Analysis of antioxidant and antigenotoxic activities of grape berry extracts.

Maria Nipo de Sá Moreira

October 2018

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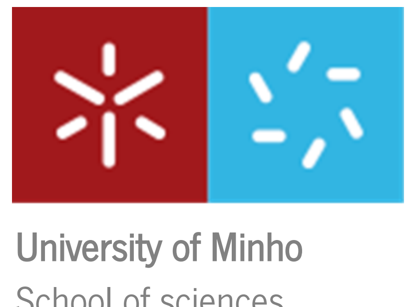

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Analysis of antioxidant and antigenotoxic activities of grape berry extracts

Master thesis
Master's in Molecular Biology, Biotechnology and Bioentrepreneurship in Plants

Work done under the guidance of
Professor Rui Oliveira and Professor Andrea Ševčovicová

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Anexo 3

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Analysis of antioxidant and antigenotoxic activities of grape berry extracts

Abstract

Vitis vinifera grape berries have attracted a lot of attention in health research due to their rich phenolic content. These compounds have biological properties, such as antioxidant, anticarcinogenic, anti-inflammatory and antimicrobial, and other protective effects against an array of stresses. Also, they showed promising results against different diseases such as diabetes, Alzheimer's, Parkinson's, cardiovascular diseases and post-traumatic stress disorder (PTSD). Red varieties are richer in these compounds due to the presence of anthocyanins but there are considerable differences also among red grape varieties. The red variety Vinhão from the Northwest of Portugal is highly concentrated in these compounds, conferring a great potential for biological activities. Bearing in mind that there is insufficient data regarding potential beneficial effects of Portuguese wines and cultivars and there is an increasingly interest in natural compounds and the reuse of agricultural wastes for other purposes. The aim of this work is to determine and compare different bioactivities of seeds and skin extracts of two 'vinho Verde' wine cultivars, Vinhão and Loureiro, a red one and a white one, respectively. And encourage the use of Portuguese wine production wastes for the application in cosmetics and nutraceuticals. To achieve these goals, total phenolic content was determined by Folin-Ciocalteu method. Furthermore, the antioxidant activity of all extracts was assessed by DPPH assay, reducing power assay, iron chelation assay and by flow cytometry. Moreover, genotoxicity and antigenotoxicity of all extracts was estimated by DNA topology assay. Two different assays were used to determine the antigenotoxic mechanism of Vinhão and Loureiro seeds extracts, the comet assay and the AMES test. Seed extracts from both varieties displayed strong reducing power and DPPH scavenging activities, while red Vinhão berries displayed higher activities than the white variety (Loureiro). Interestingly, all samples did not chelate iron, suggesting that this property is not involved in antioxidant activity. In addition, all extracts were antigenotoxic using DNA topology assay and comet assay with human lymphocytes. Ultimately, the grape extracts evaluated showed to have great antioxidant and antigenotoxic potential, except Loureiro berry extract. Furthermore, seeds extracts showed to be not mutagenic and they do not protect against mutations induced by diagnostic mutagens. Moreover, when compared to the literature, the values obtained by DPPH and reducing power assay for both seeds' extracts are higher than other well-known varieties tested, such as Merlot, Pinot Noir and Cabernet Sauvignon. Portuguese grape varieties grown in 'vinho Verde' region showed to be great sources of polyphenols with nutraceutical properties. Further studies must be conducted to determine antioxidant and

antigenotoxic activity *in vivo*, since results were not coincident, and to encourage the use of these extracts in nutraceuticals and cosmetics.

Key-words: Vinhão, Loureiro, 'vinho Verde', agricultural wastes, scavenging, reducing power, seeds extracts, pulp and skin extracts.

Análise da atividade antioxidante e antigenotóxica de extratos de bagos de uva

Resumo

Os bagos uva de *Vitis vinifera* têm atraído muita atenção na área da saúde devido ao seu elevado teor em polifenóis. Estes compostos possuem propriedades biológicas, como antioxidante, anticancerígena, anti-inflamatória, antimicrobiana, assim como outros efeitos protetores contra uma série de stresses. Para além disso, esta classe de compostos mostrou resultados promissores contra diferentes doenças, como diabetes, Alzheimer, Parkinson, doenças cardiovasculares e stress pós-traumático (PTSD). As variedades tintas são mais ricas devido à presença de antocianinas, mas também há diferenças consideráveis entre as variedades de uvas tintas. A variedade tinta Vinhão, do Noroeste de Portugal, é altamente concentrada nestes compostos, o que lhe confere um grande potencial para atividades biológicas. Tendo em conta que os dados relativos aos potenciais efeitos benéficos dos vinhos e castas portugueses são insuficientes e há um crescente interesse pela reutilização de resíduos agrícolas para a extração de compostos com efeitos benéficos para a saúde humana. O objetivo deste trabalho é determinar e comparar diferentes bioatividades de extratos de sementes e película de duas castas Minhotas, Vinhão e Loureiro, tinta e branca, respetivamente. E revelar novas aplicações para os resíduos da produção vinícola portuguesa. Para alcançar estes objetivos, o conteúdo total em polifenóis foi determinado pelo método de Folin-Ciocalteu. Além disso, a atividade antioxidante de todos os extratos foi avaliada por ensaio de DPPH, ensaio de poder redutor, ensaio de quelação de ferro e por citometria de fluxo. A genotoxicidade e a antigenotoxicidade de todos os extratos foram estimadas pelo ensaio de topologia de DNA e, a atividade das sementes também foi avaliada por ensaio cometa. O potencial anti mutagénico dos extratos de sementes de Vinhão e Loureiro foi determinado pelo teste AMES. Os extratos de sementes apresentaram forte poder redutor e elevada atividade de *scavenging* de DPPH, enquanto os bagos de Vinhão apresentaram atividades superiores às da variedade branca (Loureiro). Curiosamente, todas as amostras não quelaram o ferro, sugerindo que este não é o mecanismo envolvido na atividade antioxidante. Todos os extratos revelaram ser antigenotóxicos no ensaio de topologia de DNA e no ensaio cometa com linfócitos humanos. Finalmente, os extratos de sementes revelaram não ser mutagénicos e não apresentaram efeitos protetores contra mutagénios. Além disso, quando comprados com a literatura, os extratos de sementes de ambas as variedades apresentaram valores superiores para o ensaio de DPPH e de poder redutor do que as sementes de outras variedades conhecidas, como Merlot, Pinot Noir e Cabernet Sauvignon. As castas portuguesas cultivadas na região dos 'vinhos Verdes' mostraram

ser excelentes fontes de polifenóis com propriedades nutracêuticas. No entanto, novos estudos devem ser realizados para determinar a atividade antioxidante e antigenotóxica *in vivo*, uma vez que os resultados não foram coincidentes.

Palavras-chave: Vinhão, Loureiro, 'vinho Verde', scavenging, poder redutor, resíduos agrícolas, extrato de sementes, extrato de polpa e película.

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List of abbreviations

4NOP	4-nitro-o-phenylediame
9AA	9-aminoacidine
Abs	Absorbance
ACE	Angiotensin converting enzyme
AF	Autofluorescence
ATP	Adenosine triphosphate
C	Cytosine
C-	Negative control
C+	Positive control
CAT	Catalase
CHS	Chalcone synthase
C3H	Coumarate-3-hydroxylase
C4H	Cinnamate-4-hydroxylase
COMT	Caffeic acid *O*-methyltransferase
COX 2	Cyclooxygenase 2
DCF	Dichlorofluorescein
dH_2O	Deionized water
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EDTA	Ethylenediamine tetra-acetic acid
EtOH	Ethanol
FAO	Food and Agricultural Organization of the United Nations
G	Guanine
GA	Gallic acid
GAE	Gallic acid equivalents
GSHPx	Glutathione peroxidase
GSTs	Glutathione S-transferase
H_2DCF	Dichlorodihydrofluorescein
H_2DCFA	Dichlorodihydrofluorescein diacetate
His	Histidine

IC_{50}	Concentration of inhibition needed to reduce 50 % of DPPH
LB	Loureiro pulp and skin extract
LDL	Low density lipoprotein
Leu	Leucine
LMP	Low melting point
Log	Logarithm
LS	Loureiro seeds extract
Met	Methionine
MMO	Microsomal monooxygenase
NAD	Nicotinamide adenine dinucleotide
NMP	Normal melting point
OD	Optical density
OIV	International Organisation of Vine and Wine
PAL	Phenylalanine ammonia lyase
PBS	Phosphate Buffered Saline
pDNA	Plasmid DNA
PTSD	Post traumatic stress disorder
QE	Quercetin equivalents
ROS	Reactive oxygen species
Rpm	Rotation per minute
SCGE	Single cell gel electrophoresis
SDS	Sodium dodecyl sulphate
SO	Mitochondrial succinase
SOD	Superoxide dismutase
SRT1	Sirtuin 1
SS	Stilbene synthase
SD	Standard deviation
TBE	Tris/Borate/EDTA
TCA	Trichloroacetic acid
TFC	Total flavonoid content
TPC	Total phenolic content
Tris-HCl	Tris Hydrochloride

UV	Ultra-violet
Ura	Uracile
VB	Vinhão pulp and skin extract
VS	Vinhão seeds extract
YPD	Yeast peptone dextrose

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1. Introduction

Grape vine (*Vitis* spp.) is one of the world's most significant fruit species, ranking in second in worldwide fruit production in 2014, with approximately 75 million tonnes produced, according to the Food and Agricultural Organization of the United Nations and to the International Organisation of Vine and Wine (FAO-OIV, 2016). *Vitis* (family *Vitaceae*) includes about 60 species native from the north temperate zone, however *Vitis vinifera* is the most frequently used species in wine production (Frederic, 2009), which accounts for half of the total grape production. While one third is consumed as fresh fruit and almost 20% is used as dried fruit or as grape juice. Given the versatility of grapes it is evident that the grape market plays an important role in global food consumption. Recently, with the discovery of the health benefits linked to phytochemicals, present both in grape flesh and seeds, there has been an expansion in grapes and their by-products sectors (Glampedaki & Dutschk, 2014). Tons of organic waste are generated every year by the wine making industry, since 20% of the raw material accounts as excess (Marculescu & Ciuta, 2013). So, finding alternative solutions for the exploitation and valorisation of those by-products, which would create economic, social, and environmental opportunities, will be of the great interest. Agricultural wastes, like grape seeds, have already been widely used for the extraction of natural antioxidants (Ku & Mun, 2008). For example, grape seed extracts have been used as dietary supplements (Perumalla & Hettiarachchy, 2011) and in cosmetic and personal care (Jeandet *et al.*, 2016). Lately, grapes have attracted a lot of attention in health research, partly due to their widespread consumption, but also due to the increasing concern with the ingestion of natural products and with aging and disease prevention (Pye *et al.*, 2017).

Fruits have shown to have health benefits, due to their rich phenolic content (natural plant compounds with antioxidant activity) and may be interesting tools in the prevention of diseases (Cristóbal-Luna *et al.*, 2017). These molecules have been linked to a range of bioactivities, which include antioxidant, antigenotoxic and antimutagenic, anti-inflammatory and antiviral, as well as to protective effects against UV radiation and a variety of hazardous chemicals (Nichols *et al.*, 2010; Xia *et al.*, 2010; Yilmaz *et al.*, 2004;). Also, under certain conditions, such as high concentrations, high pH and the presence of some transition metals, phenolic compounds can act as pro-oxidants (D'Angelo *et al.*, 2017). For this reason, they are able to generate cytotoxic effects in bacteria, fungi, protozoa, and cancer cells (Tomás-Barberán & Lacueva, 2012). This characteristic of polyphenols can have implication on cancer chemoprevention and chemotherapy (Léon-González *et al.*, 2015). Diseases, such as Alzheimer's, Parkinson's, cardiovascular problems, diseases of

the immune system, cancer, obesity and diabetes, are associated with an increase in oxidative stress. This oxidative stress can be reduced or even prevented with the input of natural antioxidants present in fruits (Choi *et al.*, 2012; Wang *et al.*, 2008; Zern *et al.*, 2005; Zunino, *et al.*, 2007). However, phenolic composition is highly affected by differences in grape varieties, environmental conditions and cultural practices, making extract application in the treatment of diseases controversial. Red grape products are particularly rich in polyphenols, specifically anthocyanins, which are absent in the white varieties (Dopico-García *et al.*, 2008). Moreover, there are considerable differences in anthocyanin content also among red grape varieties (Costa *et al.*, 2015).

The 'vinho Verde' region, in the northwest of Portugal (**Figure 1**), is the second largest producing area of the country, with 21000 hectares of vineyards (da Silva *et al.*, 2018). Vinhão, the most cultivated red variety of this region, exhibits a higher anthocyanin content than other known red grape varieties, such as Merlot, Pinot Noir or Cabernet Sauvignon, since Vinhão also stores anthocyanins in the pulp of the fruit (Mazza *et al.*, 1999; Ortega-Regules *et al.*, 2006). On the other hand, Vinhão variety also showed the lowest non-coloured phenolic content compared to other 'vinho Verde' varieties (Castillo-Sánchez *et al.*, 2008). In contrast, Loureiro, the most widely cultivated white variety of this region, although lacking anthocyanins, is rich in non-coloured polyphenols such as quercetin-3-*O*-glucoside, quercetin3-*O*-rutinoside, kaempferol-3-*O*-rutinoside, kaempferol-3-*O*-glucoside and epicatechin (Dopico-García *et al.*, 2007).

Figure 1. Map of the Portuguese wine regions, which include 14 different regions. In the North of the country: Vinho Verde, Trás-os-Montes, Porto, Douro, Távora and Varosa regions. The Centre of Portugal is divided in four regions: Bairrada, Beira Interior, Tejo and Lisboa. And the South is divided in three more

regions: Alentejo, Península de Setúbal and Algarve. Finally, two more regions that include Madeira and Azores (Adapted from The Portuguese Wine, 2016).

1.1. Oxidative stress

All living organisms are constantly producing reactive oxygen species (ROS), that include hydroxyl radical ($\bullet OH$), superoxide anion ($\bullet O_2^-$) and hydrogen peroxide (H_2O_2), among others. However, they are also able to remove these damaging species with either enzymatic or non-enzymatic natural defence mechanisms (Nimse & Pal, 2015). The enzymatic defences include three important enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSHPx), that can convert ROS into more stable species (**Figure 2**), while the non-enzymatic include vitamin C, vitamin E, plant polyphenols, carotenoids, and glutathione. Antioxidants can interrupt free radical chain reactions by directly reducing ROS, inactivating metal catalysts through chelation, or even regulate antioxidant defence related genes (Nimse & Pal, 2015).

$$2\,^{\cdot}O_2^{-} + 2H^{+} \rightarrow H_2O_2 + O_2 \text{ (SOD)}$$

$$2H_2O_2 \rightarrow 2H_2O + O_2 \text{ (Catalase)}$$

$$ROOH + 2GSH \rightarrow ROH + GSSG \text{ (GSHPx)}$$

Figure 2. Antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSHPx) reducing reactions (Nimse & Pal, 2015).

In low concentrations, ROS act as signalling molecules as well as gene regulating agents (Uttara *et al.*, 2009). Thus, oxidative stress can be defined by an imbalance between the systemic manifestation of ROS and a biological system's ability to normally detoxify the reactive intermediates or to repair the resulting damage. Disturbances in the normal redox state of cells can cause toxic effects, through the production of free radicals that can damage all components of the cell, including proteins, lipids, and DNA, and these effects are associated with a range of different diseases (Kim & Byzova, 2013). Mitochondria are the major contributors to endogenous ROS production due to their electron transport chain. Electrons are passed through a series of transmembrane proteins via redox reactions and, simultaneously, protons are being transported across the inner mitochondrial membrane. This creates an electrochemical proton gradient that drives the phosphorylation of adenosine triphosphate (ATP). The final electron acceptor in the electron transport chain, during aerobic respiration, is molecular oxygen (O_2), however, some of the electrons passing through the chain react prematurely with oxygen, generating $\bullet O_2^-$. Additionally, ROS can be generated from H_2O_2, in the presence of iron, via 'respiratory burst' by Fenton reaction or from H_2O_2 and O_2, by Haber-Weiss reaction (**Figure 3**; Winterbourn, 1995). ROS

can also be generated exogenously due to pollutants, tobacco, smoke, drugs, xenobiotics, or radiation (Kohen & Nyska, 2002).

$$H_2O_2 + Fe^{2+} \rightarrow {}^{\cdot}OH + OH^{-} + Fe^{3+} \quad \text{(Fenton Reaction)}$$

$${}^{\cdot}O_2^{-} + H_2O_2 \rightarrow {}^{\cdot}OH + OH^{-} + O_2 \quad \text{(Haber-Weiss Reaction)}$$

Figure 3. Fenton and Haber–Weiss reactions for ROS production.

DNA is susceptible to different types of damage and one of those is oxidative damage. ROS induced DNA damage can lead to health problems and eventually, disease. ·OH can react with DNA in two different ways (**Figure 4**): Reaction of ·OH with guanine, forming 8-hydroxyguanine which results in a wrong impairment of dsDNA, or reaction with the sugar moiety which leads to an internal cyclization, forming 8,5'-cyclopurine-2'-deoxynucleosides. This kind of interaction results in DNA strand breaks and base-free sites (Nimse & Pal, 2015).

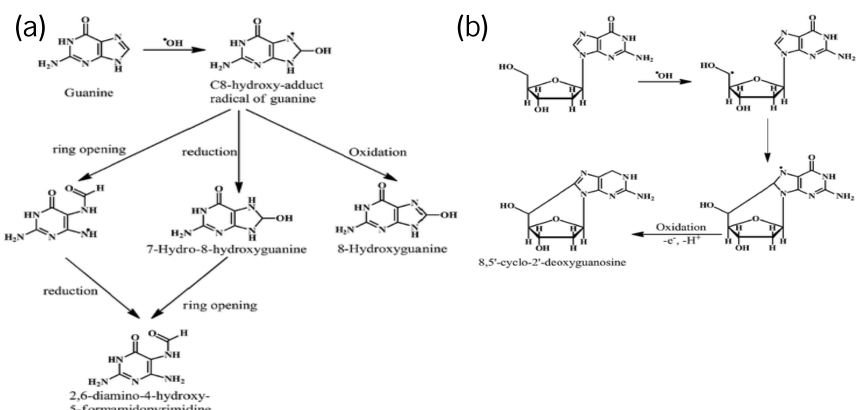

Figure 4. Mechanisms of DNA oxidation by hydroxyl radical. Reaction of ·OH with guanine and (b) reaction of ·OH with the sugar moiety of DNA (Adapted from Nimse & Pal, 2015).

As mentioned before, ROS excessive production can affect the integrity of numerous biomolecules, however polyphenols can be a new weapon in preventing this outcome. Phenolic compounds are known to have protective effects against DNA damage, and they also showed to prevent cardiovascular diseases, inhibiting LDL oxidation.

1.2. Grape phenols

1.2.1. Grape phenols classification according to structure

Polyphenols are probably the largest group of plant secondary metabolites and they are implicated in a lot of plant defence mechanisms. Phenols or phenolic compounds (one or more phenolic rings, respectively) structure consists of one or more hydroxyl groups covalently bonded to an aromatic hydrocarbon group (**Table I**). The hydroxyl group is mainly responsible for the antioxidant activity of phenolic compounds, donating hydrogen atoms to ROS, reducing them.

However, phenols' aromatic structure, with dislocated electrons, allows the stabilization of the radical formed after electron donation, consequently, ROS chain reactions are slowed or even inhibited (Cuvelier *et al.*, 1992). It is also known that the presence of a second OH, at the ortho-position, can increase the rate at which hydrogen is transferred. In addition, some phenolic compounds have an alkyl chain that connects the phenolic ring and the carboxylic or alcohol group, that may also be important in the stabilization the phenoxy radical formed after proton donation (Balasundram & Samman, 2006). Conjugation between the A and B rings allows a resonance effect of the aromatic nucleus that provides stability to the flavonoid radical (Rice-Evans *et al.*, 1996).

Table I. Classes and subclasses of phenols and their respective chemical structure and phenols commonly found in grape berries (Tsao *et al.*, 2010).

Class	Subclass	Basic Chemical Structure	Phenols found in Grape berry
Flavonoids	Anthocyanins		Delphinidin, cyanidin, petunidin, peonidin, pelargonidin and malvidin
	Flavonols		Quercetin, kaempferol and myricetin
	Flavan-3-ols		Catechin, epicatechin, epigallocatechin, epigallocatechin-3-gallate and proanthocyanidins
Phenolic acids (Non-flavonoid)	Hydroxycinnamic acid		caffeic, coumaric and ferulic
	Hydroxybenzoic acid		Gallic, gentisic and salicylic acid
Stilbenes (Non-flavonoid)	Resveratrol		Resveratrol and viniferins

Grape phenols can be classified into two major categories according to structure: flavonoid and non-flavonoid compounds. The former includes anthocyanins, flavonols and flavanols, with a structure composed of three rings ($C_6C_3C_6$). Finally, the latter, the non-flavonoid compounds, includes stilbenes, with two rings ($C_6C_2C_6$), being resveratrol the most studied compound of this class, and phenolic acids, such as hydroxy-benzoic and hydroxycinnamic acids (only one ring) ([1]Teixeira *et al.*, 2013).

1.2.2. Biosynthesis of soluble grape phenols

The biosynthesis of soluble phenols begins with phenylalanine, a product of the shikimate pathway, resulting in cinnamic acid (**Figure 5**) (Tomás-Barberán & Espín, 2001). This compound undergoes a series of transformations until it forms the precursors of phenolic acids. The incorporation of 3 molecules of malonyl-CoA, produced via the acetate pathway, with 4-coumaroyl-CoA starts the phenylpropanoid pathway and these precursors generate complex phenolic compounds, such as flavonoids or stilbenes depending on the intervening enzyme, chalcone synthase (CHS) or the stilbene synthase (SS), respectively (Flamini *et al.*, 2013). While hydroxycinnamic acids syntheses begins with the conversion of coumaroyl-CoA into caffeic acid, by coumarate-3-hydroxylase (C3H).

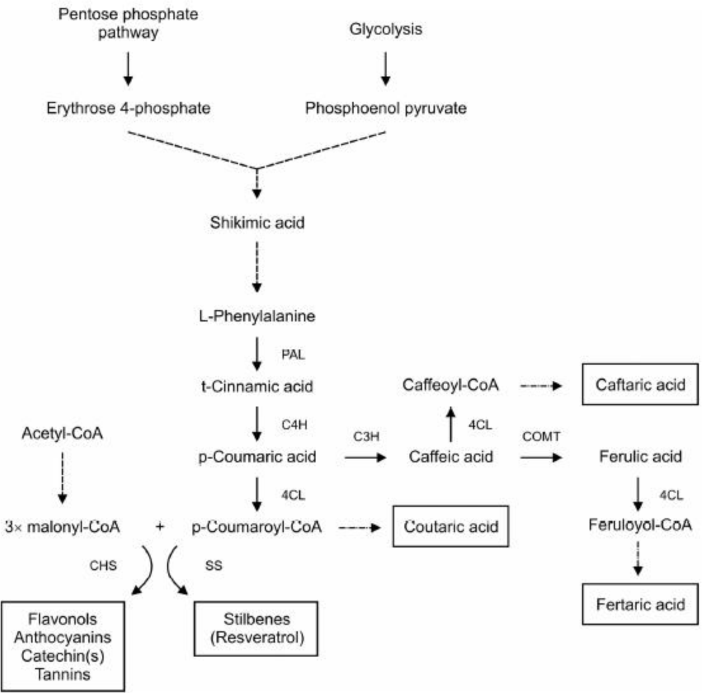

Figure 5. Biosynthetic pathway of the major soluble phenolic classes found in grape berries, including flavonoids, stilbenes and hydroxycinnamic acids. Compounds or classes that accumulate in the fruit are outlined by rectangles. **PAL**, phenylalanine ammonia lyase; **C4H**, cinnamate-4-hydroxylase; **C3H**,

coumarate-3-hydroxylase; **4CL**, 4- hydroxycinnamate:CoA ligase; **COMT**, caffeic acid *O*-methyltransferase; **SS**, stilbene synthase; **CHS**, chalcone synthase. Full lines represent direct enzymatic conversion, dashed lines represent omitted intermediates and dotted lines represent an unknown pathway (Adapted from Conde *et al*, 2007).

1.2.3. Tissue distribution of phenolics in grapes

From a winemaking perspective, the grape berry has four major types of tissue: flesh, skin, seed and pedicel, as illustrated in **Figure 6** (Cadot *et al*. 2006).

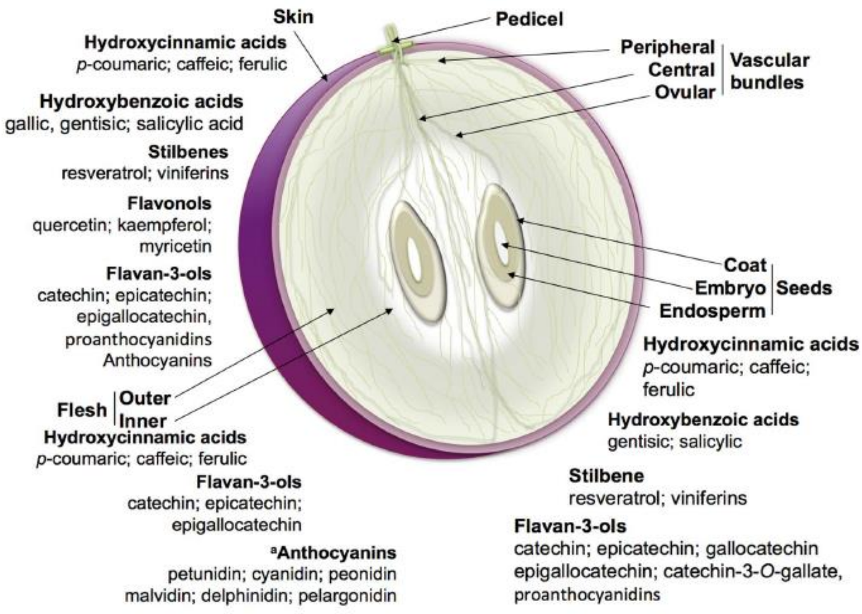

Figure 6. Schematic structure of a ripe grape berry and pattern phenolics biosynthesis distribution between several organs and tissues (indicated by arrows). [a] Anthocyanins are synthetized also in the inner flesh of some tincture varieties (Adapted from [1]Teixeira *et al*., 2013).

The exocarp, most commonly known as skin, is composed by two distinguishable tissues and it possesses between 20 and 30% of the total phenols found in grape (Fontes *et al*., 2011). The outer layer is known as cuticle, a waxy layer that inhibits the loss of water, and the inner layer, the hypodermis, contain most of the skin flavonoids, which include anthocyanins (delphinidin, cyanidin, petunidin, peonidin, pelargonidin), flavonols (quercetin, kaempferol and myricetin) and simple flavan-3-ols (catechin, epicatechin, epigallocatechin), but can also exhibit low concentrations of both stilbenes (resveratrol and viniferins) and hydroxycinnamic acids (*p*-coumaric, caffeic and ferulic acids). The pulp, which is responsible for only 10 to 20% of the phenolic composition, is also composed by an outer and inner layer, called mesocarp and endocarp respectively, and it is rich in hydroxycinnamic acids and flavan-3-ols (catechin, epicatechin, epigallocatechin and

proanthocyanidins), and some *V. vinifera* varieties also possess anthocyanins. The seeds, which are the greatest contributor to the phenolic composition (60%), contain an outer seed coat, the endosperm and the embryo, rich in hydroxycinnamic acids, stilbenes and flavanols. As said before, phenolic content depends on the characteristics of the tissue, therefore different tissue extracts exhibit various bioactivities (Yang *et al.*, 2009). For instance, grape phenolic acids are mainly found in the pulp and they are related to stress response, however flavonoids and stilbenes are predominant both in the peripheral layers of berry pericarp (skin) and in some layers of the seed coat (Montealegre *et al.*, 2006). Flavonoids act as UV and extreme temperature protectants, as well as free radical scavengers, and stilbenes are associated to antifungal capacity (Pastore *et al.*, 2017).

1.3. Phenolics protective effects and bioactivities

Phenolic compounds are known to be related to plant defence mechanisms. Due to their amphipathic characteristics, some polyphenols can scavenge aqueous free radicals, however, as the structure becomes more complex, they are also able to chelate metal ions (**Figure 7**) (Kumar & Pandey *et al.*, 2013).

Figure 7. Modes of action of flavonoids against ROS, either **(a)** by scavenging of ROS or **(b)** by metal chelation (where Me^{n+} represents metal ions) (Adapted from Kumar & Pandey, 2013).

Recent studies have demonstrated that these compounds have nutraceutical properties, such as antioxidant, antimutagenic, anticarcinogenic, anti-inflammatory, antimicrobial, antiangiogenic properties and other protective effects against an array of stresses (**Table II**).

Table II. Classes (phenolic acids, flavonoids and stilbenes) and sub-classes (anthocyanins, flavonols, flavanols, hydroxycinnamic and hydroxybenzoic acids, resveratrol and viniferins) of phenolic compounds and some of their protective roles.

Classes of phenolics	Sub-classes found in Grape berry	Some protective roles	References
Flavonoids	Anthocyanins	Beneficial effects against inflammation, obesity, diabetes, liver fibrosis and cancer due to its antioxidant properties. In plants it appears to have UV-B protective effects.	Su *et al.*, 2018; Sun *et al.*, 2018; Teng *et al.*, 2017;
	Flavonols	Antioxidant, anticancer, inhibits thrombosis and platelet activation, anti-inflammatory, hepatoprotective and neuroprotective.	Alkhalidy *et al.*, 2018; Choi *et al.*, 2015; Devi *et al.*, 2015; Hussein *et al.*, 2018; Krishnamachari *et al.*, 2002; Lesjak *et al.*, 2018; Patel *et al.,* 2018; Shih *et al.*, 2010
	Flavan-3-ols	Antibacterial, cardiac and neuroprotective, improves endothelial function and has antihypertensive properties. Also improves muscle regeneration.	Alshaibani *et al.*, 2017; Bernatova, 2018; Grzesik *et al.*, 2018; Kim *et al.*, 2017; [2]Li *et al.*, 2018; Pons *et al.*, 2016; Silva *et al.*, 2017
Phenolic acids	Hydroxycinnamic	Anti-inflammatory, anticancer and antidiabetic activity. Protection against neurotoxicity, lysosomal dysfunction, myocardial infarct, also inhibits melanogenesis.	Fuentes & Palomo, 2014; [1]Khan *et al.*, 2016; Park *et al.*, 2017
	Hydroxybenzoic		Heleno *et al.*, 2015; [2]Khan *et al.*, 2016; Kris-Etherton *et al.*, 2002;
Stilbenes	Resveratrol and viniferins	Antioxidant, anti-inflammatory, anticarcinogenic and antidiabetic properties. Chemoprotective capacity and ameliorates post-traumatic stress disorder (PTSD).	De Vries *et al.*, 2017; Dilshara *et al.*, 2014; Elshaer *et al.*, 2018; Ji *et al.*, 2018; Jimoh *et al.*, 2018; Kong *et al.*, 2018; [1]Li *et al.*, 2018; Ohara *et al.*, 2015; Öztürk *et al.*, 2017; Qi *et al.*, 2018 and Vion *et al.*, 2018.

All flavonoids have the same diphenyl propane ($C_6C_3C_6$) skeleton but the structural differences can be found in the number or the arrangement of the hydroxyl groups, as well as the alkylation/glycosylation of these groups. The glycosylation of the phenolic compounds allows a more efficient absorption by gastrointestinal track (Chen *et al.*, 2018), protects the hydroxyl groups from auto-oxidation and facilitates excretion (Scalbert *et al.*, 2002). Flavonoids can prevent lipid peroxidation, a consequence of oxidative stress, through the inhibition of highly oxidizing ROS. As flavonoids exhibit lower redox potentials, they can reduce these highly oxidizing species such as superoxide ($\cdot O_2^-$), peroxyl (HO_2), alkoxyl ($\cdot RO$), and hydroxyl radicals ($\cdot OH$) by hydrogen atom donation (Kumar *et al.*, 2013). Due to their capacity to chelate metal ions (iron, copper, etc.), flavonoids also inhibit free radical generation, through the inhibition of Fenton reaction. They can also inhibit the enzymes involved in ROS generation, such as microsomal monooxygenase (MMO), glutathione S-transferase (GSTs), mitochondrial succinoxidase (SO), NADH oxidase (Han *et al.*, 2007). This class of compounds was extensively studied showing a wide range of bioactivities, such as anti-inflammatory, antitumoral, antigenotoxic, antiplatelet and antiallergic, that are associated to their antioxidant properties (Faggio *et al.*, 2017; Park *et al.*, 2004; Raffa *et al.*, 2017; Spagnuolo & Russo, 2017). From this class of compounds, anthocyanins (delphinidin, cyanidin, petunidin and malvidin) have beneficial effects in inflammation, since they inhibit cyclooxygenase (COX 2), an enzyme involved in the inflammation process (Li *et al.*, 2017). Moreover, they help prevent obesity, protect against liver fibrosis (Sun *et al.*, 2018), cardiovascular problems and cancer (Cassidy, 2017; Morais *et al.*, 2016). Anthocyanins differ from other natural flavonoids due to their large range of colours (Gauche *et al.*, 2010). Anthocyanins that lack the *o*-diphenyl structure in the B ring (malvidin, pelargonidin and petunidin) have less efficiency toward the 2,2-diphenyl-1-picrylhydrazyl radical (•DPPH) as compared to cyanidin and delphinidin. For example, cyanidin, delphinidin, pelargonidin, petunidin, malvidin and their glycosides inhibited cell proliferation by blocking cell cycle regulator proteins (e.g., p53, p21, p27, etc.), being new weapons in cancer treatment (Teng *et al.*, 2017). Some can also protect pancreatic β-cells and stimulate insulin secretion, having huge implications on diabetes (Gowd *et al.*, 2017). Flavonols, such as quercetin and kaempferol, are linked to antioxidant, anti-inflammatory and cardioprotective properties and have beneficial effects on diabetes and neurodegenerative diseases (Alkhalidy *et al.*, 2018; Devi *et al.*, 2015; Hussein *et al.*, 2018; Krishnamachari *et al.*, 2002; Lesjak *et al.*, 2018; Patel *et al.*, 2018; Shih *et al.*, 2010). Kaempferol also inhibits thrombosis and platelet activation and has antiproliferative activity (Choi *et al.*, 2015; Liao *et al.*, 2016). Quercetin, a major flavonol commonly

found in berries, displays a wide range of healthy properties, including antioxidant, anti-inflammatory, anti-apoptotic and hepatoprotective. Additionally, flavanols, such as catechin, epigallocatechin and proanthocyanidins, have antihypertensive properties that can be associated with different biological activities, such as nitric oxide (•NO) mediated vasodilation, antioxidant capacity and an inhibition of angiotensin converting enzyme (ACE), which is a key enzyme in the control of blood pressure (Grzesik *et al.*, 2018; Pons *et al.*, 2016). Furthermore, flavanols exhibit antibacterial (Alshaibani *et al.*, 2017), cardiac, hepatic and neuroprotective effects and improve endothelial and muscle regeneration (Bernatova, 2018; Kim *et al.*, 2017; [2]Li *et al.*, 2018; Miltonprabu *et al.*, 2016; Pons *et al.*, 2016; Silva *et al.*, 2017; Han *et al.*, 2007). However, the biological effects of proanthocyanidins should not be attributed to the native compounds present in plants, but rather to their metabolized products, which are diverse phenolic acids. These metabolites are produced during fermentation by the colonic microflora and they are strongly absorbed, contrarily to normal proanthocyanidins (Renard *et al.*, 2017).

Additionally, phenolic acids (phenols with a simple C6 backbone) like hydroxycinnamic (caffeic, coumaric, ferulic, and sinapic acids) and hydroxybenzoic acids (gallic, and salicylic acids) can form complexes with metals (Yilmaz & Toledo, 2004). Despite several mechanisms, the predominant antioxidant mode of action is believed to be by radical scavenging via hydrogen atom donation (Shahidi & Ambigaipalan, 2015). Protective effects against neurotoxicity by phenolic acids have been demonstrated (Huang *et al.*, 2013), mainly due to its antioxidant properties (Khan *et al.*, 2016). Besides, some of its compounds, like coumaric and gallic acids, have anti-inflammatory, anticancer and antidiabetic activity. In addition, they also prevent lysosomal dysfunction, reduce myocardial infarct occurrence and inhibit melanogenesis (Heleno *et al.*, 2015; Khan *et al.*, 2016; Kris-Etherton *et al.*, 2002; Punithavathi *et al.*, 2011). Hydroxycinnamic acids were shown to protect endothelial tissue due to their anti-inflammatory properties (Fuentes & Palomo, 2014; Park *et al.*, 2017).

Ultimately, stilbenes (resveratrol and viniferins) are the most promising of the phenolic compounds present in grapes. For instance, a lot of studies show that resveratrol (3,4′,5-trihydroxy-trans-stilbene) plays a critical role in human health, due to its diverse biological and pharmacological actions including antioxidant, antiinflammation, anticarcinogenic and antidiabetic (Choo *et al.*, 2014; Elshaer *et al.*, 2018; Ji *et al.*, 2018). Also, this compound appears to have implications in post-traumatic stress disorder (PTSD) in model mice (Li *et al.*, 2018; Zhang *et al.*, 2017). Moreover, resveratrol has shown to protect guanine against ROS oxidation preventing DNA

damage and, consequently, cancer (Kong *et al.*, 2018). Additionally, this compound was related to prevention of obesity when mice were subjected to a fat rich diet (Jimoh *et al.*, 2018). Some reports indicate that resveratrol has an alleged role in stimulating insulin excretion, being helpful in diabetes, and it can activate an important protein, nicotinamide adenine dinucleotide (NAD+)–dependent deacetylase SIRT1, related to many neurodegenerative diseases, cancer and diabetes (Hubbard *et al.*, 2013; Öztürk *et al.*, 2017). SIRT1 controls DNA repair and apoptosis, inflammatory pathways, insulin secretion, and mitochondrial biogenesis (Li, 2013; [3]Li *et al.*, 2018). This stilbene attenuates the neurotoxic effects of ethanol, since it exhibits anti-inflammatory properties. Ethanol (EtOH) toxic mechanism of action relies on the activation of the microglia and induction of an inflammatory response, which resveratrol can inhibit (Qi *et al.*, 2018). Therefore, resveratrol could be an important tool to control these diseases and toxic effects, however, some studies report that resveratrol has a very low bioavailability reducing its biological effectiveness (De Vries *et al.*, 2017). Viniferins are resveratrol dimers and they also have implications in the treatment of diabetes (Ohara *et al.*, 2015). This compound has anti-inflammatory properties that may be important for the prevention and treatment of neurodegenerative diseases (Dilshara *et al.*, 2014; Vion *et al.*, 2018). It can also have implications in cancer treatment, since it exhibits antiproliferative activity (Cheng *et al.*, 2018; Nivelle *et al.*, 2018).

Furthermore, synergistic action of flavonoids must be also considered. For example, antiplatelet activity is enhanced by interactions between grape seeds and grape skin compounds (Shanmuganayagam *et al.*, 2002). Several studies reported that synergistic effects of the most potent anticarcinogenic polyphenols (e.g. curcumin, resveratrol and quercetin) with other less potent anticarcinogenic polyphenols, increased therapeutic effects (Brglez *et al.*, 2016).

1.4. Aim

Bearing in mind that there is insufficient data regarding potential beneficial effects of Portuguese wines and cultivars and there is an increasingly interest in natural compounds and re-use of agricultural wastes. The aim of this work is to determine different bioactivities of seeds and pulp and skin extracts of two Portuguese wine cultivars, Vinhão and Loureiro, a red one and a white one respectively. The bioactivities assessed include antioxidant, antigenotoxic and antimutagenic. Also, evaluate and compare total phenolic and flavonoid contents of all four extracts and correlate with the different bioactivities measured. This work can improve the potential introduction of these extracts in pharmaceuticals, cosmetics and nutraceuticals, creating new applications for these varieties grown in Portugal, as well as their wine production wastes. To achieve the proposed goals, different assays were used:

1- Folin-Ciocalteu method for the determination of the total phenolic content of both berry (pulp and skin) and seeds extracts, of Vinhão and Loureiro cultivars. Also, assess total flavonoid content of all extracts by a colorimetric method;
2- Furthermore, evaluation of the antioxidant activity of all extracts by DPPH assay, reducing power assay and by flow cytometry;
3- Determination of grape extracts' chelation capacities;
4- Moreover, estimation of the genotoxicity and antigenotoxicity of all extracts by DNA topology assay;
5- Measurement of the antigenotoxic and antimutagenic potential of Vinhão and Loureiro seeds extracts by comet assay and AMES test, respectively.

2. Materials and Methods

2.1. Extract preparation

Two cultivars from 'vinho Verde' region in Portugal were selected for this study due to their contrasting characteristics. On one hand, Vinhão, dark coloured rich in anthocyanins cultivar, and 'Loureiro', a white one from the same region. Grapes were collected from a vineyard near Braga, Portugal in September 2017. Initially, the seeds (9.43 g of Vinhão and 7.27 g of Loureiro) from both cultivars, were separated from the rest of the fruit (27.92 g of Vinhão and 24.84 g of Loureiro) and both parts were deep frozen in liquid nitrogen and turned into a powder with the IKA A 11 basic Analytical mill. Extraction of the phenolic compounds was made with 40 ml 70% (v/v) ethanol to each sample, sonication for 5 min and incubation at room temperature for 60 h with agitation (200 revolutions per minute - rpm). Afterwards, the four extracts were filtrated by low pressure vacuum and the solvent was evaporated in a rotary evaporator at 48°C. The resulting powder was dissolved in a small amount of water and it was lyophilized to remove the excess water. Four extract stock solutions (50 mg/mL) were prepared with 50 mg lyophilized material in 1 mL ethanol 70%. Two for 'Vinhão' cultivar, Vinhão pulp and skin (VB) and Vinhão seeds (VS) extracts, and two for 'Loureiro' cultivar, Loureiro pulp and skin (LB) and Loureiro seeds (LS) extracts.

2.2. Total phenolic content (TPC) evaluation by Folin-Ciocalteu method

10 µl of each extract (50 mg/mL, for VB and LB, and 5 mg/mL, for VS and LS) and 100 µL of 1:10 Folin-Ciocalteu reagent were mixed into a 96 well microplate and the mixture was left to react for 5 min. The same thing was done for five different concentrations of gallic acid (50, 100, 250, 500 and 750 µg/mL). Then, 80 µL of 1 M Na_2CO_3 were added to each well and the plate was kept in the dark for 1 hour. The absorbances of the mixtures were read with a Synergy™ HTX Multi-Mode Microplate Reader at 760nm. A standard calibration curve was elaborated with the absorbances of each concentration of gallic acid tested (**Appendix A**). The different absorbances corresponding to the different extracts were used to calculate the concentration of extract in gallic acid equivalents (GAE) and total phenolic content (TPC) was calculated according to the formula:

$$TPC = Csample \frac{V\ extract}{m\ lyophilized\ material}$$

Where, C_{sample} represents the concentration in mg of GAE/mL calculated with the standard calibration curve, $V_{extract}$ is the volume of solvent, in mL, used to dissolve a certain mass, in mg of lyophilized extract ($m_{lyophilized\ material}$).

2.3. Total flavonoid content (TFC) evaluation by colorimetric method

Total flavonoid content determination was performed as Li *et al.* (2015) with some modifications. Briefly, 125 µL of each extract (50 mg/mL, for VB and LB, and 5 mg/mL, for VS and LS) were separately mixed with 75 µL of 5 % $NaNO_2$ and the mixture was left to react for 6 min. 150 µL of 10% $AlCl_3$ where added to each tube to allow the formation of the complex, which was left to react for 6 min. 750 µL of NaOH were added to each sample and, finally, water was added until a final volume of 2.5 mL. The same thing was done for seven different concentrations of quercetin (5, 10, 25, 50, 100, 250, 500, 750 and 1000 µg/mL). The absorbance of the reaction was recorded at 420 nm. A standard calibration curve was elaborated with the absorbances of each concentration of quercetin tested (**Appendix B**). The different absorbances corresponding to the different extracts were used to calculate the concentration of extract in quercetin equivalents (QE) and total flavonoid content (TPC) was calculated according to the formula:

$$TFC = Csample \frac{V\ extract}{m\ lyophilized\ material}$$

Where, C_{sample} represents the concentration in mg of QE/mL calculated with the standard calibration curve, $V_{extract}$ is the volume of solvent, in mL, used to dissolve a certain mass, in mg, of lyophilized extract ($m_{lyophilized\ material}$).

2.4. Determination of antioxidant activity and chelation properties

2.4.1. 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay

The procedure was performed as Mishra *et al.* (2012) with some modifications. 950 µL of 0.1 mM DPPH ethanolic solution were added to 50 µL of different concentrations of the four extracts in 70% ethanol (50, 100, 250, 500, 750, 1000 µg/mL). Similarly, samples with different concentrations (50, 100, 250, 500, 750, 1000 µg/mL) of a standard compound (gallic acid) were prepared. A blank and a negative control were prepared with 50 µL of 70 % ethanol plus 950 of 100% ethanol, and with 50 µL of 70 % ethanol plus 950 of DPPH, respectively. Solutions were incubated for 20 min in the dark at room temperature and the absorbance of the samples was measured at 517 nm with a Genesys 20 spectrophotometer. The percentage of DPPH scavenging activity was calculated according to the following equation:

$$\%\ Radical\ scavenging\ activity = \frac{Abs\ control - Abs\ sample}{Abs\ control} x100$$

Where, $Abs_{control}$ corresponds to the measurements of the negative control, while Abs_{sample} is the absorbance measured for each sample tested including the standard compound.

2.4.2. Reducing power assay (RP)

Reducing power assay was performed as Vijayalakshmi *et al.* (2016) with a few alterations. 100 µL of different concentrations of the ethanolic extracts (500, 750, 1000, 1500, 2500, 5000 µg/mL, for VB and LB, and 10, 25, 50, 100, 250 and 500 µg/mL, for VS and LS) were added to 500 µL of 0.2 M sodium phosphate buffer (0.14 M NaCl, 2.7 mM KCl, 0.01 M Na_2HPO_4 and 1.8 mM KH_2PO_4, pH 6.6) and 500 µL of 1% ferricyanide. The same was done for the negative control which contained only ethanol 70 %, and for the standard compound, different concentrations of gallic acid (10, 25, 50, 75, 100, 250, 500, 750 and 1000 µg/mL). The mixtures were incubated for 20 min at 50 °C. Afterwards, 500 µL of 10% TCA were added and the samples were centrifuged for 10 min at 3000 rpm with an Eppendorf 5804 Benchtop Centrifuge. Then, 500 µL of the supernatant was removed and added to 0.1 mL of 0.1 % ferric chloride and 500 µL of water. After waiting 10 min the absorbance of the different samples was read at 700 nm with a Genesys 20 spectrophotometer. The results were converted into plots of the absorbance versus concentrations of the different extracts.

2.4.3. Iron chelation assay

The assay was performed as Lim *et al.* (2007) with some modifications, where 100 µL of plant extract (500, 750, 1000, 1500, 2500, 5000 µg/mL, for VB and LB, and 10, 25, 50, 100, 250 and 500 µg/mL, for VS and LS) were added to 50 µL 2 mM ferrous chloride and 200 µl of 5 mM ferrozine solution. The same thing was done for the negative control, with 100 µL ethanol 70 %, and for the standard compound, 100 µL of different concentrations of gallic acid (10, 25, 50, 100, 250, 500 and 1000 µg/mL). The solutions were mixed thoroughly and incubated in the dark at room temperature for 10 min. The absorbance was read at 562 nm with a Genesys 20 spectrophotometer.

The percentage of inhibition of ferrozine–Fe^{2+} complex formation was calculated resorting to the equation:

$$\%\ inhibition\ of\ complex\ formation = \frac{Abs\ control - Abs\ sample}{Abs\ control} x100$$

Where, $Abs_{control}$ corresponds to the measurements of the negative control, while Abs_{sample} is the absorbance measured for each sample tested including the standard compound.

2.4.4. Flow cytometry

A pre-culture of BY4741 *Saccharomyces cerevisiae* strain, *MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*, obtained from a stock culture on solid YPDA medium (1% of yeast extract, 2% peptone, 2% dextrose and 2% agar), was prepared with 5 mL of liquid YPD medium (1% of yeast extract, 2% peptone and 2% dextrose) and incubated overnight at 30°C, 200 rpm. The pre-culture was diluted with fresh YPD medium to an OD_{600} of 0.1, for a final volume of 10 mL, and incubated under the same conditions until an OD_{600} between 0.4 and 0.8 (more than 3 h). The cells were then harvested by centrifugation at 5000 rpm and 4 °C for 2 min, with Eppendorf 5810 centrifuge, and washed twice with the same volume of fresh PBS (137 mM NaCL, 2.7 mM KCl, 4.3 mM Na_2HPO_4, pH 7.4). The cell suspension was diluted to an OD_{600} of 0.02 to the same final volume and 500 µL were kept aside to determine cells autofluorescence (AF). 100 µL of 50 µM 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) were added to the cell suspension which was incubated at 30 °C, 200 rpm for 1 h in the dark. Cells were harvested again by centrifugation at 5000 rpm and 4 °C for 2 min, washed and resuspended in the same volume of fresh PBS. 450 µL of cell suspension were transferred into each aliquot containing 25 µL of extract (25, 50, 100, 250 or 500 µg/mL, for VB or LB, or 1, 10, 25, 50, 100 µg/mL, for VS or LS) and 25 µL of H_2O_2. For the controls the cell culture was added to solvents only (25 µL of ultra-pure water and 25 µL of ethanol 70%), corresponding to the negative control, and another with 25 µL H_2O_2 and 25 µL of ethanol 70%, which corresponds to the positive control. The treatments lasted 20 min in the dark at 30 °C and 200 rpm. The samples were then analysed by flow cytometry in a CytoFlex Beckman Coulter cytometer, with a 525/40 BP fluorescence channel and an excitation wavelength of 488 nm.

2.5. Determination of antigenotoxic activity

2.5.1. DNA topology assay

To evaluate the genotoxicity of the extracts, 1 µL of plant extract (500, 750, 1000 or 1500 µg/mL, for VB or LB, or 50, 100, 250 or 500 µg/mL, for VS or LS) was added, separately, to 7 µL of dH_2O, 1 µL of 0.5X TBE buffer (0.9 M H_3BO_4, 0.9 M Tris-HCl and 0.02 M EDTA) and 1 µL of 1:10 plasmid DNA pBR322 (stock solution 1mg/mL). For the antigenotoxicity, 1 µL of 1 mM $FeSO_4$ was added to the mixture for the same final volume. Controls were made in the presence and absence of $FeSO_4$. All the samples were left to incubate for 15 min and were then mixed with 2 µL 6x gel loading dye (2.5% Ficoll®-400, 11 mM EDTA, 3.3 mM Tris-HCl, 0.017% SDS and 0.015% bromophenol blue pH 8) and submitted to an 1% agarose gel electrophoresis at 50 V for 90 min.

In the preparation of the agarose gel 10 µL of 10000x in water GelRed were added to mark the DNA and the gel was observed under UV light with a VWR GenoSmart Gel documentation system.

2.5.2. Comet assay

The alkaline comet assay was performed as described by Wojewódzka *et al.* (2002) with some modifications. Blood was obtained by capillary puncture from one female subject (age 22, non-smokers), divided into 30 µL aliquots with 1 mL of PBS (137 mM NaCL, 2.7 mM KCl, 4.3 mM Na_2HPO_4, pH 7.4) and kept on ice for 30 min. Lymphocytes were isolated adding 100 µL of Histopaque 1077 (Sigma) to each aliquot, which creates a density gradient through centrifugation at 200 *g*, 2 °C for 3 min (lymphocytes are present in the middle layer formed, called buffy layer). 200 µL of the buffy layer formed were added to another aliquot with 1 mL of fresh PBS, proceeding to centrifugation, at the same conditions as before, and discarding of the supernatant to isolate the lymphocytes. Next, 140 µL of 1.5% Genbiotec low melting point agarose dissolved in PBS were added, slowly, to each aliquot containing the lymphocytes, then divided into to two drops which were placed onto a previously coated glass slide, with 0.5 % normal melting point agarose dissolved in water, and the drops were covered with coverslips. After waiting 10 min at 4 °C, the coverslips were removed and 1 mL of extract (100, 250, 500, 750 or 1000 µg/mL, for VB or LB, or 10, 50, 100, 250 or 500 µg/mL, for VS or LS) was added on top the slides, two slides for each concentration (one for genotoxicity assessment and another for antigenotoxicity evaluation). Two slides were kept on PBS to serve as controls. The treatment lasted 1 h and by 55 min 1 mL of 0.1 M H_2O_2 was added to the positive control slide and half of the slides already subjected to the extracts. Thereafter, slides were washed with PBS and submerged in 100 mL of lysis solution (2.5 M NaCl, 10 mM Tris, 130 mM EDTA) and 1 mL of Triton 100x, for 1 h at 4 °C. The slides were washed again with PBS and transferred to an electrophoresis tank filled with electrophoresis solution (NaOH 10 M, EDTA 200 mM). The tank was kept at 4 °C for 20 minutes and the electrophoresis was performed for 30 min at 25 V and between 260 and 320 mA. Afterwards, the slides were kept on PBS for 5 min, subsequently for 5 min in deionized water and they were left to dry for 24 h. 10 µL of 100mM GelRed were added to each slide, the slides were covered and visualized on an Olympus BX51 fluorescence microscope, with 40x magnification. The size of the lymphocytes and the distance of migrated DNA (size of the tales) were evaluated with *CometScore*.

2.5.3. Determination of antimutagenic activity: Ames test

All strains were originally derived from *Salmonella typhimurium* LT2, however they possess different types of mutation in the histidine operon, as can be seen in **Table III**, *his*C6610 and *his*O1242 for TA97, *his*D3052 for TA98 and *his*G46 for TA100. Both TA97 and TA98 show a frameshift type of mutation, the first is characterized by a run four GC and the second by a run of three G. On the other hand, TA100 has a run of six C in the histidine gene. These DNA sequences work as mutational hot-spots, allowing the evaluation of a compound's mutagenicity, since it restores the wild-type genotype (Imray & Macphee, 1976; Levin *et al.*, 1986). In addition to the histidine mutation the standard tester strains contain other mutations that greatly increase their ability to detect mutagens (Chłokiewicz *et al.*, 2005).

Table III. *Salmonella typhimurium* strains TA97, TA98 and TA100 genotypes, types of mutation in the histidine gene and DNA target (Aiub *et al.*, 2004).

Strain of *Salmonella typhimurium*	Genotype	Type of mutation	DNA target	Mutagen used
TA97	*hisC6610 hisO1242 rfa ΔuvrB chl bio* pKM101	frameshift	CGCGCGCG	9AA
TA98	*hisD3052 Rfa ΔuvrB chl bio* pKM101	frameshift	GGG	4NOP
TA100	*hisG46 rfa ΔuvrB chl bio* pKM101	Base pair substitution	CCCCCC	NaN_3

Pre-cultures of TA97, TA98 and TA100 *Salmonella typhimurium* strains were cultured overnight in 25 mL of LB medium (1% Bacto tryptone, 0.5% Bacto yeast extract and 17 mM NaCl), at 37 °C. To evaluate de mutagenicity of Vinhão and Loureiro extracts, 100 µL of each culture (TA97, TA98 and TA100) (approximately $1x10^8$ cells) were separately pipetted into 3 mL top agar (0.6% agar, 0.09 M NaCl and 72 µM of ampicillin) tubes liquified (autoclaved) in hot water bath (57 °C) and the mixture was vortexed. Before pouring the top agar with bacteria into labelled minimal glucose agar plates (0.2% glucose, 57 mM K_2HPO_4, 1 mM $C_6H_8O_7.H_2O$, 1.7 mM $MgSO_4$, 17 mM $NaHNH_4(PO_4.4H_2O)$ and 1.5 % agar), 50 µL of extract (5, 50 and 500 µg/mL for VS and LS) were added to the mixture. The top agar was gently distributed over the surface of the plate and it was left to dry for 10 min (Maron & Ames, 1983). To evaluate the extracts' antigenotoxicity, different known mutagens were also added to the top agar before pouring. For each strain one specific mutagen was used, 9-aminoacridine (9AA), for TA97, 4-nitro-*o*-phenylenediame (4NOP) for TA98 and sodium azide (NaN_3) for TA100. Different volumes of mutagen were added, 100 µL, 50 µL

and 20 µL, respectively, for a final concentration of 50 µg/plate. The plates were incubated for 48h at 37 °C, after which the colonies were counted. Control plates of bacteria with no treatment (C-) and control plates for each strain in the presence of the respective mutagen (C+) were also prepared (Miadokova *et al.*, 2010).

2.6. Statistical analysis

Three replicates were made for all experiments and the results were analysed with ANOVA statistical analysis of *GraphPad Prism 7*. From where, P, which is used to quantify the idea of statistical significance, and F value, that indicates the ratio of the variance of the group means to that of the pooled within group variance, were retrieved. In the comet assay, the statistical analysis corresponds to 20 lymphocytes measured in one replicate. The statistical analysis is represented either by different letters when there are significant differences between samples measured or by * when there are significant differences between samples, $P<0.05$, and ns when the difference is not significant, $P>0.05$

2.6.1. T-test analysis

A t-test is a type of inferential statistic which is used to determine if there is a significant difference between the means of two groups which may be related in certain features. This analysis was used to compare total polyphenolic and flavonoid contents for each extract and for the comparison of IC50 values between extracts of the same variety and between extracts of the same tissue of both varieties.

2.6.2. Two-way ANOVA Bonferroni's test

The two-way analysis of variance is a multiple comparison analysis that allows the evaluation of the influence of two different categorical independent variables on one continuous dependent variable. Particularly, it was used to study the effect of treatment used and the concentrations tested for each treatment on a dependent variable (e.g. % of reduced DPPH). If multiple hypotheses are tested, the chance of a rare event increases, and therefore, the likelihood of incorrectly rejecting a null hypothesis increases. Bonferroni correction compensates for that increase by testing each individual hypothesis. Bonferroni test allows the evaluation of significant differences between each treatment and each concentration used, with less error. This analysis was carried out for DPPH, reducing power and iron chelation assays.

2.6.3. One-way ANOVA Dunnett's test

Dunnett's test is a multiple comparison analysis used to compare each treatment with a single control. This test was used in flow cytometry, comet assay and Ames test, since cells treated with extract only were compared to the negative control and cells treated with genotoxic agent and extract were compared to the positive control.

2.6.4. *CytExpert*

The *CytExpert* software allows the flow cytometer's operation, the collection of experiment data, and the analysis of the results. This software creates statistical tables for each sample comparing them to control samples. In this case, samples and negative control (cells without treatment) were compared to positive control (cells treated with genotoxic agent). Results are presented in percentage of cells left to the positive control (cells with less fluorescence=cells less oxidized).

3. Results and discussion

3.1. Determination of total phenolic and flavonoid content (TPC and TFC)

The absorbances measured for each extract concentration were used to calculate the extract's concentration of polyphenols in gallic acid equivalents (GAE) and total phenolic content (TPC) in mg of GAE/ g of lyophilized extract. Also, total flavonoid content (TFC) was evaluated, which was calculated in a similar way, but, instead of GA as standard compound, quercetin was used. Standard deviations for each extract are also presented in **Table IV**. C_{sample} values were determined from the linear regression of gallic acid calibration curve (y=0.0024x-0.0104; r^2 = 0,9998) and TFC from the calibration curve of quercetin (y = 0.001x + 0.032; r^2 =0.9862) and expressed in mg of Equivalents/g of lyophilized extract.

Table IV. Total phenolic content (TPC), in mg of gallic acid equivalents (GAE) per gram of lyophilized material, and total flavonoid content (TFC), in quercetin equivalents (QE) per gram of lyophilized material for Vinhão pulp and skin extract (VB), Loureiro pulp and skin extract (LB), Vinhão seeds extract (VS) and Loureiro seeds extract (LS). Also, percentage of TFC/TPC values for each extract. SD from the mean, n = 3. Statistical analysis is represented by different letters when there are significant differences between samples, P<0.05, and the same letter when the difference is not significant, P>0.05. t-Test analysis between extracts from the same variety and extracts from the same tissue of different varieties.

Sample	TPC mg GAE/g lyophilized extract	TFC mg of QE/ g of lyophilized extract	TFC/TPC (%)
VB	18.68±0.41[a]	13.66±2.05 [a]	73.13
LB	1.58±0.36[b]	0.87±1.55 [b]	55.06
VS	209.38±6.39[c]	218.20±8.37[c]	100
LS	239.36±9.43[d]	220.30±8.53[c]	92.04

The P is used to quantify the idea of statistical significance of variance of the group means to that of the pooled within group variance. Loureiro pulp and skin (LB) extract showed a significant lower TPC value (1.58±0.41 mg of GAE/g extract) than the rest of the extracts, while the red variety, Vinhão pulp and skin extract (VB; 18.68±0.36 mg GAE/g extract), showed almost 12x higher TPC value than LB (P<0.0001). Both seeds extracts demonstrated to have a significantly higher TPC value than pulp and skin extracts (209.38±6.39 mg of GAE/g extract for VS and 239.36±9.43 mg of GAE/g extract for LS; P<0.0001). There were also significant differences in

phenolic content between the seeds extracts of the two varieties, the Loureiro seeds showing higher content of these compounds (P=0.0104). Likewise, LB showed the lowest flavonoid content, of 0.87±1.55 mg QE/g of lyophilized extract, significantly lower than VB (P=0.001), 13.66±2.05 mg QE/g of lyophilized extract, however, both significantly different of seeds extracts (P<0.0001). VS and LS showed a flavonoid content of 218.28±8.37 and 220.31±8.53 mg QE/g of lyophilized extract, respectively, and there are no significant differences between TFC values of seeds extracts (P=0.7772).

Furthermore, if we evaluate the factor TFC/TPC in percentage (**Table IV**), LB shows the lowest percentage of flavonoid content in total phenols (55%). On the other hand, VB has 73% of flavonoids in total phenols, showing significant differences between quantifications (P=0.0141). Also, both seeds extract showed 100% or almost of TFC/TPC, suggesting that their phenols content is almost exclusively flavonoids. However, differences between TFC and TPC values for LB, VS and LS are not significant (**Figure 8**) (P=0.479, P=0.220 and P=0.0604, respectively). According to the literature, red varieties are known to have higher TPC values than white varieties, mostly due to the presence of anthocyanins ([1]Teixeira *et al.*, 2013). Also, the 30% left of phenolic content in VB might be attributed to phenolic acids present in the pulp of the fruit (Klapa, 2015). Loureiro variety is known to have certain compounds, such as quercetin-3-*O*-glucoside, quercetin3-*O*-rutinoside, kaempferol-3-*O*-rutinoside, kaempferol-3-*O*-glucoside and epicatechin, which may contribute to flavonoid content (Dopico-García *et al.*, 2007; Mazza *et al.*, 1999; Ortega-Regules *et al.*, 2006). However, LB must have phenolic acids also present in the pulp of the fruit, such as gallic acid and stilbenes, as trans-resveratrol, that contribute to almost 50% of the total phenolic content such as demonstrated by Klapa, 2015. Montealegre *et al.* (2006) reported that seeds consist almost exclusively of flavan-3-ols. This explains the high percentage of TFC/TPC values for VS and LS (Klapa, 2015).

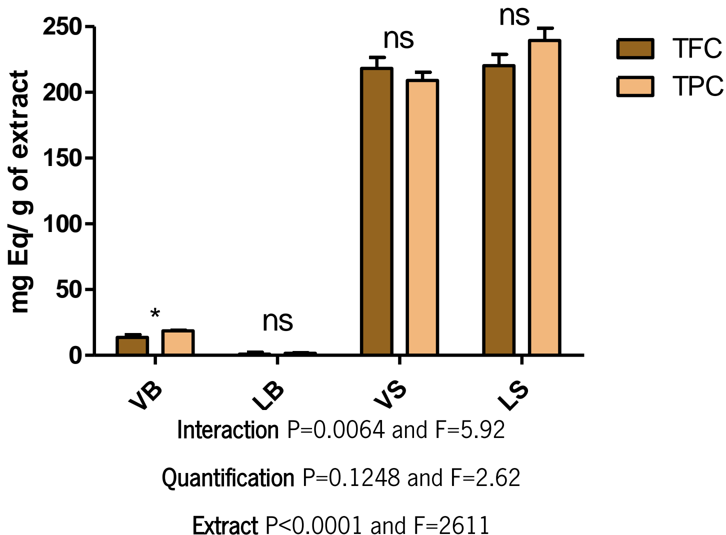

Figure 8. Total phenolic content (TPC) and total flavonoid content (TFC) in mg of gallic acid equivalents or quercetin equivalents, respectively, per g of lyophilized extract of Vinhão and Loureiro pulp and skin (VB and LB, respectively) and seeds extracts (VS and LS, respectively). Error bars denote the SD from the mean, n = 3. Statistical analysis is represented by * when there are significant differences between samples, P<0.05, and the ns when the difference is not significant, P>0.05. t-Test analysis between types of quantification for each extract.

All extracts, except Loureiro pulp and skin extract, seem to have higher phenolic content than other varieties already tested. Adding that seeds extracts have almost twice as much polyphenols as seeds extracts from varieties grown in Jordan, which include Scion, Baladi, Suri Baladi, among others (El-Elimat *et al.*, 2018). Also, VB extract, which is composed by skin and pulp, showed higher TPC values than skin extracts from other well-known varieties, such as Merlot, Pinot Noir, Sangiovese and Cabernet Sauvignon (Guendez, 2005; Rockenbach *et al.*, 2011). This might be due the presence of anthocyanins in the pulp that can contribute to phenolic content. Therefore, Vinhão cultivar wastes can be great sources of polyphenols for cosmetic and nutraceutical applications.

3.2. Evaluation of antioxidant and chelation properties

3.2.1. ROS scavenging activity - DPPH assay

DPPH assay is the most used assay to estimate proton donation capacity of a compound/extract. DPPH, 2,2-diphenyl-1-picrylhydrazyl, is a stable free radical purple solution, that turns yellow when reduced (**Figure 9**).

Figure 9. Principle of DPPH radical scavenging capacity assay (Adapted from [2]Teixeira *et al.*, 2013).

The results were expressed in xy plots (**Figure 10**) to obtain linear regression equations for the IC_{50}, the concentration needed to reduce 50% of the DPPH (**Table V**), calculation. Also, a column plot was elaborated to compare the antiradical activity of the extracts with gallic acid.

In **figure 10 a, b, c** and **d**, it is possible to analyse any dose/effect relation, through the slope of the linear regression. For instance, LB (**Figure 10b**) showed a slope not significantly different from zero, suggesting that this extract does not exhibit any scavenging activity (P=0.0788). On the other hand, all extracts showed a slope significantly different from zero (P<0.0001). Furthermore, a multiple comparison analysis between all extracts and GA (**Figure 10 e**) revealed that VS and LS have the same scavenging potential of GA, at 500 µg/mL or higher. Also, VS and LS did not show any significant differences in reducing capacity for the same concentrations. However, seeds extracts seem to have a significantly higher scavenging capacity than berry extracts.

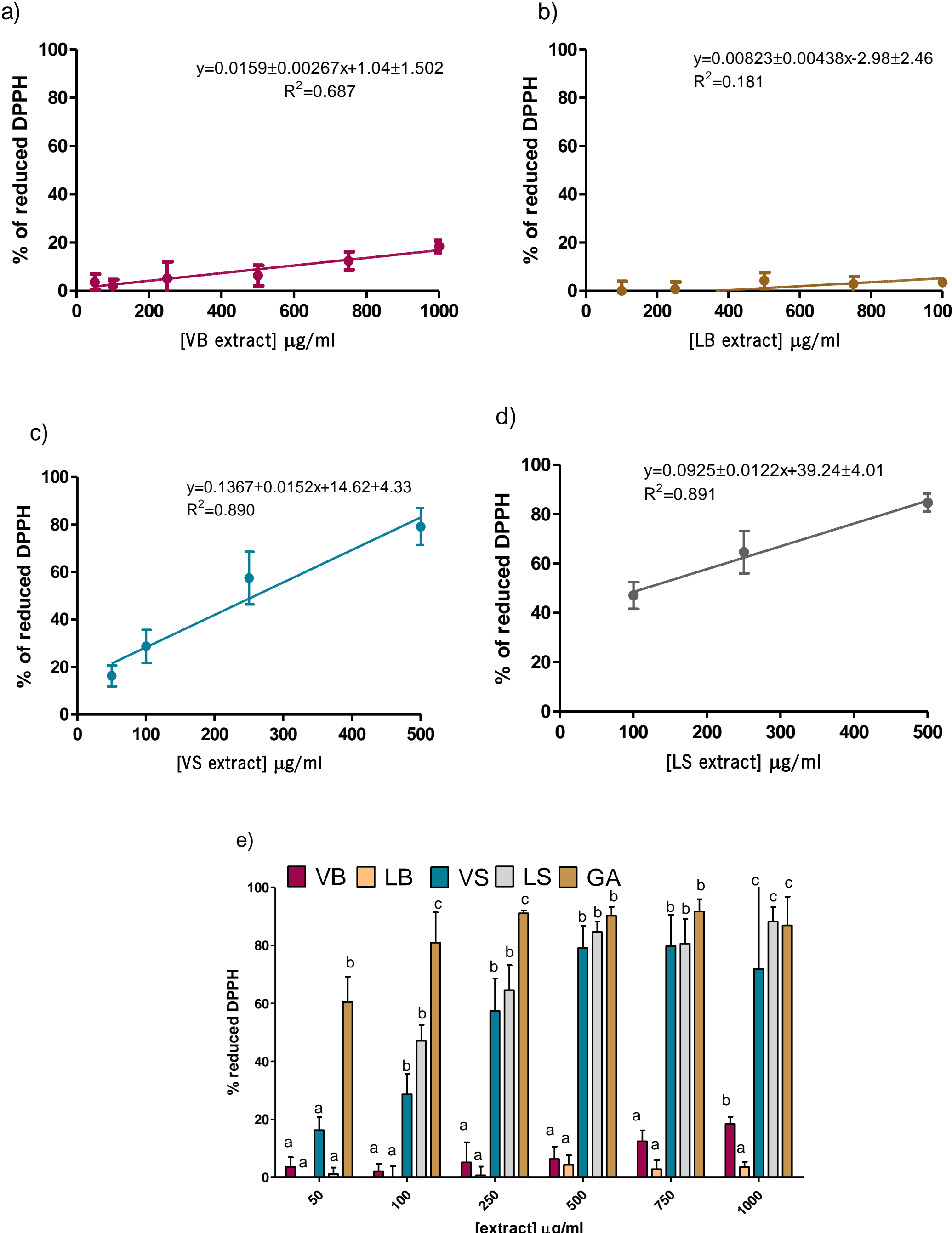

Figure 10. Scavenging activity of all extracts (50, 100, 250, 500, 750 and 1000 µg/mL), with the respective linear regression equation, of **(a)** Vinhão pulp and skin extract (VB), **(b)** Loureiro pulp and skin extract (LB), **(c)** Vinhão seeds extract (VS), **(d)** Loureiro seeds extract and **(e)** graphical comparison of all extracts with the standard compound gallic acid (GA). Gallic acid linear regression equation is y=0.409±0.157x+40.07±12.44. Error bars denote the SD from the mean, n = 3. Statistical analysis is represented by different letters when there are significant differences between samples, P<0.05, and the same letter when the difference is not significant, P>0.05.

Loureiro pulp and skin extract (LB) showed an IC_{50} of 6.02±3.84mg/mL, significantly higher than Vinhão pulp and skin extract, of 3.11±0.30 mg/mL (P=0.0437; **Table V**). Contrarily, Loureiro seeds (LS) extract showed an IC_{50}, of 0.247±0.207, significantly lower than Vinhão seeds extract, of 0.349±0.068, suggesting a higher scavenging activity (P=0.0016). These results correlate with the polyphenolic and flavonoid quantification, since pulp and skin extract from the red variety showed higher TPC and TFC values than the white variety. Moreover, Loureiro seeds extract showed a significantly higher TPC and TFC values than Vinhão seeds extract. Seeds extracts from both varieties showed lower IC_{50} values than pulp and skin extracts (P<0.0001 for Vinhão extracts and P=0.0054 for Loureiro extracts). There were also recorded significant differences between all extracts and the standard compound (P<0.05), however Loureiro seeds extract showed less difference than the remain extracts (P=0.0108 for LS and P<0.005 for the remain extracts). Also, seeds extracts showed almost twice the scavenging activity of seeds extracts from other varieties, such as Cabernet Sauvignon and Merlot (Guendez, 2005). Which is in accordance to the comparison of TPC results with other known varieties, which is also twice as high.

Table V. Values of IC_{50} in mg/mL calculated for Vinhão pulp and skin extract (VB), Loureiro pulp and skin extract (LB), Vinhão seeds extract (VS), Loureiro seeds extract and gallic acid (GA), a standard compound with the respective SD from the mean, n = 3. Statistical analysis is represented by different letters when there are significant differences between samples, P<0.05.

Sample	IC_{50} mg/ml
VB	3.11±0.30 [a]
LB	6.02±3.84 [b]
VS	0.349±0.068 [c]
LS	0.247±0.207 [d]
GA	0.029±0.021 [e]

3.2.2. Reducing power properties

The reducing power assay is based on the ability of an antioxidant molecule to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) (Moein *et al.*, 2008), which allows the formation of a coloured complex (blue or green) between the antioxidant, potassium ferricyanide, trichloroacetic acid and Fe^{2+} (Zhang *et al.*, 2011). This leads to an increase in absorbance, which is measured at 700 nm by UV-Spectrophotometry. A rise in absorbance correlates with an increased antioxidant activity.

Results of reducing power, measured as Fe^{3+} reduction, are represented in **Figure 11**. For both pulp and skin extracts results are not shown, since the slope was not significantly different from zero (P=0.073 for VB and P=0.117 for LB), suggesting that these extracts do not exhibit reducing power capacity. In **Figure 11**, it is possible to observe extracts' reducing capacity and through the slopes of the linear regressions it is possible to compare activities between extracts and the standard compound. Both seeds extracts showed a slope significantly different from zero (P<0.0001), suggesting some reducing power capacity (**Figure 11a and b**). However, Vinhão seeds extract showed 1/6 of the gallic acid activity, while Loureiro seeds extract showed ¼ of the reducing capacity of the standard compound and, since gallic acid is an isolated compound, these results suggest a high reducing power capacity by the extracts evaluated. This correlates with phenolic quantification, since ¼ of the lyophilized material is polyphenols (**Table IV**). Also, both seeds extracts seem to show the same reducing power capacity as seeds methanolic extracts from Bangalore blue grapes, typically grown in India (Jayaprakasha *et al.*, 2001).

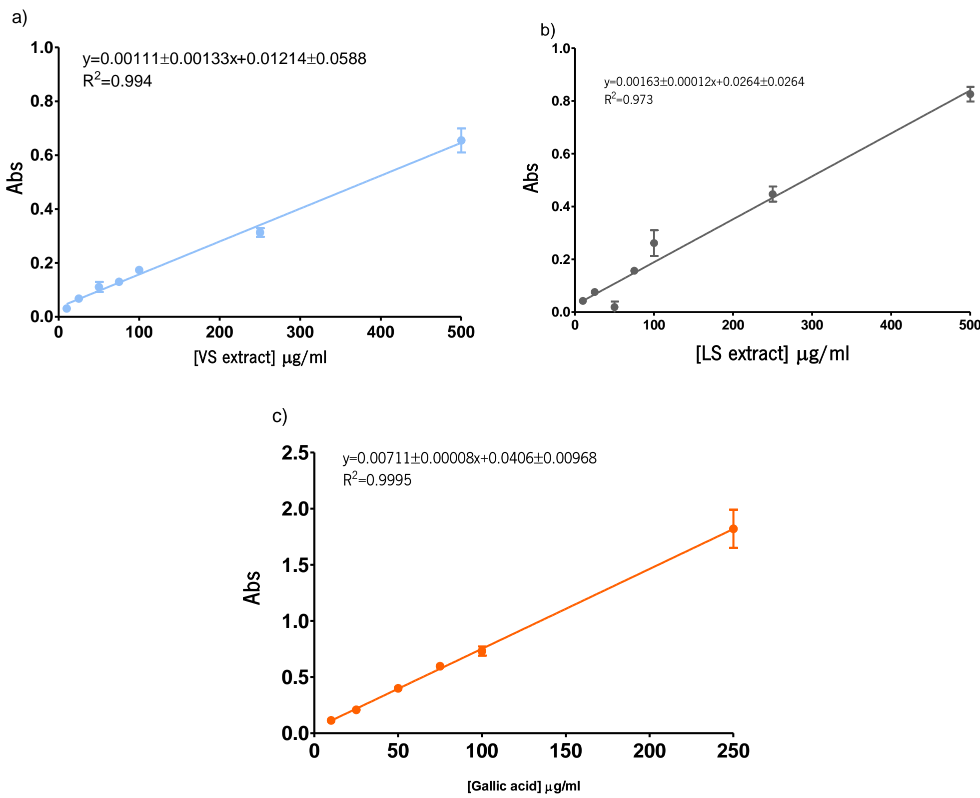

Figure 11. Reducing power vs extract concentration (10, 25, 50, 75, 100, 250 and 500 µg/mL) for **(a)** Vinhão seeds extract (VS), **(b)** Loureiro seeds extract (LS) and **(c)** a standard compound **GA** with the respective linear regression equation. Error bars denote the SD from the mean, n = 3.

3.2.3. Chelating properties

Other antioxidant mode of action relies on the formation of complexes between the antioxidant and metal ions, inhibiting the transfer of electrons, thus arresting the oxidation process. One method to determine this chelating activity is the iron chelation assay and it is based on ferrozine's ability to quantitatively chelate with Fe^{2+} and form a red coloured complex. This reaction is limited in the presence of other chelating agents, which results in a decrease of the red colour of the ferrozine-Fe^{2+} complex. Measurement of the colour reduction estimates the chelating activity to compete with ferrozine for the ferrous ions.

As shown in **figure 12**, all extracts did not show any chelating properties contrarily to the standard compound, EDTA, that showed a percentage of chelation dependent of the concentration tested. According to Symonowicz & Kolanek (2012), the molecular structure of flavonoids allows the formation of flavonoid-metal complexes, therefore, seeds extracts should have had some chelating activity (**Figure 12b**), since they are rich in flavonoids (see **Table IV**).

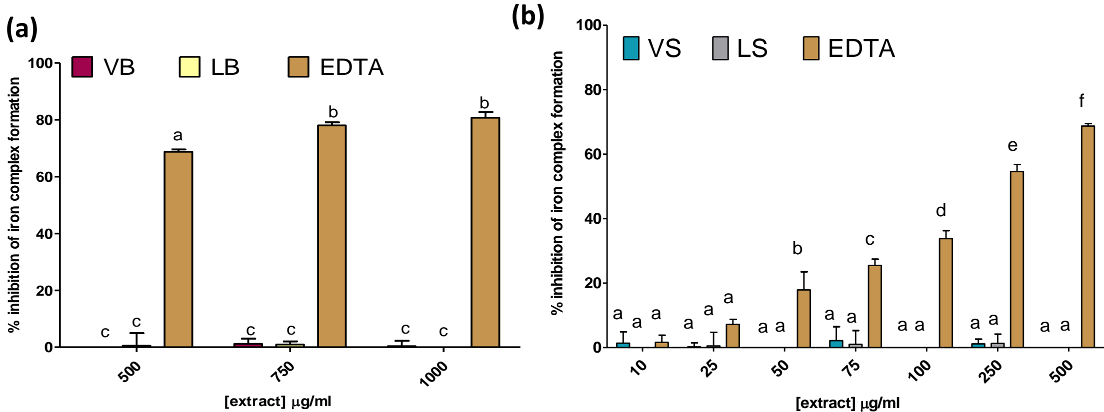

Figure 12. Chelating capacity vs extract concentration (500, 750, 1000 µg/mL) for **(a)** Vinhão pulp and skin extract (VB) and Loureiro pulp and skin extract (LB) and (10, 25, 50, 75, 100, 250 and 500 µg/mL) for **(b)** Vinhão seeds extract (VS) and Loureiro seeds extract (LS). Both plots also show EDTA % of inhibition for the same concentrations as standard. Error bars denote the SD from the mean, n = 3. A Two-way ANOVA Bonferroni post-test analysis was also done. Statistical analysis is represented by different letters when there are significant differences between samples and GA, $P<0.05$, and the same letter when the difference is not significant, $P>0.05$.

3.2.4 *in vivo* antioxidant activity – flow cytometry

Antioxidant activity *in vivo* can be measured by flow cytometry, a widely-used assay for analysing simultaneously multi-parameters of cells flowing in a suspension. This technique allows the expression of cell surface and intracellular molecules, the characterization and definition of

different cell types in a heterogeneous cell population and the analysis of cell size and volume. It is predominantly used to measure fluorescence intensity produced by fluorochromes, which bind or react to intracellular molecules. For instance, 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA) is a nonpolar compound that can easily permeate the cell membrane, where nonspecific esterases cleave the lipophilic acetate groups resulting in a polar compound, 2,7-dichlorodihydrofluorescein (H_2DCF), that is trapped inside the cell. The non-fluorescent H_2DCF, when oxidized by intracellular ROS, is converted to a fluorescent form, dichlorofluorescein (DCF) (λ_{exc}= 498 nm; λ_{emi}= 522 nm) (Gomes *et al.*, 2005). As can be seen in **Figure 13**, the treatment with peroxide results in a shift to the right, increase in fluorescence. When yeast cells are treated with extract and hydrogen peroxide, simultaneously, and there is a decrease in fluorescence compared to oxidized cells, the extract/compound has antioxidant activity.

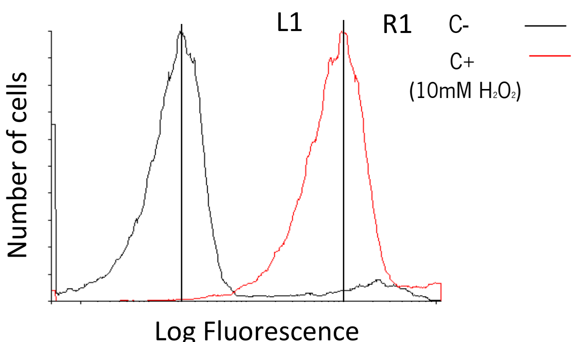

Figure 13. Flow cytometry resulting histogram of number of yeast cells versus Log of fluorescence, using the redox-sensitive fluorescent probe dichlorofluorescein, for the negative control (C-), cells untreated, and positive control (C+), cells treated with 10 mM H_2O_2. The gate **L1** represents the percentage of cells with less fluorescence than the positive control and the gate **R1** represents the percentage of cells with higher fluorescence levels than the positive control.

The results of flow cytometry were analysed with CytExpert software, creating density plots, histograms and statistical columns for each sample (**Appendix C**). Where a first gate (L1) was created for percentage of cells with less fluorescence than the positive control and a second gate (R1) for percentage of cells with higher fluorescence levels than the positive control, represented in **Table VI.**

Table VI. Intracellular oxidation of cells exposed to 10mM H_2O_2 measured by flow cytometry with redox-sensitive fluorochrome H_2DCFDA for different concentrations (1, 10, 25, 50 and 100 μg/m) of Vinhão pulp and skin (VB) and Loureiro pulp and skin (LB) extracts. With L1 and R1 values, were L1 represents percentage of cells with less fluorescence than the positive control and R1 the percentage of cells with higher fluorescence levels than the positive control. Multiple comparison One-way ANOVA Dunnett's analysis is represented by *, when there are significant differences between samples and the C+, P<0.05 (N=4).

Sample	[extract] μg/mL	L1	R1
C^-	———	98.20±2.22 ***	1.80±2.22 ***
C^+	———	50.56±1.23	49.44±1.23
VB	100	63.79±21.61 ns	36.21±21.61 ns
	50	53.12±13.85 ns	46.88±13.85 ns
	25	56.95±16.17 ns	43.05±16.17 ns
	10	61.54±4.4 ns	38.46±4.4 ns
	1	59.20±14.64 ns	40.80±14.64 ns
LB	100	58.94±9.59 ns	41.06±9.59 ns
	50	50.22±36.82 ns	49.78±36.82 ns
	25	52.30±15.47 ns	47.70±15.47 ns
	10	58.91±15.38 ns	41.09±15.38 ns
	1	52.95±19.76 ns	47.05±19.76 ns

According to the results, both berry extracts seem to have antioxidant activity *in vivo*, since there is a shift to the left in comparison to the positive control. This shift is represented by a percentage of cells, with less fluorescence than the cells under H_2O_2-induced oxidative stress, above 50 %. A small shift in percentage of cells represents a huge difference in fluorescence since results are expressed as Log of fluorescence.

Table VII. Intracellular oxidation of cells exposed to 10mM H_2O_2 measured by flow cytometry with redox-sensitive fluorochrome H_2DCFDA for different concentrations (0.1, 1, 10, 25 and 50 μg/mL) of Vinhão seeds (VS) and Loureiro seeds (LS) extracts. With L1 and R1 values, were L1 represents percentage of cells with less fluorescence than the positive control and R1 the percentage of cells with higher fluorescence levels than the positive control. Multiple comparison One-way ANOVA Dunnett's analysis is represented by * when there are significant differences between samples and the C+, P<0.05 (N=2).

Sample	[extract] μg/mL	L1	R1
C^-	———	98.21±2.22 ***	1.79±2.22 ***
C^+	———	50.56±1.23	49.44±1.23
VS	50	33.48±26.34 ^{ns}	66.52±26.34 ^{ns}
	25	57.67±16.87 ^{ns}	42.33±16.87 ^{ns}
	10	46.68±12.43 ^{ns}	53.32±12.43 ^{ns}
	1	70.14±18.91 ^{ns}	29.86±18.91 ^{ns}
	0.1	53.25±18.75 ^{ns}	46.75±18.75 ^{ns}
LS	50	42.46±19.37 ^{ns}	57.54±19.37 ^{ns}
	25	44.91±21.92 ^{ns}	55.09±21.92 ^{ns}
	10	55.36±25.03 ^{ns}	44.64±25.03 ^{ns}
	1	48.03±15.33 ^{ns}	51.97±15.33
	0.1	49.59±20.77 ^{ns}	50.41±20.77 ^{ns}

On the other hand, both seeds extract seem to have pro-oxidant activity, since there is a shift to the right comparing to the positive control, resultant of the increase in fluorescence of cells due to intracellular oxidative stress. Loureiro seeds extracts (LS) appears to have pro-oxidant effects for all concentrations tested, but Vinhão seeds extract exhibits antioxidant activity at low concentrations. This correlates to what have been described in the literature, that polyphenols at high concentrations can act as pro-oxidants. Nevertheless, as differences in percentages are not significant, compounds present in the extract may not be entering the cell, due to polarity, and therefore cannot act against oxidative damage induced by H_2O_2.

3.3. Evaluation of antigenotoxic activity

3.3.1. Antigenotoxic activity - DNA topology assay

Genotoxicity is the ability of a chemical agents to damage DNA molecules either causing mutations or structural alterations of the molecule. One preliminary test to determine if an extract is genotoxic or exhibits any antigenotoxicity is based on the mobility of different DNA conformations in an agarose gel. Iron sulphate solution will form ROS, through Fenton reaction, and these ROS will damage the plasmid DNA, originating linear or nicked DNA. These three DNA forms, including the intact plasmid, will migrate at different velocities allowing the evaluation of any extract protection against ROS oxidation of DNA molecules (Horvathova *et al.*, 2014).

As can be seen in **Figure 14**, negative control band corresponds to supercoiled conformation and the extra band in the positive control represents relaxed pDNA. For the genotoxicity evaluation, left side (**Figure 14a** and **c**), the bands resemble the negative control suggesting that the extracts don't have genotoxic effects on pDNA. Likewise, in the presence of iron all extracts seem to protect pDNA fro ROS oxidation, since bands for all concentrations are similar to the negative control (**Figure 14b** and **d**). Since none of the extracts showed any chelation properties, the mechanism of action underlined must be the reducing capacity of the extracts. ROS are normally formed in the presence of iron, however, in the presence of extract, ROS are reduced, stopping ROS chain reaction and protecting DNA from further damage. However, Loureiro berry extract did not show any reducing capacities too, so another mode of action must be happening. One explanation can be the formation hydrogen bonds between pDNA and extracts' polyphenols, inhibiting ROS action through encapsulation of the DNA molecule (Shin *et al.*, 2015; Xu *et al.*, 2018).

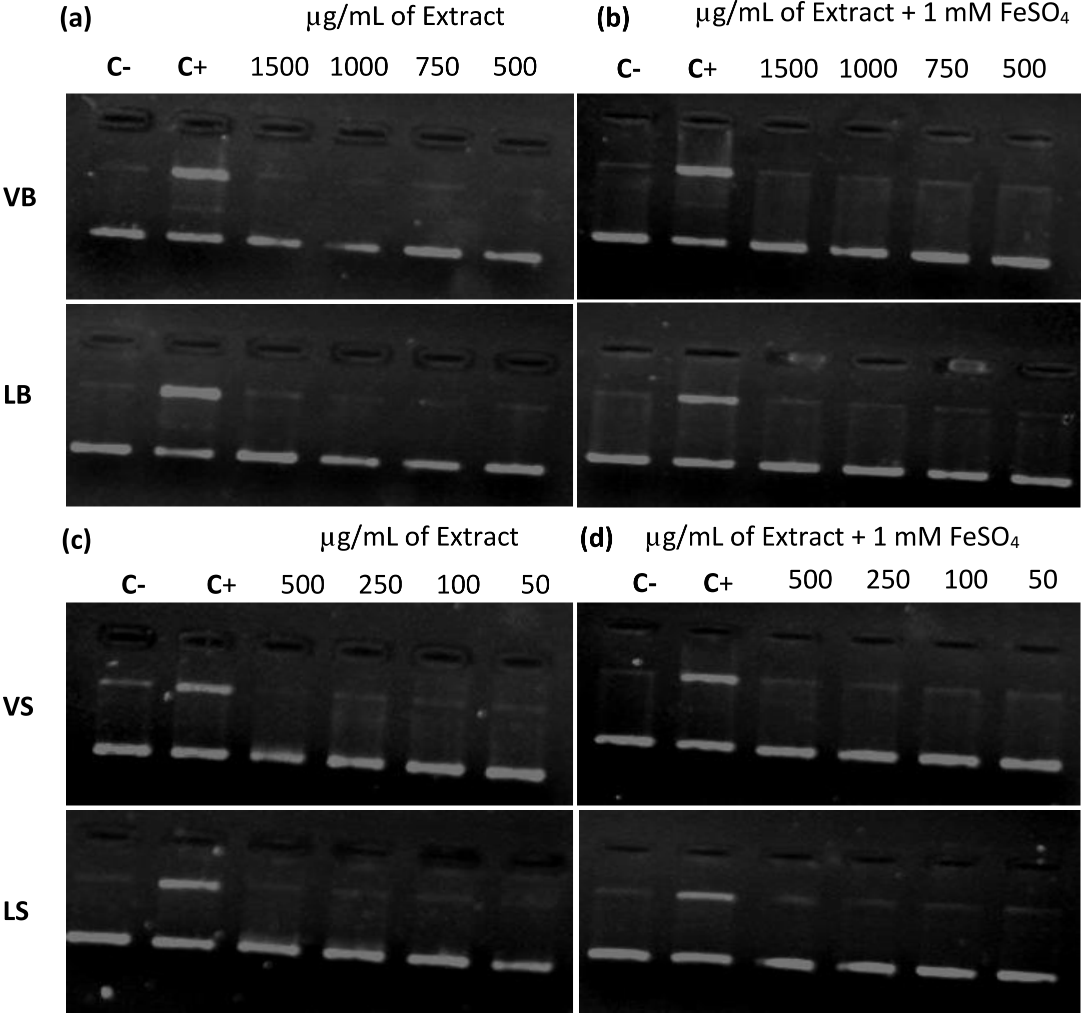

Figure 14. DNA topology assay for all extracts with plasmid pBR31438, in which C- represents the intact plasmid, without treatment, and the C+ represents plasmid (pDNA) treated with 1 mM $FeSO_4$. Where, **(a)** and **(c)** represent pDNA treated with extract (500, 750, 1000 and 1500 µg/mL, for Vinhão pulp and skin (VB) and Loureiro pulp and skin (LB) extracts, and 50, 100, 250 and 500 µg/mL for Vinhão seeds (VS) and Loureiro seeds (LS) extracts). And **(b)** and **(d)** represent pDNA treated with the same concentrations of extract in the presence of 1 mM $FeSO_4$.

3.3.2. Antigenotoxic activity - Comet assay

The comet assay was used to confirm whether antigenotoxic activity is also observable when the mechanism of the cell is implicated, since there were promising results in DNA topology assay for all extracts. The comet assay or single cell gel electrophoresis (SCGE) assay is a popular tool for the measurement of DNA damage (strand breaks) in individual cells (Anderson *et al.*, 1994; Tice *et al.*, 2000). Cells, immobilized in a thin agarose layer, are pre-incubated with the test solution and then with hydrogen peroxide, a strong genotoxic agent. After cell lysis and electrophoresis, damaged DNA will migrate forming a “cloud” behind the cell that, when dyed with GelRed, resembles a comet, while intact DNA will remain in the nucleoids (**Figure 15**). Through statistical

analysis of the cloud length it is possible to determine if the sample tested exhibits any antigenotoxic effect against hydrogen peroxide. This method only allows the evaluation of the extract protection against conformational changes induced by genotoxic agents; however, mutations can also be happening.

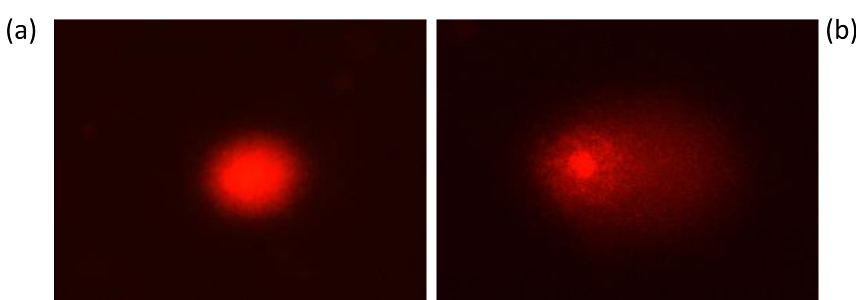

Figure 15. Comet assay fluorescence microscopy images with 40x amplification of lymphocytes' DNA marked with GelRed (a) without treatment and (b) treated with 1 mM H_2O_2 for 5 min.

It is possible to see that there are differences in tail length when cells are pre-treated with seeds extracts before exposing them to the genotoxic agent (H_2O_2) (**Figure 16 and appendix D and E**). Lymphocytes not exposed to the genotoxic agent show a mean of tail length of 0.9 μm, while lymphocytes exposed to H_2O_2, show a mean of 14.3 μm. For VS extract, tail lengths were 1.3, 1.1 and 1.2 μm for 50, 100 and 250 μg/mL of extract, respectively (**Figure 16a**). Values are similar to the C-, which suggests that Vinhão seeds extract does not exhibit genotoxic activity in the concentrations tested. On the other hand, in the presence of H_2O_2, VS seems to protect cells from oxidative stress, reducing DNA damage. For the same concentrations of extract, tail lengths were 1.6, 1.7 and 2.1 μm, respectively. For LS extract the same occurred (**Figure 16b**), the extract seems to not have genotoxic effects and protects cells from H_2O_2 induced DNA damage. With values of tail length of 1.2, 1.1, 1.1 and 0.8, for 50, 100, 250 and 500 μg/mL of LS extract, respectively, and 6, 1.8, 1.5 and 2.4 for the same concentrations in presence of H_2O_2, respectively. There are significant differences between the lowest concentration tested and the remain, suggesting a lower protection at lower concentrations. However, there are still significant differences when compared to the C+, revealing some protection effects at this concentration. These results are in accordance to the literature, since seeds extracts are mainly composed by proanthocyanidins and these compounds have been reported as antigenotoxic agents (Llópiz *et al.*, 2004). Also, grape juices seem to protect H_2O_2-induced DNA damage in human lymphocytes and lymphoblastoid cells (Razo-Aguilera *et al.*, 2011; Sugisawa *et al.*, 2004). Also, they are in accordance to previous results, such as TPC, TFC, antioxidant activity and DNA topology. Both seeds extracts showed a high TPC and

TFC values, which correlates to a high antioxidant activity, resulting in greater protection against H_2O_2-induced DNA damage in lymphocytes. Contrarily to flow cytometry results, both seeds extracts showed some protection against H_2O_2 induced oxidative stress, which might be the result of pre-treatment with extract for 55 min before exposing cells to H_2O_2. This may lead to the activation of antioxidant defence pathways by compounds present in the extract, which is not possible to happen in flow cytometry since H_2O_2 and extract are co-incubated for 15 min.

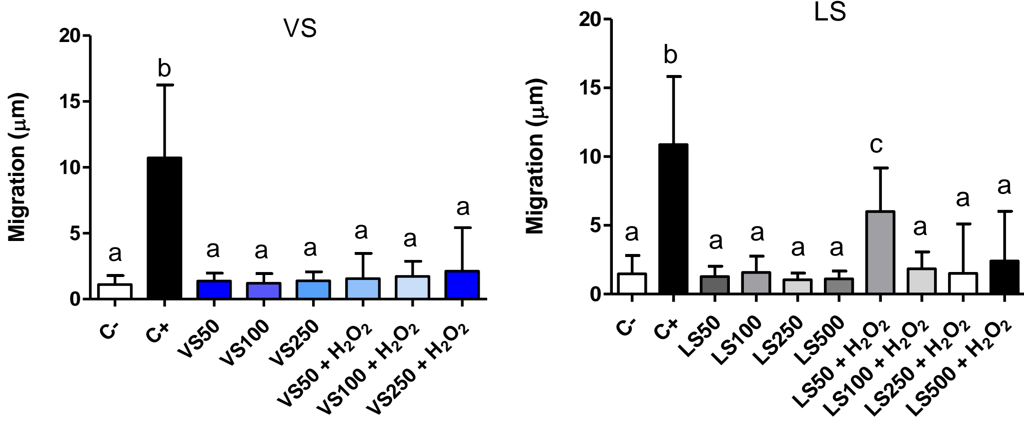

Figure 16. Genotoxicity and antigenotoxicity assessment, through DNA migration in μm, of **(a)** Vinhão seeds extract (VS) and **(b)** Loureiro seeds extract (LS), by comet assay. In each plot, the first two columns are the negative control (C-), that corresponds to lymphocytes with no treatment, and the positive control (C+), treatment with 10mM H_2O_2 for 5min. Then, columns for lymphocytes treated with 50, 100, 250 and 500* μg/mL of each extract tested and columns for treatment with extract for 1h, for the same concentrations, and 10mM of H_2O_2 for 5min. *Only for LS extract. Multiple comparison One-way ANOVA Dunnett's analysis is represented by different letters when there are significant differences between samples and the C+, $P<0.05$ (N=20).

Results of flow cytometry were not consistent with comet assay results, however cells used in both assays are different since comet assay was performed with lymphocytes and flow cytometry with yeast cells. So, differences in results can be due to different underlying mechanisms of cells. Moreover, pre-incubation with extract must be conducted to assess if the extracts ability to protect cells from oxidative stress relies on the activation of antioxidant defence mechanisms.

3.3.3. *In vivo* antimutagenic activity- AMES test

The AMES test allows the antimutagenicity evaluation of a sample. The AMES test uses bacteria *Salmonella typhimurium* modified strains that lack histidine synthesis related genes, relying on medium supplemented with histidine to survive. These strains are susceptible to mutations in the

histidine gene, recovering the histidine function. Therefore, in the presence of a mutagenic compound the strain will recover the genotype and will be able to proliferate in medium lacking histidine. However, in the presence of an antimutagenic agent this phenotype is not reverted. Different strains are susceptible to different kinds of mutations, for example TA98 and TA97 strains exhibit a frameshift type of mutation, on the other hand TA100 is more sensitive to base pair substitution type of mutations (see **2.4.3.**). The number of colonies for each treatment were recorded to create graph plots of number of colonies versus treatment used (**Figure 17**). Specific mutagens were used for each strain, 9-aminoacridine (9AA) for TA97, 4-nitro-o-phenylenediame (4NOP) for TA98 and sodium azide (NaN_3) for TA100.

For TA97 and TA98 it is not possible to determine whether the extracts are either mutagenic or antimutagenic since there are no significant differences between the negative and the positive control. However, for TA100, both extracts do not exhibit mutagenic effects, since there are no significant differences when compared to the negative control. The same can be said for their antimutagenic properties, since there are no significant differences between treatments and the positive control for all concentrations tested. TA100 strain is susceptible to base-pair substitution types of mutations, so Vinhão and Loureiro seeds extracts seem to not protect DNA against this type of mutations. However, further studies must be conducted in order to determine if Vinhão and Loureiro seeds extracts have antimutagenic properties. Some studies reported protective effects of, both red and white, grapes extract against H2O2 induced mutations in TA102 (Rybková *et al.*, 2016; Stagos *et al.*, 2006).

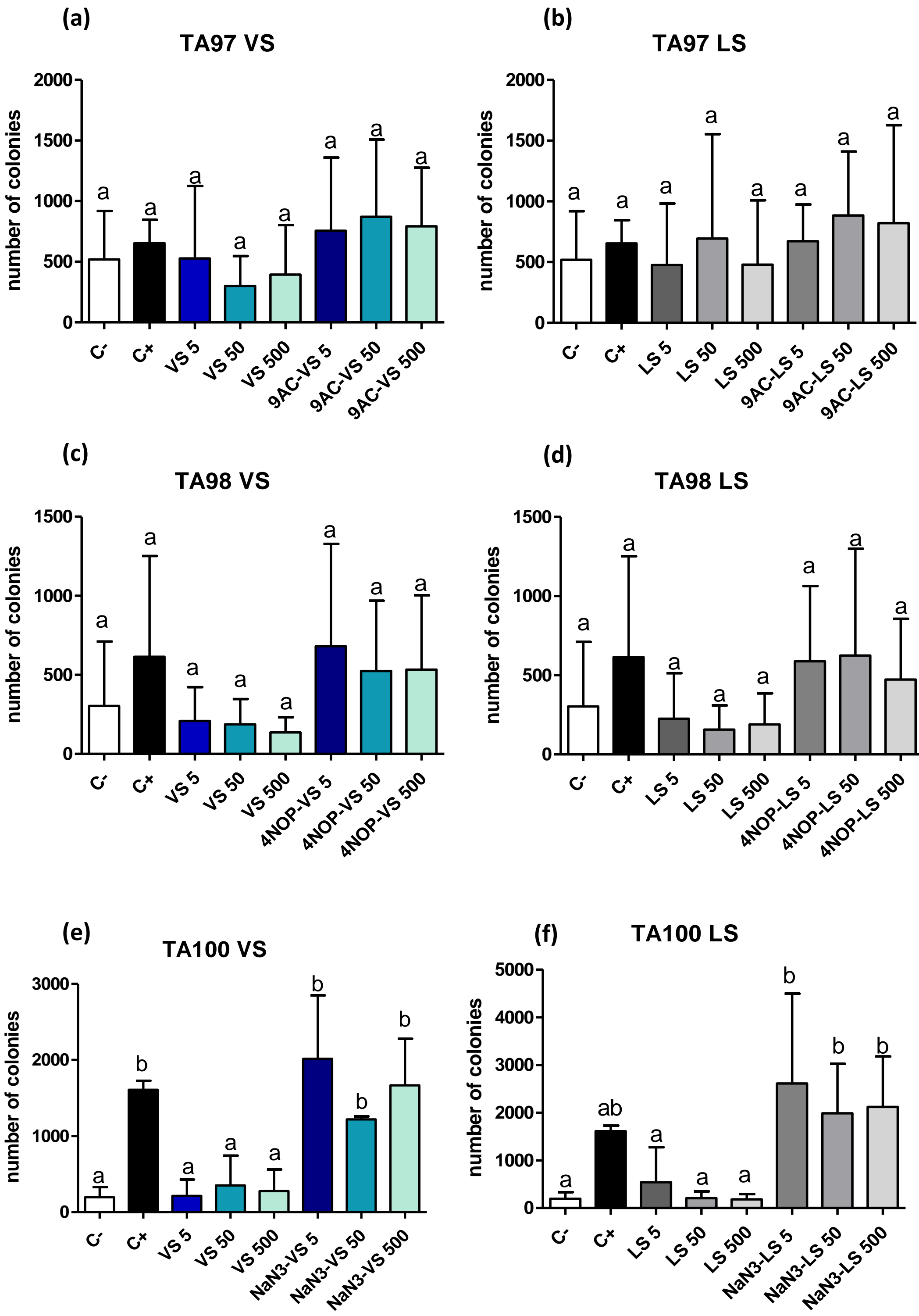

Figure 17. Mutagenicity and antimutagenicity assessment by AMES test for seeds extracts. Number of colonies vs treatment for TA97 (**a** and **b**), TA98 (**c** and **d**) and TA100 (**e** and **f**) *S. typhimurium* strains for Vinhão and Loureiro seeds extracts (VS and LS). In each plot the first two columns are the negative control (C-), that corresponds to bacteria with no treatment, and the positive control (C+), treatment with 50 µg/plate of the corresponding mutagen for each strain. 9-aminoacridine (9AA), for TA97, 4-nitro-o-phenylenediame (4NOP) for TA98 and sodium azide (NaN_3) for TA100. Then, three columns of colonies of

bacteria treated with 5, 50 and 500 µg/mL of each extract tested and three columns of treatment with extract, for the same concentrations, and the respective mutagen. Error bars denote the SD from the mean, n = 3. Multiple comparison One-way ANOVA Dunnett's analysis is represented by different letters when there are significant differences between samples and the C+, $P<0.05$ (N=2).

4. Conclusions and future perspectives

Seed extracts have a higher TPC and TFC values than pulp and skin extracts, as well as percentage of TFC/TPC. Furthermore, Vinhão berry extract showed to have a higher TPC than Loureiro berry extract. Accordingly, seed extracts from both varieties displayed strong reducing power and DPPH scavenging activities, while red Vinhão berries displayed higher activities than the white variety (Loureiro). Interestingly, all samples did not chelate iron, suggesting that this property is not involved in antioxidant activity. Contrarily to what is proposed in the literature, that most flavonoids can act as metal chelating agents. In addition, all extracts were antigenotoxic using DNA topology assay and both seeds extracts showed some protection against H_2O_2 induced DNA damage by comet assay with human lymphocytes. As Loureiro berry extract did not show any scavenging, reducing or chelating properties, another mechanism of action must be underlined in the protection of DNA against H_2O_2. So, further studies must be conducted with this extract to evaluate if it has any antigenotoxic potential and which compounds are involved in this protection. Results of flow cytometry were not consistent with the rest of the results, so pre-incubation with extract must be conducted to assess if the extracts ability to protect cells from oxidative stress relies on the activation of antioxidant defence mechanisms. Antimutagenic activity was not recorded for the mutagens used, but maybe all extracts can protect against H_2O_2 induced mutations, which is also a new possibility to be tested. Also, additional assays must be conducted using human cell lines or *in vivo* models for the confirmation of the beneficial activities with potential human applications.

Ultimately, the grape extracts evaluated showed to have great antioxidant and antigenotoxic potential, expect Loureiro berry extract. Furthermore, seeds have more compounds with antioxidant properties and red varieties have greater reducing capacity than white varieties. Moreover, seeds' extracts from both varieties showed higher antioxidant activity than seeds extracts from other well-known varieties, such as Merlot, Pinot Noir and Cabernet Sauvignon. Portuguese grape varieties grown in vinho Verde region showed to be great sources of polyphenols with nutraceutical properties, specially the seeds, which are the major constituent of wine industry wastes. Further studies must be conducted to determine antioxidant and antigenotoxic activity *in vivo*, since results were not coincident.

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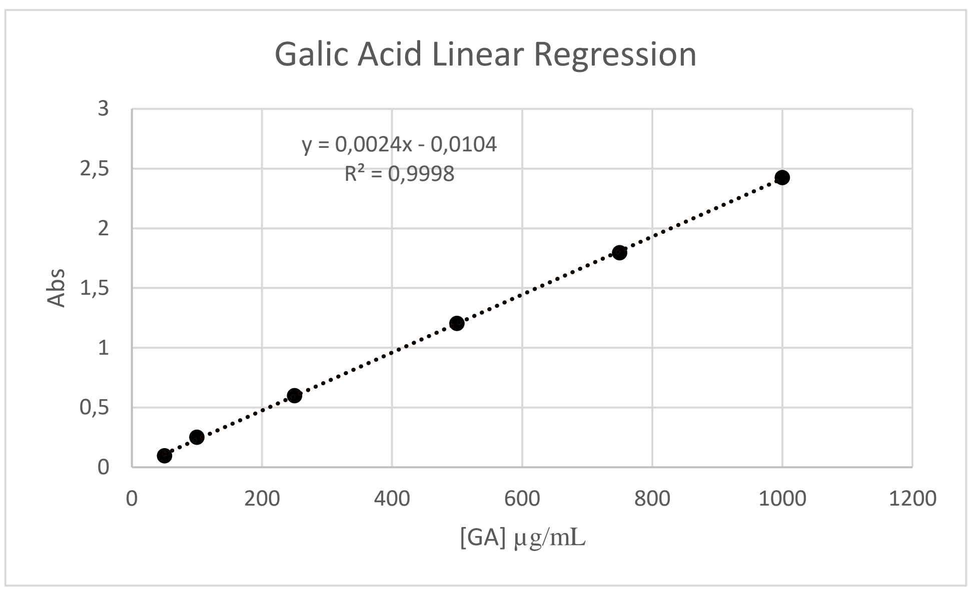
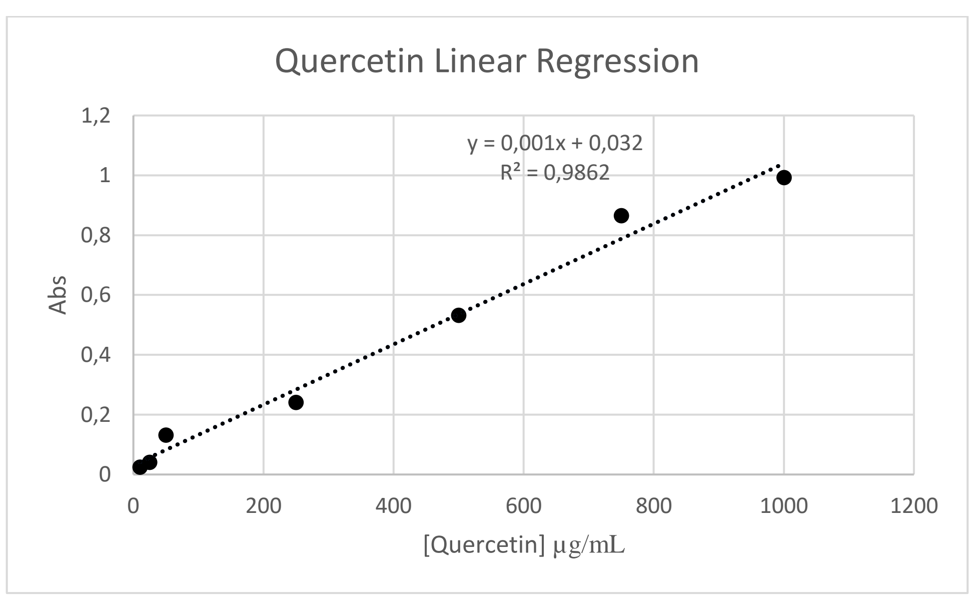
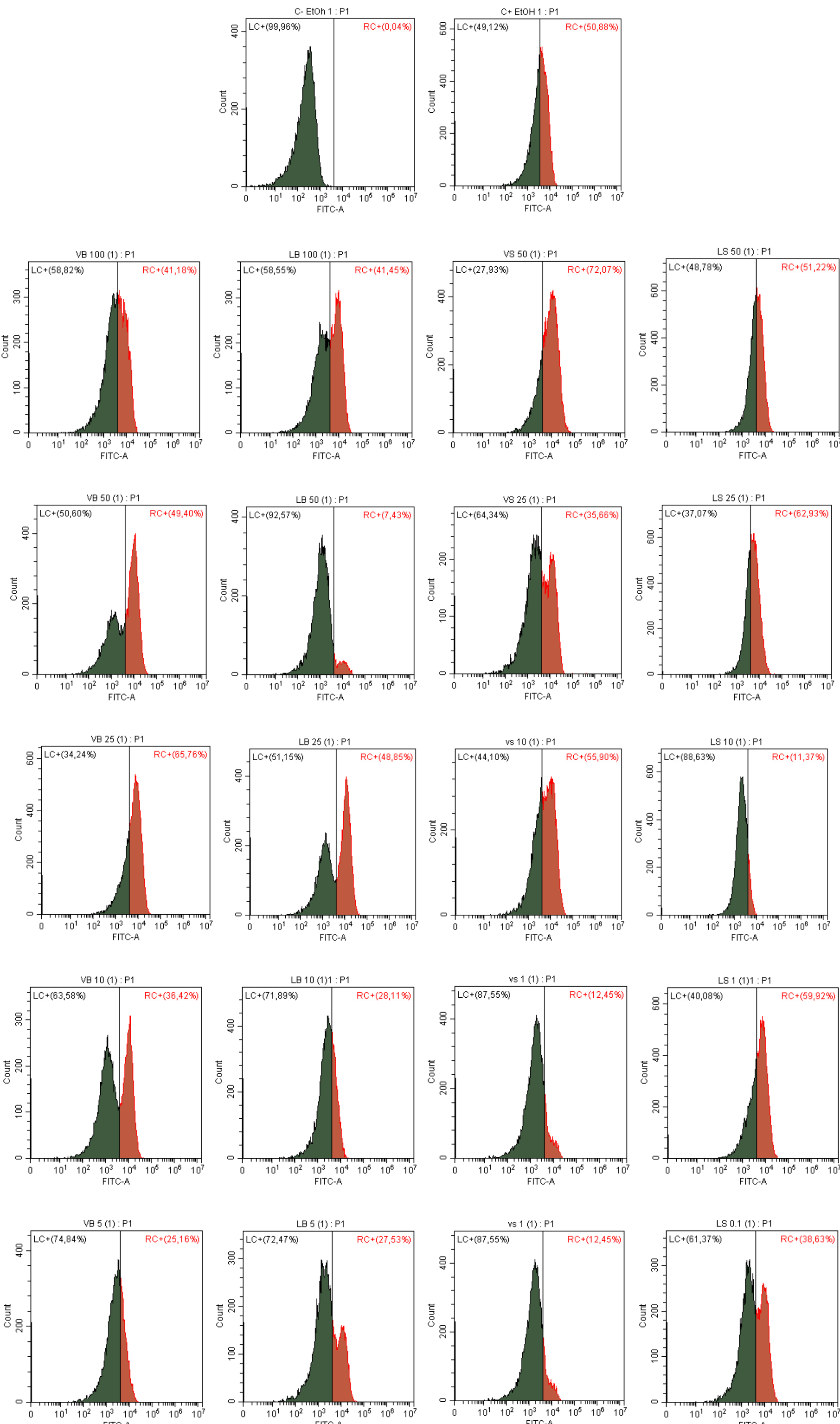
Appendix A- Gallic acid calibration curve.



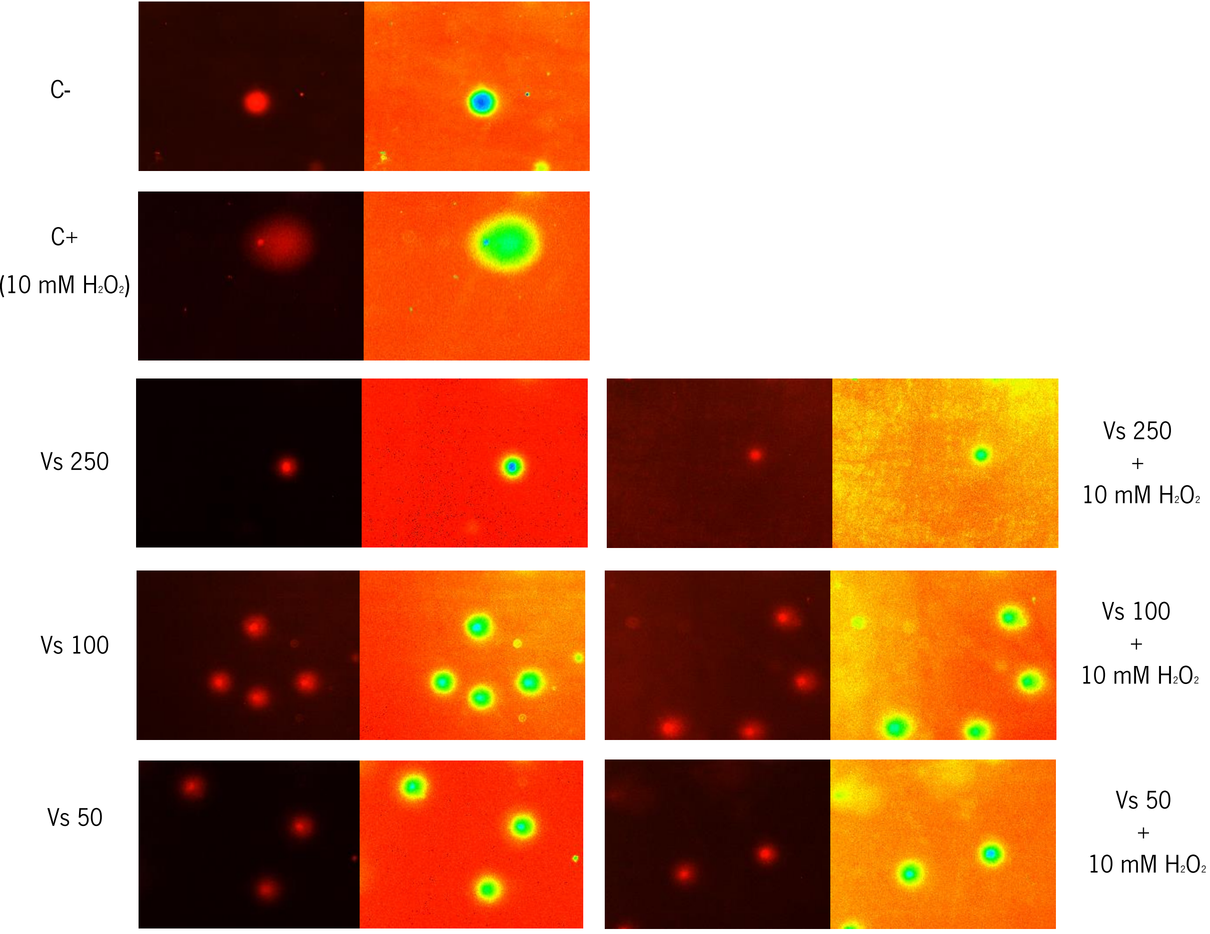
Appendix B - Quercetin calibration curve.



Appendix C – Flow cytometry histograms of number of cells versus Log of fluorescence.



Appendix D - Comet assay, for Vinhão seeds extract (LS), fluorescence microscopy images with 40x amplification of lymphocytes' DNA marked with GelRed and pictures of *CometScore* software.



Appendix E - Comet assay, for Vinhão seeds extract (LS), fluorescence microscopy images with 40x amplification of lymphocytes' DNA marked with GelRed and pictures of *CometScore* software.