds relative to control (**Figure 3.7**).

Besides individual compounds, the evaluation of the concomitant effect of individual phenolics and vitamins C and E showed that no combination improved significantly the biological activity displayed by the individual phenolics, providing values from 1.5 to 2.0-folds higher than the control. Interestingly, though combinations of quercetin-3-*O*-glucuronide with vitamins C and E provided the best capacities to increase the level of GSH, both of them were much lower than the isolated phenolics. This fact is in agreement with previous reports available in the literature that have pointed out the possibility of vitamin E working as a pro-oxidant depending on the dosage by disrupting the natural balance of the antioxidant system and thus augmenting the vulnerability to oxidative damage and inhibiting human cytosolic *S*-transferases (Van Haaften *et al.,* 2003; Miller III *et al.,* 2005). Thus, although not so high concentrations were tested in the present work, the joint application with phenolics may entail a similar effect, which should be further tested in order to fine-tune the optimal formulation of new products.

The $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ IC_{50} of individual compounds, polyphenolic extract (2.00 µmol/L), and vitamins C and E (0.20 µmol/L) were also evaluated on their capacity to modulate lipid peroxidation events. Through this assay, no significant differences were

found ($p > 0.05$) between all treatments applied neither relative to the control under basal conditions (**Figure 3.7**).

On the other hand, the evaluation of the target individual phenolics on the capacity to prevent lipid peroxidation under the effect of H_2O_2 showed that, even though quercetin-3-*O*-glucuronide and malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside displayed significantly higher capacity than malvidin-3-*O*-glucoside (27.8 % lower, on average), all of them were featured by a lower potential in comparison with whole polyphenolic extracts, vitamins C and E, and the dedicated combinations tested (**Figure 3.6**) under oxidative condition. Indeed, a significant averaged increase of fluorescence of 2.8-folds relative the untreated control (**Figure 3.7**), which corresponds a proportionally lower lipid peroxidation (Dominguez-Perles *et al.,* 2016). These results once more proved the synergic effects of the addition of both vitamins C and E to the isolated phenolic compounds and also to the whole extract. This works as a way to increase the biological uses of grape stems extracts. Our results come once again in accordance with what is described in bibliography, namely Liao and Yin that proved that the enhancement of phenolic compounds with vitamin C and E is more effective in protecting the lipid peroxidation than the phenolic compounds by themselves (Liao & Yin, 2000).

The integration of the information provided by the separate oxidative markers under evaluation (ROS, GSH, and LP) towards the calculation of the global intracellular redox balance according to Du Plessis et al. (Du Plessis *et al.*, 2010), informed on a lack of significant oxidative stress decrease in cells treated with phenolic compounds (aided or not by vitamins C and E) when grown under basal conditions in comparison with untreated cells. However, in oxidative environments, all treatments provided a significant protection against oxidative stress, which under basal conditions corresponded to a value of 4.18. Under the latter conditions, although no significant differences were stated between treatments, the most efficient one was malvidin-3-*O*-glucoside plus vitamin C, which exhibited an oxidative index 16-fold lower than the control (**Figure 3.7**).

These results showed that besides the fact that the grape stems remain as one of the less studied and reused by-products of vinification processes there are important biological properties that can make these residues constitute interesting candidates to obtain new resources of antioxidant and anti-inflammatory compounds. As previously shown by Dominguez-Perles *et al.,* extracts of grape stems have shown a good antioxidant activity, likewise our extract have also showed a good antioxidant activity either alone and in combination with vitamins E and C. Nevertheless, the isolated phenolic

compounds showed a much greater capacity alone and also when working synergistically with the vitamins, which indicates that there is more from the grape stems that their activity as one.

4. Conclusions

To date, phenolic compounds have been largely related to the prevention of oxidative events *in vitro* by cumulative evidences on their capacity to scavenge free radicals or chelate transition metal ions. However, the development of new combinations of bioactive compounds with even augmented biological activity remains to be explored, specially using more informative biological models. In this concern, our results suggest that individual phenolics isolated from grape (*Vitis vinifera* L.) stems could be successfully combined with vitamin featured by antioxidant activity, in order to obtain higher capacities to prevent the deleterious effects associated with redox unbalance in cells. Hence, although minor differences were observed for the isolated phenolics obtained from grape stems concerning oxidative stress, taken together the depth evaluation on radical scavenging power, anti-inflammatory capacity, and modulation of redox balance disturbance markers (ROS, GSH, and lipid peroxidation), it seems evident that the final combination will be closely dependent on the chemical features of the phenolic compound under evaluation. In this concern, malvidin-3-*O*-glucoside and quercetin-3-*O*-glucuronide are successfully aided by vitamins E and C, respectively, and these combinations provide a well-balanced set of biological actions and low side effects, supporting their utilization for the prevention of oxidative insults in skin cells, for instance by being integrated in the formulation of new cosmetic products. Nevertheless, it should also be emphasized that some phenolics-antioxidant vitamins interactions occur, demanding a deeper knowledge on the effective dosages and the molecular characterization of the eicosanoids (isoprostanes and prostaglandins) specifically related with their modulatory effect on oxidative stress and inflammation.

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Chapter IV

Anti-apoptotic effect of phenolic compounds isolated from grape stems polyphenolic extracts on human keratinocytes

Anti-apoptotic effect of phenolic compounds isolated from grape stems' polyphenolic extracts on human keratinocytes[†]

ABSTRACT

Phenolics compounds present in the grape stems have shown a variety of properties being one of them the anti-apoptotic activity. In this work, the polyphenolic compounds extracted from grape stems were tested to confirm their anti-apoptotic potential using the Western Blot technique. The individual compounds and their combination with vitamins E and C where tested to evaluate the extent in which they and their combinations can modulate the levels of cleaved caspse-3, one of the caspases responsible for the apoptosis. The results obtained through this experiment confirmed that phenolic compounds from the grape stems have in fact anti-apoptotic properties being the quercetin-3-*O*-glucoronide and malvidin-3-*O*-(6-O-caffeoyl)-glucoside the ones that show a bigger potential. These results jointly with those on the capacity to protect cells against oxidative stress would allow the information required to make rational decisions regarding the optimal (qualitative and quantitative) combinations of antioxidant compounds for practical applications towards obtaining valuable functional products.

Keywords: *Apoptosis, phenolic compounds, Western Blot, cleaved caspase-3.*

[†] ***Abbreviations:*** *DPPH, 2,2-diphenyl-1-picrylhidracyl radical; ABTS, 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt; Tris/HCl, Tris(hydroxymethyl) aminomethane hydrochloride; SDS, sodium dodecyl sulfate; AMPS, ammonium persulfate; TEMED, tetramethylethylenediamine; PVDF, Hybond®-Polyvinylidene difluoride; TBS-T, Tris-buffered saline-Tween; RT, room temperature; CA, California; WB, western blot; Mv-3-O-glc, malvidin-3-O-glucoside; Q-3-O-glc, quercitin-3-O-glucoronide; Mv-3-O-(6-O-caff)-glc, malvidin-3-O-(6-O-caffeoyl)-glucoside; ROS, reactive oxygen species.*

1. Introduction

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 (Gupsta, 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).

These mechanisms of cell death are triggered by specialized intracellular proteases that cleave specific sequences of cytoplasmatic proteins. These proteases have a cysteine at their active site and cleave their target protein at specific aspartic acid site, this is why they are denominated caspases. Apoptotic caspases are synthesized in the cells as inactive precursors (pro-caspases) and are only activated during apoptosis, being classified in initiator and executioner caspases (**Figure 4.1**). The formers, which exist as inactive soluble monomers in the cytosol, begin the apoptotic process when apoptotic signals trigger the assembly of large protein platforms, bringing multiple initiator caspases together into larger complexes. In these complexes pairs of caspases associated into dimers result in protease activation, by cleaving its partner in a specific site in the protease domain, which stabilizes the active complex. These mechanisms are required for their proper function in cells (Alberts *et al.*, 2014).

The main function of the initiator caspases is to activate the executioner caspases, which also exist as inactive dimmers, which are rearranged towards the active conformation when cleaved by an initiator caspase at a site in the protease domain. Merits to be mentioned that one initiator caspase complex is capable to activate many executioner caspases, amplifying the proteolytic cascade. Once activated, the executioner caspases catalyze the widespread protein cleavage events that will result in the cell's death by the apoptotic pathway (Alberts *et al.*, 2014).

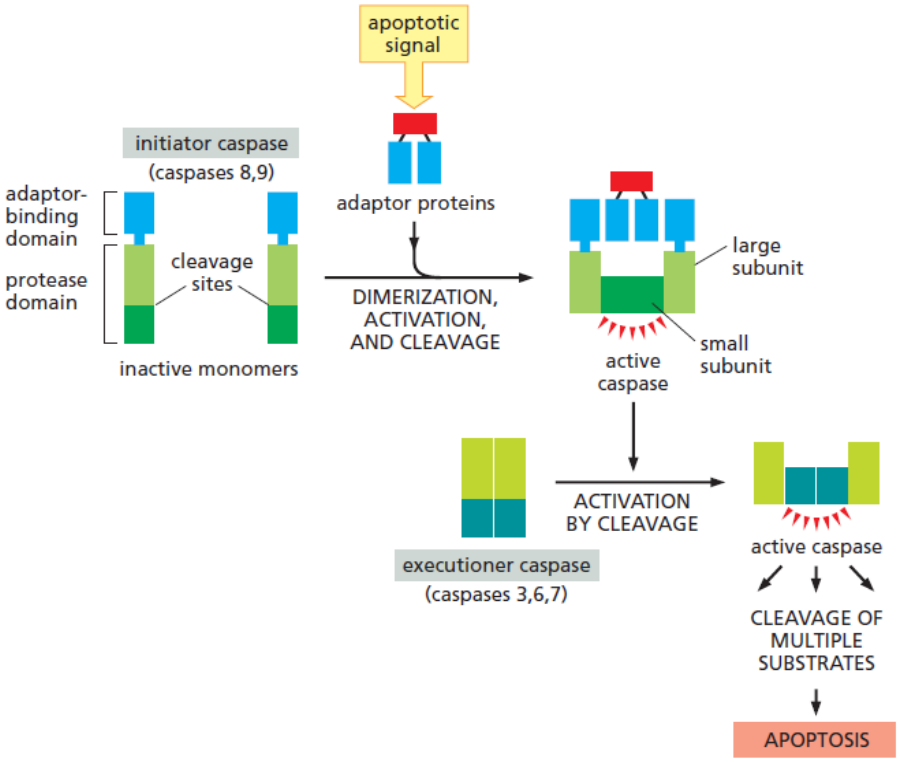


Figure 4.1. Caspase activation during apoptosis (adapted from Alberts *et al.*, 2014).

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 *et al.*, 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.

Not only the caspases are involved in the apoptotic process, the Bcl-2 family members are also a part of the apoptotic events. There are 3 groups of Bcl-2 family

members: the anti-apoptotic(Bcl-2, Bcl-x_L, Mcl-a, Bcl-w and A1), pro-apototic (Bax, Bak and Bok) and also the pro-apoptotic "BH3 only" (Bim, Bid, Bik, Bmf, Bad, Hrk, BNIP3) proteins (Gupsta, 2003).

The permeabilization of the mitochondrial membrane is controlled by the Bcl-2 family, especially Bax and Bak, that initiate the permeabilization by forming along with other proteins of this family what seems to be a channel in the membrane. In apoptosis, Bax is translocated from cytosol to the mitochondrial membrane to form a dimer or oligomers of high order. Other proteins of this family can also associate with the mitochondrial membrane such as Bak and Bim (Zamzani et al., 2001; Gupta, 2003).

During apoptosis, Bad is dephosphorylated and translocated to the other membrane of mitochondria. Once there interacts with Bcl-x_L blocking the anti-apoptotic function of this protein (Gupsta, 2003).

In this chapter, it will be evaluated the anti-apoptotic effects of the previously isolated and characterized phenolic compounds (from grape stems' polyphenolic extracts), already proved on their capacity to prevent oxidative stress on human keratinocytes exposed to oxidative environments (Chapter 2). Anti-apoptotic activity is evaluated in HaCaT cells, by monitoring diverse molecular markers (Caspase-3, BAX and Bcl-2) using the western blot methodology. However only the results regarding the activity of cleaved caspase-3 are described because it was not possible to have conclusive results regarding the other apoptotic markers (Bax and Bcl-2).

2. Material and methods

2.1. Western Blot

HaCaT cells, Cell Lines Services (CLS) Eppelheim, Germany) (Boukamp *et al.*, 1988) were seeded in 6-well plates (3.0×10^5 cells/well) and grown in the already described culture media and growth conditions (see section 2.5 of Chapter 2). After 48 hours, culture media was removed and replaced by fresh medium containing 2 µmol/L grape stems' polyphenolic extract and the isolated phenolic compounds (at each IC_{50}, concentration that inhibited 50 % of DPPH and ABTS), and vitamins C (0.02 µmol/L) and E (0.2 µmol/L), and cells were cultured for further 36 h in the presence of these compounds. Then, cells were centrifuged at 500*g* for 5 min, at 4 °C and supernatants were discarded. Cells were lysed with 0.125 M Tris/HCl buffer (pH 6.4), 10 % glycerol, 4 % sodium dodecyl sulfate (SDS), 4 M urea, 10 % β-mercaptoethanol, and 0.001 % bromophenol blue (de Luna *et al.*, 2006; Domínguez-Perles *et al.*, 2016), which

constitutes both the lysis buffer and the loading buffer for the SDS-page electrophoresis. Suspended cell lysates were boiled for 3 min at 95 °C and then, after cooling, centrifuged at 9500 x *g* for additional 3 min.

For electrophoretic separation of the target proteins, the polyacrylamide gel was prepared by mixing 4.65 mL of water, 2.65 mL of 30 % acrylamide/bis mix, 2.50 mL of 1.50 M Tris-HCL (pH 8.8), 0.10 mL of 10 % (w/v) SDS, 0.10 mL of 10 (w/v) ammonium persulfate (AMPS), and 0.004 mL of TEMED. Twenty microliters of the supernatants were applied to each line of the polyacrylamide gel and run for 10 min at 50 V and then for about 1 h at 100 V, using a Mini-Protean Tetra cell (BioRad, Portugal).

After electrophoresis, proteins were transferred to previously equilibrated Hybond®-Polyvinylidene difluoride (PVDF) membranes (Amersham GE, acquired from VWR), during 2 h at 100 V, on ice. After transfer, unspecific biding sites on the blots were blocked with 5.0 % defatted powder milk in TBS-T buffer (20 mM Tris-HCl, 137 mM NaCl, 0.1 % Tween-20; pH adjusted to 7.6). Blocked blots were incubated with primary mouse anti-human β-actin (Santa Cruz Biotechnology, CA, USA), and rabbit anti-human cleaved caspase-3 (Cell Signaling Technology, Enzifarma, Portugal) monoclonal antibodies at 1:750 and 1:2000 dilutions in TBS-T buffer, respectively, for 2 hours, at RT. After incubation, PVDF membranes were washed in washing buffer (TBS-T) 3 times for 5 minutes each, at RT under horizontal agitation. After washing, membranes were incubated with the secondary antibodies goat anti-rabbit or goat anti-mouse linked to alkaline phosphatase (Amersham, GE Healthcare), at a 1:10000 dilution TBS-T buffer for 1 h, at RT. After incubation, PVDF membranes were washed in washing buffer (TBS-T) 3 times for 5 minutes each at RT in agitation. Immunoreactive bands were visualized, after incubation with ECF substrate (Amersham, GE Healthcare), with a Gel Doc™EZ Gel Documentation System (BioRad, Portugal) and the relative amount of protein was normalized with resort to the β-actin (constitutive molecule) expression level, using the Image Lab 5.1 software's (BioRad Laboratories, Portugal).

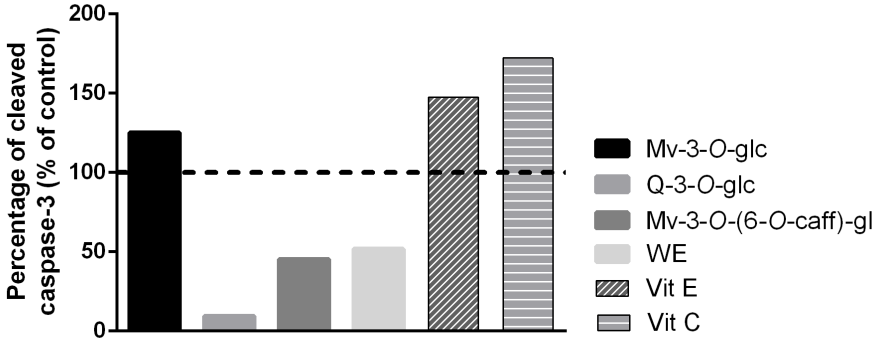
2.2. Statistical analyses

The results were subjected to an analysis of variance (ANOVA) and a multiple range test (Tukey's test) using SPSS statistic 21.0 software package (SPSS Inc., Chicago, USA). Significant differences between the biological activity of the compounds and combinations assessed were set at $p < 0.05$

3. Results and discussion

Results on the level of proteins related to apoptosis retrieved from the WB assays indicated a low occurrence of cleaved caspase-3 (directly related with apoptotic events) in cells treated with individual phenolics and whole polyphenolic extract of grape stems relative to control cells, exception made to malvidin-3-*O*-glucoside. In this regard, quercitin-3-*O*-glucoronide was the compound showing the most relevant anti-apoptotic activity, since contributed to a decrease in the percentage of cleaved caspase-3 by almost 90.0 % regarding the control group (**Figure 4.2)**. This fact is in agreement with previous descriptions that indicate that phenolic compounds, like flavonoids, trigger apoptosis in a selective manner, inducing apoptosis in cultured cancer cells. This activity has been suggested as a consequence of the capacity of these compounds to modulate key elements in cellular signal transduction pathways linked to apoptosis like caspases and *Bcl-2* genes, which expresion ratio in normal cell remain in much lower level compared with malignant cells (Ramos, 2007). For instance quercitin showed an apoptotic effect in aggressive PC-3 and moderately DU-145 prostate cancer cell lines but it did not have an effect in poorly aggressive LNCaP prostate cancer cells or in normal fibroblasts (Ramos, 2008). Phenolic compounds have been shown as competent to mediate apoptosis due to morphological alterations and DNA fragmentation, activation of caspase-3, caspase-7, and caspase-9, release of cytochrome-*c* and apoptosis-inducting factor (AIF) or activation of the mithocondrial pathway, which consists in loss of the mitocondrial membrane potential. All this occours in association with down-regulation of antiapoptotic proteins like the Bcl-2 (Yang *et al.*, 1998; Babich *et al.*, 2005; Ramos, 2008).

A



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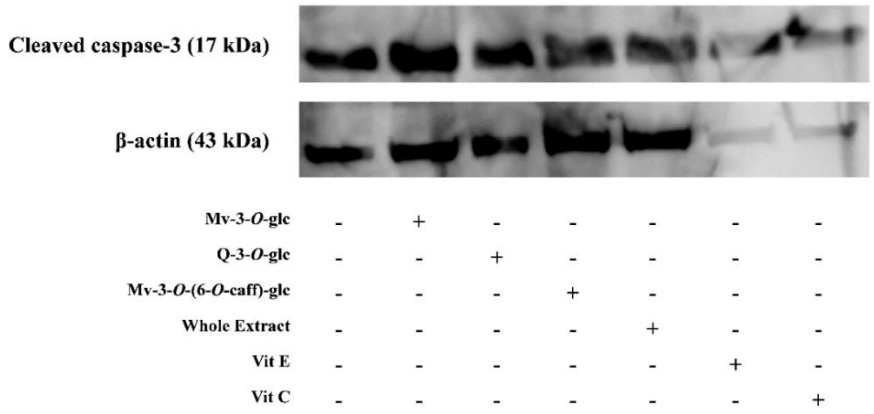

Figure 4.2. A- Percentage of cleaved caspase-3 of all the individual phenolic compounds and vitamins E and C. **B-** Western Blot membrane of the individual compounds and vitamins.(One blot analyzed)

Despite this, and like our own data show (**Figure 4.2**), quercetin can inhibit apoptosis in some non-tumorgenic cells. It was shown that quercetin inhibits the hydrogen peroxide (H_2O_2) induced apoptosis of mesangial cells, fibroblasts and epithelial cells. This inhibitory effect of quercetin on cell apoptosis can occur via intervention in the activator protein (Ap-1) pathway, the crucial signalling route for the H_2O_2 induced apoptosis (Ishikawa & Kitamura, 2000).

Other studies have also shown that the addition of quercetin is responsible for significant reduction of the expression of caspases 3, 8, and 9 in H_2O_2 induced apoptosis. It seems that quercetin significantly reduced the indicated caspase enzyme activity protecting the cells aginst the apoptosis (Chen *et al.*, 2006).

Interestingly, it was observed an increase of the expression of cleaved caspase-3 in HaCaT cells treated with vitamins E and C (by 47.5 and 72.3 %, respectively, relative to the control) (**Figure 4.2**). These results, that could be in contradiction with previous data reported in the literature on the anti-apoptosis activity of the referred vitamins (Haendeler *et al.*, 1996), might be related with the effective concentrations applied in the treatments featured, which would constitute excessive vitamin levels exerting pro-oxidant behavior, thus, triggering apoptotic events *in vitro* (Rietjens *et al.,* 2002). In this sense, this pro-apoptotic activity of vitamins, only seen at high concentrations, can ultimately be the cause of this augmented apoptosis via intrinsic pathway (through activation of pro-caspase 3, augmenting the levels of cleaved caspase-3), which is an indication, *a priori*, to develop further investigations that provide the actual concentrations of antioxidant vitamins that allow to reduce oxidative stress without induction of augmented levels of the pro-apoptotic caspase-3. At these concentrations of vitamins, the ROS levels (**Figure 3.7**) were not statistically different from control, thus a distinct mechanism might be involved which is worth to explore.

When evaluating the effect of the combination of individual phenolics/polyphenolic extracts of grape stems with the antioxidant vitamins (C and E), it was observed that almost all combinations exerted the capacity to lower the expression of cleaved caspase-3 relative to the control, with the only exception of cells treated with whole polyphenolic extract supplemented with vitamin E, where the occurrence of cleaved caspase-3 increased by 65.8 % relative to control cells. A detailed examination of the capacity to modulate cleaved caspase-3 concentration informed that, in general, combinations of individual phenolics with vitamin C were more efficient on this activity, providing an average decrease of 33.6 % in comparison with controls. This was also true when considering the combination of whole phenolic extracts and vitamin C, which reduced the cleaved caspase-3 level by 5.6 %, on average (**Figure 4.3**).

The evaluation of the combinations between individual phenolics and vitamin E, showed a significant decrease of the cleaved caspase-3 levels relative to the control (by 24.52%, on average (**Figure 4.3**).

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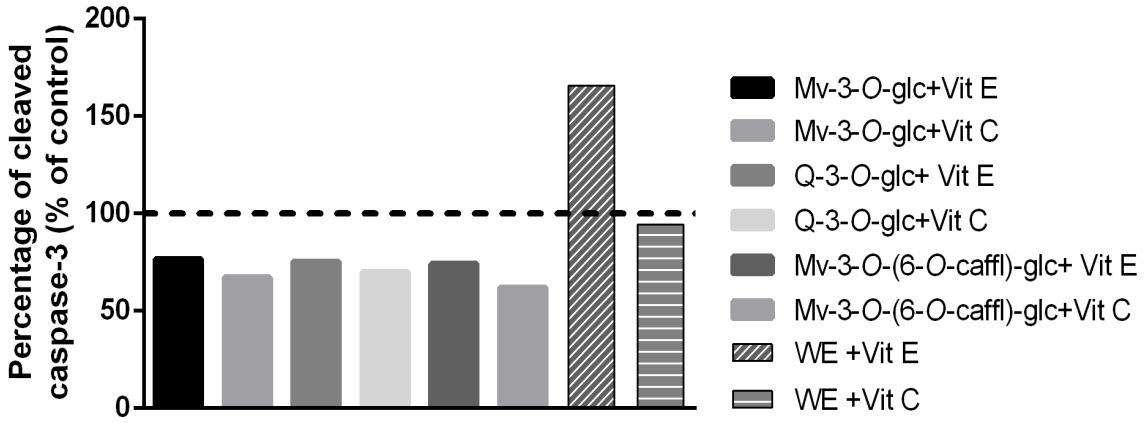

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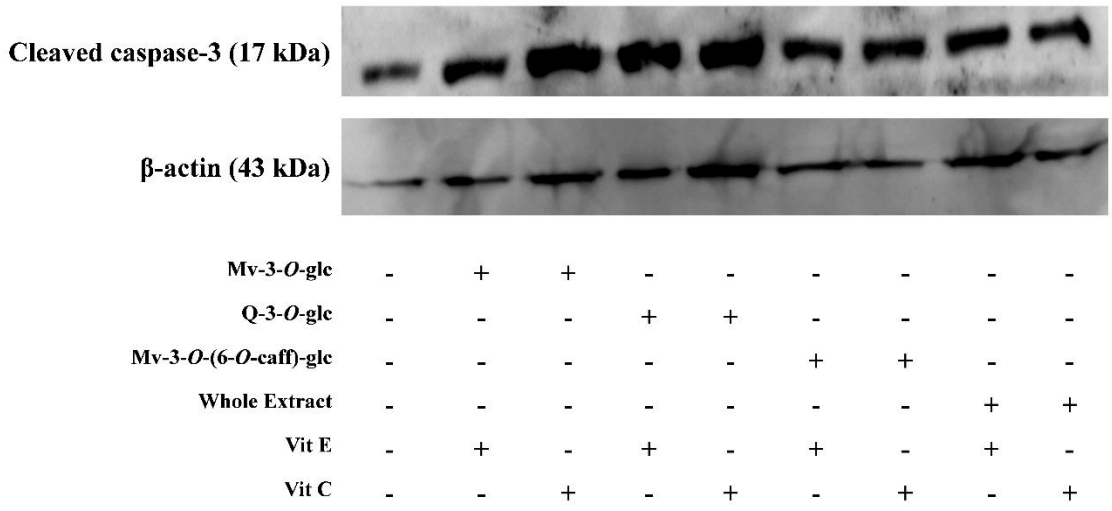

Figure 4.3. A- Percentage of cleaved caspase-3 expressed with the different phenolic compounds combined with vitamin E and C. **B-** Western Blot membrane of the individual compounds combined with vitamins E and C.(one blot analyzed)

These results evidenced that the individual phenolic compounds previously isolated have in fact an anti-apoptotic activity because they are able to reduce the percentage of cleaved capase-3 control wise. From all the isolated compounds the one that had the most reduction of cleaved caspase-3 was the quercitin-3-*O*-glucuronide with 90.7 % less expression of cleaved caspase-3 than control group. The addition of vitamins didn't prove to be positive despite when comparing to the percentage of the control group there is in fact a general reduction in the expression of the cleaved caspase-3, the individual compounds demonstrate a better capacity alone. This can be related with the concentration of both vitamins that was causing an increase stress, responsible to augment

the apoptosis levels instead of helping reducing them. When comparing the addition of the two vitamins (E and C) with the isolated compounds and whole extract, vitamin C exhibited a better effect with a decrease of cleaved caspase-3 by 33.6 %, on average, relatively to control group.

These data further support the interest to develop proper combinations of phenolic compounds to obtain the best anti-apoptotic activity, even though these results have to be managed in combination with those on the activity of the treatments under evaluation to prevent oxidative stress and inflammation when exposed to oxidative environments. Anyway, it was observed that the tested compounds and their combination do not have any pro-apoptotic activity (tentatively, since it was monitored the level of pro-apoptotic proteins and not the occurrence of apoptotic events itself), but even protect the cell from apoptosis by lowering the percentage of cleaved caspase-3, which is in agreement with previous descriptions in available in the literature (Nair *et al.*, 2004; Ramos, 2007), and further support the development on additional assays on the biological excellence of the grape stems' phenolics and their practical applications.

4. Conclusion

Results from this assay evidenced that the phenolic compound present in grape stems can protect normal cells from apoptosis, by lowering the concentration of cleaved caspase-3 present in cells. In this concern, the isolated compounds, quercetin-3-*O*-glucoronide and malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside were identified as the most competent to modulate the level of proteins related to programmed cells death. However, additional studies are required, increasing the panel of pro- and anti-apoptotic proteins monitored, to further confirm this fact. Anyhow, the jointly interpretation of the capacity to lower the level of cleaved caspase-3 with previous data obtained in the present work on the ability of the target compounds/extracts to modulate abnormal ROS, glutathione, lipid peroxidation events and inflammation severity stresses the potential of the grape stems as a source of multifunctional phenolic compounds with highly valuable biological activities.

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Chapter V

General Conclusions and Future Perspectives

General Conclusions

1. General Conclusions

The wastes associated with the vinification process can represent a huge issue to the environment and as well to the companies that have to spend money to get rid of those unwanted wastes.

Grape stems, besides being a major residue resultant from the vinification, is still one of the less studied and therefore one of the less valorized by-products. This material contains significant amounts of biologically active phenolic compounds. In this work, the assessment of grape stems of the red cultivar 'Sousão' retrieved accurate information on its considerable content of phenolics and the consequent radical scavenging antioxidant capacity of their hydromethanolic extracts.

To further use this material and take advantage of its content in radical scavenging compounds it is essential to identify the main phenolic compounds present, as well as to isolate and purify them. In this regard, the most abundant phenolic compounds in grape stems of 'Sousão', obtained at purities higher than 95 %. were caftaric acid, malvidin-3-*O*-glucoside, quercetin-3-*O*-glucuronide, malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside, and Σ-viniferin.

The results obtained suggest that individual phenolics isolated from grape stems could be successfully combined with vitamin featured by antioxidant activity, in order to obtain higher capacities to prevent the deleterious effects associated with redox unbalance in cells.

Malvidin-3-*O*-glucoside and quercetin-3-*O*-glucuronide are successfully aided by vitamins E and C, respectively, providing these combinations a well-balanced set of biological actions and low side effects, supporting their utilization for the prevention of oxidative insults in skin cells.

The results obtained evidenced that there are important biological properties attributable to the phenolic compounds present in grape stems, which turns this material into an interesting stuff to be further processed towards isolated compounds that can be used (alone or combined with in combination with antioxidant vitamins) in the development of new functional products.

Phenolic compounds present in grape stems also have an anti-apoptotic potential, proved by the decrease of the levels of cleaved caspase-3 in cells treated with isolated

phenolic compounds alone or in combination with vitamins E and C. From all combinations on test the ones with the best anti-apoptotic potential were quercetin-3-*O*-glucoronide and malvidin-3-*O*-(6-O-caffeoyl)-glucoside with vitamin C.

This study as a whole proved that grape stems have a huge potential as a source of bioactive compounds, that have multiple properties from the protection against oxidation to the anti-apoptotic potential and the capacity to reduce inflammation, allowing the reduction of the wastes and in that way have a reduction on the negative environmental effects of the accumulation of such wastes.

Other important factor that this research shows is that this natural compounds can be combine with well-known vitamins (E and C) to increase their capacities/activities. In fact, at the concentrations used in this investigation, the major part of the isolated phenolic compounds had better results than the vitamins. This proves once again how useful the stems can be providing the content in phenolic compounds that can be used as alternatives to some vitamins.

Future Perspectives

2. Future perspectives

Besides all the tests and results presented in this work, there is much more that can be done to further prove the utility of grape stems as a source of bioactive compounds, namely:

- Using different extracts from stems of distinct cultivars which would allow to identify new compounds or to find if there is stems with higher concentrations of phenolic compounds of interest;
- Testing combinations of different individual compounds to verify if there are any synergistic effects;
- Combining individual phenolics with important biological activities one to another to further increase/potentiate those capacities;
- Using different cell lines, such as cancer cell lines, to find the potential anticancer activity of the phenolic compounds.
- Further studies on the relationship between apoptosis and phenolic compounds from grape stems, by studying various apoptotic markers, either pro-apoptotic and anti-apoptotic.