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ORIGINAL PAPER

Extracts from berries of Portuguese *Vitis vinifera* L. varieties Vinhão and Loureiro exhibit powerful antioxidant, antihaemolytic, and cytotoxic activities

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Abstract

Grapes (*Vitis vinifera* L.) are widely consumed fruits known for their diverse bioactive compounds. Vinhão and Loureiro are the major red and white grape varieties, respectively, of the largest Portuguese wine region: Vinho Verde. Large amounts of residues are produced in wine making that potentially are rich in bioactive compounds, so this study aimed at valorizing the residues from these grape varieties for further applications. The grape extracts were obtained from the seeds and from the pulp and skin together for characterization. The phenolic composition differed across these parts, with the seeds being particularly rich in polyphenols and flavonoids. Both varieties exhibited high antioxidant activity; seed extracts stood out, showing the strongest radical scavenging ability (IC_{50} = 0.22 ± 0.08 for 2,2-diphenyl-1-picrylhydrazyl and 0.004 ± 0.001 mg mL^{-1} for thiobarbituric acid reactive substances assays). The Loureiro seed extract showed higher antihaemolytic properties (IC_{50} = 4.81 ± 0.22 µg mL^{-1}) by protecting erythrocyte membranes from oxidative damage. The seed extracts displayed cytotoxic potential on tested tumor cells, particularly with the Loureiro variety. Overall, the results suggest that both varieties have high antioxidant properties, with the seed extracts showing the most promising bioactivities. Although further characterization is required in terms of bioactivity in *in vivo* experimental models, these findings contribute to the potential utilization of grape extracts and their waste products, providing new applications for Portuguese grape varieties in various applications, such as pharmaceuticals, cosmetics, and nutraceuticals.

Keywords Bioactivities · Antioxidant activity · Seed extracts · Vinhão grape variety · Loureiro grape variety

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Abbreviations

AAPH	2,2′-Azobis(2-methylpropionamidine) dihydrochloride
DMEM	Dulbecco's modified Eagle's medium
DPPH	2,2-diphenyl-1-picrylhydrazyl
FBS	fetal bovine serum
GA	gallic acid
GAE	gallic acid equivalents
GI_{50}	growth inhibition 50%
IC_{50}	half-maximal inhibitory concentration
LB	Loureiro pulp and skin extract
LPS	lipopolysaccharide
LS	Loureiro seeds extract
OxHLIA	oxidative hemolysis inhibition assay
PBS	phosphate-buffered saline
QE	quercetin equivalents
RP	reducing power capacity
SEM	standard error of the mean
SRB	sulforhodamine B
TBARS	thiobarbituric acid reactive substances
TCA	trichloroacetic acid
TFA	trifluoroacetic acid
TFC	total flavonoid content
TPC	total phenolic content
UPLC	ultra performance liquid chromatography
VB	Vinhão pulp and skin extract
VS	Vinhão seeds extract

Introduction

Grapes (*Vitis vinifera L.*) are among the most popular fruits consumed worldwide. They contain a wide range of beneficial phytochemicals, such as flavonols, anthocyanins, proanthocyanidins, stilbenes, and phenolic acids, which are distributed differently across the grape's skin, pulp, and seeds [1]. In 2022, global grape production reached 80.1 million tons, averaging 11 tons per hectare [2]. Europe contributed around 37% of this output, followed by Asia at 34% and America at 19%. This production was supported by approximately 7.3 million hectares of vineyard area worldwide [2].

Research has shown that fruits contain compounds that can positively impact health, largely due to their phenolic content [3–6]. This makes them potential sources of bioactive substances useful in disease prevention. With growing awareness of the benefits of phytochemicals found in both grape pulp and seeds, there has been increased interest in utilizing grapes and their derivatives in innovative ways [1–7]. These compounds are associated with various biological effects, including antioxidant, anti-inflammatory, antimutagenic, and antiviral properties, as well as protection against UV radiation and harmful chemicals [8–10].

Winemaking is a seasonal activity that generates large volumes of waste following each grape harvest. In 2018 alone, the industry was responsible for producing approximately 10 million tons of by-products [11]. One of the main residues is grape pomace - also known as bagasse - which includes components like seeds, skins, stems, pulp, and leaves. These materials are rich in polyphenolic compounds, a diverse group of bioactive molecules. Among them are phenolic acids (e.g., hydroxybenzoic and hydroxycinnamic acids), flavonoids (such as flavan-3-ols, anthocyanins, proanthocyanidins, flavones, and flavonols), stilbenes and anthocyanins. The abundance of these compounds in grape waste highlights its potential for value-added applications [12]. Therefore, exploring sustainable ways to repurpose these by-products could offer economic, environmental, and social benefits.

Located in the northwest of Portugal, the Vinho Verde Region - historically known as Entre-Douro-e-Minho - is the country's largest designated wine-producing area and ranks among the biggest in Europe [13]. Portugal is home to a broad spectrum of grape varieties, with 34 types each occupying at least 1% of the national vineyard area [14]. In 2022, the Vinhão and Loureiro grapes represented 2% and 4% of Portugal's vineyard surface, and 0.06% and 0.1% globally [2, 14]. Within the Vinho Verde Region, however, these two varieties dominate: Vinhão leads among red grapes with 15.1% (4.33 kha), while Loureiro tops the white varieties with 29.6% (7.24 kha) of the regional vineyard area [15]. Vinhão is notable for its high anthocyanin levels, which are present not only in the skin but also in the pulp [16, 17]. Loureiro, though lacking anthocyanins, contains significant amounts of non-pigmented polyphenols like quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside, kaempferol-3-*O*-rutinoside, kaempferol-3-*O*-glucoside, epicatechin, and gallic acid (GA) [18, 19]. Both varieties are recognized for their antioxidant, anti-inflammatory, antimicrobial, and anticancer properties [18, 20]. While other grapes such as *Vitis labrusca* [21], and the *V. vinifera* varieties Muscat of Alexandria [22], Cabernet Sauvignon [23], Pinot Noir, Marselan, and Tamyanka [24] have also shown strong bioactive potential, there is still limited comparative data on the phenolic content and biological effects of red versus white grape types. This highlights the importance of distinguishing between grape varieties to better identify those with the greatest potential as source of bioactive compounds [25].

Considering the unique characteristics of Portuguese grape cultivars and the growing interest in natural bioactive compounds and sustainable reuse of agricultural by-products, this study focuses on evaluating the biological

properties of two native wine grape varieties: Vinhão (red) and Loureiro (white). Given that phenolic profiles vary not only between grape types but also among different plant tissues, the research includes extracts from seeds as well as the edible portions - pulp and skin. The findings aim to support the potential use of these extracts in sectors such as pharmaceuticals, cosmetics, and nutraceuticals, offering innovative applications for both the grapes and the residues from wine production.

Materials and methods

Cells and chemicals

The sheep red blood cells were obtained from Innovative Research, Inc., Novi, Michigan, USA; IC100-0210. The human tumor cell lines, AGS (gastric adenocarcinoma), CaCo-2 (colorectal adenocarcinoma), MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), non-tumor cells line VERO (monkey kidney) and RAW 264.7 (murine macrophage) cell lines were obtained from the Leibniz Institute DSMZ, German Collection of Microorganisms and Cell Culture. Lipopolysaccharide (LPS), sulforhodamine B (SRB), trichloroacetic acid (TCA), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), fetal bovine serum (FBS), standards used to analyze the chemical composition of the extracts, and H_2O_2 were purchased from Sigma-Aldrich, Germany. Ethanol, Folin-Ciocalteu reagent, GA, Na_2CO_3, $AlCl_3$, trifluoroacetic acid (TFA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Tris–HCl buffer, $FeSO_4$, $FeCl_3$, ascorbic acid, thiobarbituric acid, ferricyanide and Dulbecco's modified Eagle's medium (DMEM) were purchase from Merck, Darmstadt, Germany. Agarose was purchased from Genbiotec, Argentina; penicillin and streptomycin, ellipticin, dexamethasone, and 100x water GelRed were obtained from Quimigen, Madrid, Spain. Griess Reagent System kit and acetic acid were bought from Acros Organics, Geel, Belgium.

Preparation of extracts

This study selected two grape cultivars from Portugal's Vinho Verde region due to their distinct characteristics: *V. vinifera* 'Vinhão', a dark-skinned variety rich in anthocyanins, and *V. vinifera* 'Loureiro', a white grape. Grapes were harvested near Braga in September 2017. Seeds (9.43 g from Vinhão and 7.27 g from Loureiro) were separated from the pulp and skin (27.92 g from Vinhão and 24.84 g from Loureiro), then frozen in liquid nitrogen and ground using an IKA A 11 basic Analytical mill. Phenolic compounds were extracted following a modified version of the method described by Krasteva et al. [24]. Each sample was mixed with 40 mL of 70% ethanol, sonicated for 5 min, and incubated at approximately 25 °C for 60 h with agitation. The extracts were filtered under low-pressure vacuum, and the solvent was removed using a rotary evaporator at 48 °C. The resulting dry extracts were rehydrated with water and lyophilized. Four stock solutions (50 mg mL^{-1} in 70% ethanol) were prepared: VB and VS for Vinhão pulp/skin and seeds, respectively, and LB and LS for Loureiro pulp/skin and seeds. All extracts were stored at − 20 °C in the dark.

Total phenolic content

The total phenolic content (TPC) was determined using the Folin-Ciocalteu assay [26]. In this procedure, 10 µL of each sample extract (concentrations: 50 mg mL^{-1} for VB and LB; 5 mg mL^{-1} for VS and LS) was combined with 100 µL of diluted Folin-Ciocalteu reagent (1:10) in a 96-well microplate. The mixture was allowed to react for 5 min. A similar process was followed for GA, the reference compound, at five concentrations: 50, 100, 250, 500, and 750 µg mL^{-1}. Subsequently, 80 µL of 1 M Na_2CO_3 was added to each well. The plate was then incubated in the dark at ambient temperature (approximately 25 °C) for 1 h. Absorbance readings were taken at 760 nm using a Synergy™ HTX Multi-Mode Microplate Reader. A calibration curve was constructed using the absorbance values of the GA standards. These values were then used to estimate the phenolic content of the extracts in terms of GA equivalents (GAE). The TPC was calculated using the following equation:

$$TPC = \frac{C_{sample}\ x\ V_{extract}}{m_{lyoplizied\ material}}$$

In this formula, C_{sample} is the concentration in mg of GAE mL^{-1} derived from the calibration curve, $V_{extract}$ is the volume of solvent used (in mL), and $m_{lyophilized\ material}$ is the mass of the freeze-dried extract (in mg).

Total flavonoid content

The quantification of total flavonoid content (TFC) was carried out following the method described by Matić et al. [27], with slight adjustments. In summary, 100 µL of each extract (VB and LB at 50 mg mL^{-1}; VS and LS at 5 mg mL^{-1}) was individually combined with 100 µL of a 10% (*w/v*) $AlCl_3$ solution. The resulting mixtures were allowed to stand for 1 h to enable complex formation. This procedure was also applied to a series of quercetin standard solutions at concentrations of 5, 10, 25, 50, 100, 250, 500, 750, and 1000 µg mL^{-1}. Absorbance measurements were taken at 420 nm, and a calibration curve was constructed using the

absorbance values of the quercetin standards. These values were then used to determine the flavonoid content of the extracts, expressed in quercetin equivalents (QE). The TFC was calculated using the following equation:

$$TFC = \frac{C_{sample}\ x\ V_{extract}}{m_{lyophilized\ material}}$$

In this formula, C_{sample} refers to the concentration of QE in mg mL^{-1} obtained from the calibration curve, $V_{extract}$ is the volume of solvent used (in mL), and $m_{lyophilized\ material}$ is the mass (in mg) of the freeze-dried extract.

Ultra performance liquid chromatography analysis of phenolic compounds

For the ultra-performance liquid chromatography (UPLC) analysis, the dried extracts were dissolved in a solution of ethanol and water (80:20 *v/v*). In the case of anthocyanin extraction, 0.5% TFA was added to the solvent. The final concentration of the solution was adjusted to 10 mg mL^{-1}, and the samples were filtered through a 0.2 µm membrane before being transferred into 1 mL HPLC vials for phenolic profiling. The identification of phenolic compounds, including both anthocyanins and non-anthocyanins, was performed using a Dionex Ultimate 3000 UPLC system (Thermo Scientific, San Jose, CA, USA) coupled with an electrospray ionization mass spectrometer (Linear Ion Trap LTQ XL, Thermo Scientific), operating in negative ion mode for non-anthocyanins and positive ion mode for anthocyanins. For non-anthocyanin compounds, separation was achieved using a Waters Spherisorb S3 ODS-2 C18 column (4.6 × 150 mm, 3 µm), with a gradient elution involving 0.1% formic acid in water and acetonitrile. Detection wavelengths were set at 280, 330, and 370 nm. Anthocyanins were analyzed using a reverse-phase AQUA® C18 column (5 µm, 150 × 4.6 mm; Phenomenex, Torrance, CA, USA), with a mobile phase consisting of 0.1% TFA in water and pure acetonitrile, and detection at 520 nm. The system used nitrogen as the sheath gas at 50 psi, with a spray voltage of 5 kV, a source temperature of 325 °C, and a capillary voltage of − 20 V. The tube lens offset was maintained at − 66 V. Mass spectra were recorded across a range of *m/z* 100 to 1800 [28], and data were processed using the Xcalibur® software (Thermo-Finnigan, San Jose, CA, USA). Phenolic compounds were identified based on retention times, absorption spectra, and mass spectral data, which were compared with literature values and, when available, with reference standards. Standards were not injected into the MS system; identification relied on MS/MS fragmentation patterns and retention time matching. Quantification was performed using calibration curves generated from appropriate standards and detected via a diode array detector (DAD). When specific standards were unavailable, quantification was based on a standard from the same phenolic class. Results were expressed as milligrams per gram of dry extract (mg g^{-1}), reported as the mean ± standard deviation from three independent replicates.

Antioxidant capacity

The antioxidant potential of the extracts was assessed using four distinct in vitro methods. The results were expressed as IC_{50} values (mg mL^{-1}), indicating the concentration required to achieve 50% inhibition of oxidative activity.

2,2-Diphenyl-1-picrylhydrazyl radical scavenging activity assay

The ability of the extracts to neutralize free radicals was assessed using the DPPH method, adapted from the protocol by Yamauchi et al. [29]. In this assay, 950 µL of a 0.1 mM ethanolic DPPH solution was mixed with 50 µL of each extract at varying concentrations (50, 100, 250, 500, 750, and 1000 µg mL^{-1}), prepared in 70% ethanol (*v/v*). Solutions at the same concentrations were prepared with GA as a reference standard. Control samples included a blank (50 µL of 70% *v/v* ethanol with 950 µL of absolute ethanol) and a negative control (50 µL of 70% *v/v* ethanol with 950 µL of DPPH solution). All mixtures were incubated in the dark at room temperature for 20 min. Absorbance was then measured at 517 nm using a Genesys 20 spectrophotometer. The radical scavenging activity (RSA) was calculated using the formula:

$$\%\ RSA = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} x100$$

Here, $Abs_{control}$ refers to the absorbance of the negative control, and Abs_{sample} corresponds to the absorbance of each tested sample, including the standard. The percentage of DPPH inhibition was used to determine the IC_{50} value (mg mL^{-1}), representing the concentration required to inhibit 50% of the DPPH radicals, calculated via non-linear regression.

Thiobarbituric acid reactive substances

The inhibition of lipid peroxidation was assessed by monitoring the reduction of thiobarbituric acid reactive substances (TBARS), following the method outlined by De Leon and Borges [30], with results expressed as IC_{50} values (mg mL^{-1}), indicating the concentration needed to achieve 50% antioxidant activity. To begin, the extracts were

homogenized using a Polytron in chilled Tris–HCl buffer (20 mM, pH 7.4) at a 1:2 (*w/v*) ratio. The homogenate was centrifuged at 3000 × *g* for 10 min, and varying concentrations (0.1 mL) of the resulting supernatant were mixed with 0.2 mL of a solution containing 10 µM $FeSO_4$ and 0.1 mM ascorbic acid. This reaction was incubated at 37 °C for 1 h and then halted by adding 0.5 mL of 28% (*w/v*) TCA, followed by 0.38 mL of 2% (*w/v*) thiobarbituric acid. The mixture was heated at 80 °C for 20 min. After cooling, the samples were centrifuged again at 3000 × *g* for 10 min to remove precipitated proteins. The absorbance of the supernatant, which reflects the formation of the malondialdehyde-TBA complex, was measured at 532 nm. The percentage of TBARS inhibition was calculated using the formula:

$$\%TBARS = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} x100$$

Here, $Abs_{control}$ refers to the absorbance of the negative control, and Abs_{sample} corresponds to the absorbance of each tested sample, including the standard. The IC_{50} value was then determined using non-linear regression, as described above.

Reducing power capacity

The reducing power (RP) of the extracts was evaluated using a modified version of the method described by Zhou et al. [31]. Reaction mixtures were prepared by combining 100 µL of each extract at various concentrations (VB and LB: 500, 750, 1000, 1500, 2500 and 5000 µg mL^{-1}; VS and LS: 10, 25, 50, 100, 250 and 500 µg mL^{-1}) with 500 µL of 0.2 M sodium phosphate buffer (containing 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% (*w/v*) potassium ferricyanide. A negative control was prepared using 70% (*v/v*) ethanol in place of the extract. Standard solutions of GA at concentrations 10, 25, 50, 75, 100, 250, 500, 750, and 1000 µg mL^{-1} were prepared using the same protocol. All mixtures were incubated at 50 °C for 20 min. After incubation, 500 µL of 10% (*w/v*) TCA was added, and the samples were centrifuged at 3000 × *g* for 10 min using an Eppendorf 5804 centrifuge. From the supernatant, 500 µL was transferred and mixed with 0.1 mL of 0.1% (*w/v*) $FeCl_3$ and 500 µL of deionized water. After a 10-min incubation, absorbance was measured at 700 nm using a Genesys 20 spectrophotometer. A calibration curve was constructed using the absorbance values of the GA standards. These values were used to determine the concentration of phenolic compounds in the extracts, expressed as gallic acid equivalents (GAE). The reducing power was calculated using the formula:

$$RP = \frac{V_{extract}\, x\, C_{sample}}{m_{lyophilized\ material}}$$

In this equation, C_{sample} is the concentration of GAE in mg mL^{-1} derived from the standard curve, $V_{extract}$ is the volume of solvent used (in mL), and $m_{lyophilized\ material}$ is the mass of the freeze-dried extract (in mg).

Antihaemolytic activity

The OxHLIA assay was used to evaluate the extracts' ability to prevent oxidative damage to red blood cells. A 200 µL portion of a 2.8% (*v/v*) sheep erythrocyte suspension in phosphate-buffered saline (PBS, pH 7.4) was mixed with 400 µL of one of the following: (i) extract solutions in PBS at concentrations ranging from 6 to 1000 µg mL^{-1}, (ii) PBS alone as a control, (iii) deionized water to induce complete hemolysis, or (iv) trolox (7.81–250 µg mL^{-1}) as a positive control. After a 10-min pre-incubation at 37 °C with shaking, 200 µL of 160 mM AAPH (in PBS) was added to each mixture. Absorbance readings were taken at 690 nm approximately every 10 min using a Bio-Tek ELX800 microplate reader until full hemolysis occurred [32]. The results were expressed as IC_{50} values (µg mL^{-1}) at time intervals of 60 and 120 min, representing the concentration of extract required to preserve 50% of the erythrocytes over those durations. The percentage of intact cells (P) was calculated using the following equation:

$$\%P = \frac{Abs_{sample\ t} - Abs_{hem}}{Abs_{sample\ t0} - Abs_{hem}} x100$$

In this formula, $Abs_{sample\ t}$ and $Abs_{sample\ t0}$ refer to the absorbance of the sample at time (t) and at the start of the experiment, respectively, while Abs_{hem} corresponds to the absorbance of the fully hemolyzed sample at time zero.

Cytotoxic activity

Cytotoxic effects of the extracts were tested against four human cancer cell lines: AGS, CaCo-2, MCF-7, and NCI-H460. The SRB assay was performed based on the method described by Vajrabhaya and Korsuwannawong [33], with slight modifications. Cells were cultured in RPMI 1640 medium supplemented with 10% (*v/v*) FBS, 100 U mL^{-1} penicillin, and 100 µg mL^{-1} streptomycin. Cell cultures growth was monitored using a phase-contrast microscope. Cells were sub-cultured and seeded into 96-well plates at a density of 1.0×10^4 cells per well. After treatment with varying concentrations of the extracts, the cells were incubated for 72 h. To fix the cells, 100 µL of cold 10% (*w/v*) TCA was

added, followed by a 60-min incubation at 4 °C. Plates were then rinsed with deionized water and air-dried. Next, 100 µL of SRB solution (0.1% *w/v* in 1% *v/v* acetic acid) was added to each well, and the plates were left at room temperature for 30 min. Excess dye was removed by washing with 1% (*v/v*) acetic acid. The bound SRB was then solubilized using 200 µL of 10 mM Tris buffer, and absorbance was measured at 540 nm using a Biotek ELX800 microplate reader. The cytotoxicity results were expressed as GI_{50} values, which represent the concentration of extract (in µg mL^{-1}) required to inhibit 50% of culture growth. Ellipticin was used as the positive control.

Anti-inflammatory activity

The effect of the extracts on NO production was assessed in RAW 264.7 murine macrophage cells, using nitrite levels in the culture medium as an indicator [34]. Cells were cultured in DMEM supplemented with 10% (*w/v*) heat-inactivated FBS and 2 mM glutamine, maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were seeded into 96-well plates at a density of 150,000 cells per well and allowed to adhere overnight. The following day, they were exposed to different concentrations of each extract for 1 h. After this pre-treatment, cells were stimulated with 1 µg mL^{-1} LPS for 18 h. To measure NO production, the Griess Reagent System kit was used. A 100 µL aliquot of the culture supernatant was transferred to a new plate in duplicate, mixed with the Griess reagent, and incubated at room temperature for 5–10 min. Nitrite levels were quantified by measuring absorbance at 515 nm using a microplate reader, and values were compared against a standard curve. The results were expressed as IC_{50} values (µg mL^{-1}), representing the concentration of extract required to inhibit 50% of NO production. Dexamethasone (50 µM) served as the positive control.

Statistical analysis

All data are presented as the mean ± standard error of the mean (SEM) from three independent experiments. Statistical analysis was performed using analysis of variance (ANOVA). A significance threshold was set at $P \leq 0.05$. Depending on the type of data, either two-way ANOVA (used for TPC, TFC, and cytotoxicity assays) or one-way ANOVA (applied to all other assays) was conducted, followed by Tukey's post hoc test for multiple comparisons. All analyses were carried out using Prism® 8 software (GraphPad Software, La Jolla, CA, USA). IC_{50} values were determined through non-linear regression analysis using the same software. These values represent the concentration of extract required to achieve 50% inhibition in the respective biological assays. Specifically, for antioxidant (DPPH, TBARS), anti-hemolytic (OxHLIA), and anti-inflammatory assays, IC_{50} indicates the concentration needed to reduce radical activity, lipid peroxidation, hemolysis, or nitric oxide production by half. In cytotoxicity assays, the GI_{50} value refers to the extract concentration that inhibits 50% of tumor cell culture growth *in vitro*.

Results and discussion

Total polyphenol and flavonoid content

Extracts from *V. vinifera* seeds and the edible parts - pulp and skin - were chemically analyzed to explore their potential biological properties, particularly as by-products of the winemaking process. The grapevine is known to be rich in phenolic compounds, especially concentrated in the fruit's peel, pulp, and seeds [35, 36]. The TPC was quantified using a calibration curve of GA, with results expressed in milligrams of GA equivalents per gram of lyophilized extract. As indicated in Table 1, statistically significant differences ($P < 0.05$) were observed among all extract types. Notably, seed extracts exhibited significantly higher polyphenol levels ($P < 0.0001$), with the LS extract showing the greatest concentration.

Since flavonoids are a subgroup of polyphenols known for their antioxidant properties, their concentration in the grape extracts was measured to evaluate potential antioxidant effects. The TFC was calculated using a quercetin-based calibration curve (Figure S2) and expressed in milligrams of QE per gram of lyiophilized extract (Table 1). The grape variety itself did not significantly affect flavonoid levels ($P > 0.05$); however, notable differences were observed between different extract types within each variety ($P < 0.0001$). Seed extracts contained substantially higher flavonoid concentrations (229.8 ± 7.05 mg QE g^{-1}

Table 1 Total polyphenol content (TPC) and total flavonoid content (TFC) of seeds and pulp and skin extracts from Vinhão and Loureiro varieties

Extract	TPC (mg GAE $g^{-1}_{\text{lyophilized extract}}$)	TFC (mg QE $g^{-1}_{\text{lyophilized extract}}$)
Vinhão pulp and skin (VB)	18.51 ± 0.79^{cA}	15.60 ± 0.81^{bA}
Vinhão seeds (VS)	226.2 ± 2.18^{bA}	227.6 ± 7.39^{aA}
Loureiro pulp and skin (LB)	1.40 ± 0.11^{dA}	1.803 ± 0.78^{bA}
Loureiro seeds (LS)	257.6 ± 3.62^{aA}	229.8 ± 7.05^{aB}

Results are the mean ± SEM from at least three independent replicas and expressed in gallic acid equivalents (GAE) for TPC and quercetin equivalents (QE) for TFC. Two-way analysis of variance was performed by ANOVA, different lowercase letters in the same column indicate significant differences ($P \leq 0.05$) in each extract and different capital letters indicate significant differences ($P \leq 0.05$) at each assay by Tukey test.

for Loureiro, LS, and 227.6 ± 7.39 mg QE g^{-1} for Vinhão, VS) compared to pulp and skin extracts, which had much lower values: 1.803 ± 0.78 mg QE g^{-1} for Loureiro, LB, and 15.60 ± 0.81 mg QE g^{-1} for Vinhão, VB. These findings reinforce the idea that grape seeds are significantly richer in polyphenols and flavonoids than the edible parts. Similar results were reported by Xia et al. [37], who also assessed TPC and antioxidant activity using DPPH assays on grape skins and seeds from various cultivars.

Based on the TPC measured across the entire fruit, Table 1 shows that Loureiro grapes contain more phenolics than Vinhão grapes. Interestingly, Coelho et al. [18], who studied the same varieties, reported that red grape bagasse had higher antioxidant activity and phenolic levels than white grape bagasse. This discrepancy may be due to the fact that their study analyzed bagasse - comprising pulp, skin, and seeds - collected during red wine production. This material is left behind after the juice is extracted and often includes grape skins and stems. In some cases, partial fermentation of the bagasse can occur, increasing its alcohol and tannin content compared to bagasse from white wine production. In contrast, the current study used separated grape components that had not undergone juice extraction, which could explain the higher phenolic content observed in the white grape samples.

A comparison of TFC and TPC for each extract (Table 1) revealed that LS showed a statistically significant difference ($P < 0.01$), with TPC at 257.6 ± 3.62 mg GAE g^{-1} and TFC at 229.8 ± 7.05 mg QE g^{-1}. This suggests the presence of additional polyphenolic compounds beyond flavonoids. Similar findings were reported by Chengolova et al. [38], who observed higher TPC than TFC in seeds from several grape varieties - including Pinot Noir, Cabernet Sauvignon, Marselan, and Tamyanka - grown in Bulgaria's Danube region. Their study also confirmed that grape seeds contain more phenolics and flavonoids than skins, aligning with the current results. Conversely, the LB, VB, and VS extracts showed no significant difference between TPC and TFC ($P > 0.05$), indicating that their polyphenol content is primarily composed of flavonoids. This may be due to varietal differences, as previous research on Virginia-grown grapes (Chambourcin, Viognier, Cabernet Franc, and Vidal Blanc) also reported significant variation in TPC and TFC among bagasse extracts [39].

UPLC analysis of phenolic compounds

The phenolic profiles of seed extracts and those derived from pulp and skin were analyzed using UPLC, with results presented in Table 2 for Loureiro and Table 3 for Vinhão. These tables include retention times (Rt), maximum absorption wavelengths (λmax), and mass spectrometry data. In LS, type-B procyanidins were the dominant compounds (Table 2). Fragmentation patterns indicated multiple catechin units lonked via type-B interflavan bonds, with breakdown products suggesting catechin monomers (289 u). Peaks 1, 4, and 7 ($[M-H]^-$ at *m/z* 1153) were identified as tetramers, while peaks 2, 3, 5, 6, 9, and 10 ($[M-H]^-$ at *m/z* 865) corresponded to trimers, and peak 8 ($[M-H]^-$ at *m/z* 1441) to a pentamer. Galloylated derivatives were also detected, adding galloyl groups to catechin structures and enhancing their complexity and potential bioactivity. Peaks 11 ($[M-H]^-$ at *m/z* 1305), 12 ($[M-H]^-$ at *m/z* 1017), and 13 ($[M-H]^-$ at *m/z* 729) were tentatively assigned as monogalloyl procyanidin tetramer, trimer, and dimer, respectively. In addition to LS, the extract LB revealed phenolic acids - peak 1 ($[M-H]^-$ at *m/z* 153; protocatechuic acid) and peak 2 ($[M-H]^-$ at *m/z* 367; 3-*O*-feruloylquinic acid) - alongside flavan-3-ols in peaks 3 through 6.

The phenolic composition of VS was thoroughly examined, revealing a wide range of polymeric procyanidins (Table 3). Among the identified compounds were B-type procyanidin hexamers (peak 1, $[M-H]^-$ at *m/z* 174), pentamers (peaks 2 and 10, $[M-H]^-$ at *m/z* 1441), tetramers (peaks 5, 9, and 12, $[M-H]^-$ at *m/z* 1153), and trimers (peaks 3, 4, 6, 7, 8, and 11, $[M-H]^-$ at *m/z* 865). More complex structures were also detected, including monogalloylated derivatives: a tetramer (peak 13, $[M-H]^-$ at *m/z* 1305), a pentamer (peak 14, $[M-H]^-$ at *m/z* 1593), and trimers (peaks 15 and 16, $[M-H]^-$ at *m/z* 1017). In the Vinhão pulp and skin extract, VB, nine anthocyanins were tentatively identified, including one delphinidin, three cyanidin glycosides, one petunidin, two peonidins, one pelargonidin glycoside, and one malvidin derivative. These compounds are primarily glycosylated forms of common anthocyanidin aglycones, which enhances their solubility and stability, contributing to the vivid coloration and potential health-promoting properties of grapes.

In LS (Table 2), quantitative analysis revealed the presence of several proanthocyanidins: procyanidin dimers (23.84 ± 0.97 mg g^{-1}), trimers (89.94 ± 1.26 mg g^{-1}), tetramers (46.69 ± 0.89 mg g^{-1}), and pentamers (12.26 ± 0.45 mg g^{-1}). These findings align with previous studies that have highlighted the dominance of procyanidins in grape seed extracts [40, 41]. In LB, compounds such as gallocatechin (0.56 ± 0.01 mg g^{-1}), 3-*O*-feruloylquinic acid (0.18 ± 0.01 mg g^{-1}), epigallocatechin (0.078 ± 0.001 mg g^{-1}), and protocatechuic acid (0.068 ± 0.002 mg g^{-1}) were identified. These molecules have been previously reported in grape tissues, with flavan-3-ols like epigallocatechin found in skins and seeds [42], gallocatechin detected in grapes [43], and 3-*O*-feruloylquinic acid observed in red grape skins [44]. Notably, Pešić et al. [45] suggested that protocatechuic acids (phenolic acids) play a major role in the antioxidant activity of grape skin extracts. The current results support

Table 2 Retention time (Rt), wavelengths of maximum absorption in the visible region (λmax), mass spectral data, and identification of phenolic compounds Loureiro seeds (LS) and pulp and skin (LB) extracts

Loureiro						
Peak	Rt (min)	λmax	$[M-H]^-$ m/z	MS^2 (m/z)	Tentative identification	Quantification (mg g^{-1})
LS						
1	4.71	279	1153	865(5),863(15),577(59),575(19),289(32),287(9)	B-type procyanidin tetramer[1]	4.61±0.16
2	5.4	280	865	739(11),713(14),695(20),577(33),449(73),425(18), 407(57),289(7),287(85)	B-type procyanidin trimer[1]	11.15±0.33
3	6.18	280	865	739(10), 713(14), 695(18),577(27),449(43),425(52), 407(25),289(11),287(18)	B-type procyanidin trimer[1]	12.37±0.18
4	6.62	280	1153	865(10),863(15),577(61),575(25),289(40),287(11)	B-type procyanidin tetramer[1]	16.65±0.06
5	8.35	280	865	739(8),713(18),695(19),577(31),451(95),425(27), 407(92),289(25),287(3)	B-type procyanidin trimer[1]	11.01±0.25
6	9.42	280	865	739(10),713(13),695(20),577(35),451(8),425(47), 407(100),289(52)0.287(6)	B-type procyanidin trimer[1]	10.39±0.11
7	10.84	280	1153	865(7) 863(18),577(60),575(27),289(41),287(11)	B-type procyanidin tetramer[1]	10.53±0.07
8	12.36	280	1441	1153(10),865(8),577(30),289(94),287(31)	Procyanidin pentamer[1]	12.26±0.45
9	14.98	280	865	739(9),713(12),695(22),577(15),449(64),425(9), 407(11),289(32),287(55)	B-type procyanidin trimer[1]	18.46±0.83
10	16.7	280	865	739(13),713(15),695(21),577(18),449(34),425(15), 407(12),289(65),287(5)	B-type procyanidin trimer[1]	11.38±0.08
11	17.82	280	1305	1017(51),729(32),577(28),567(19),441(20),289(2), 287(3)	Procyanidin tetramer monogallate[1]	14.9±0.75
12	18.98	280	1017	891(20),865(23),847(12),771(4),739(5),695(9), 577(18),407(3),289(11)	Procyanidin trimer monogallate[1]	15.18±0.51
13	21.04	280	729	577(100), 559(41), 407(77), 285(13)	Procyanidin dimer monogallate[1]	10.7±0.07
14	22.2	280	577	451(28),425(35),407(100),289(62),287(9)	B-type procyanidin dimer[1]	13.13±0.9
					Total procyanidin dimers	23.84±0.97
					Total procyanidin trimers	89.94±1.26
					Total procyanidin tetramers	46.69±0.89
					Total procyanidin pentamers	12.26±0.45
					Total proanthocyanidins	172.73±2.67
LB						
1	4.22	314	153	109(100)	Protocatechuic acid[2]	0.068±0.002
2	5.63	316	367	193(100)	3-*O*-Feruloylquinic acid[3]	0.18±0.01
3	6.79	279	305	261(41),221(34),219(65),179(100),165(13)	Gallocatechin[1]	0.56±0.01
4	8.73	276	305	261(41),221(34),219(65),179(100),165(13)	Epigallocatechin[1]	0.078±0.001
5	10.02	287	575	449(70),423(60),407(100),289(10),287(15),285(5)	A-type procyanidin dimer	0.105±0.002
6	19.57	277	865	739(11),695(18),577(11),449(54),425(100),407(16), 289(22),287(51)	B-type procyanidin trimer[1]	0.107±0.001
					Total phenolic Acids	0.248±0.011
					Total flavan-3-ol	0.852±0.011
					Total phenolic compounds	1.1±0.022

Calibration curves used for each compounds: (1) (+)-catechin (y = 84950x – 23200, R^2 = 0.98; LOD = 0.17 µg mL^{-1}; LOQ = 0.68 µg mL^{-1}); (2) protocatechuic acid (y = 214168x + 27102, R^2 = 0.9999; LOD = 0.14 µg mL^{-1}; LOQ = 0.52 µg mL^{-1}) and (3) ferulic acid (y = 633126x – 185462, R^2 = 0.9990; LOD = 0.20 µg mL^{-1}; 1.01 µg mL^{-1})

this conclusion, as the low concentration of protocatechuic acid in the Loureiro pulp and skin extract correlates with its reduced antioxidant capacity and free radical scavenging ability (see Table 4, Sect. 3.3.1).

In the Loureiro seed and pulp/skin extracts, LS and LB, respectively (Table 2), as well as in the Vinhão seed extract, VS (Table 3), B-type procyanidin trimers were identified. These compounds are among the most common proanthocyanidins found in food sources, whereas A-type variants are relatively rare [46]. As a subclass of flavonoids, proanthocyanidins are recognized for their potent antioxidant capabilities, effectively neutralizing various free radicals and reactive nitrogen species [47]. The high concentrations of B-type procyanidin trimers − 92.5 ± 2 mg g^{-1} in VS and

Table 3 Retention time (Rt), wavelengths of maximum absorption in the visible region (λmax), mass spectral data, and identification of non-anthocyanins phenolic compounds in Vinhão seeds (VS) and anthocyanins phenolic compounds in pulp and skin (VB) extracts

Vinhão						
Peak	Rt (min)	λmax	$[M-H]^-$ m/z	MS^2 (m/z)	Tentative identification	Quantification (mg g^{-1})
VS						
1	4.26	264	1745	1441(20),1153(11),865(15),577(36),289(71), 287(12)	B-type proanthocyanidin hexamers[1]	3.05±0.07
2	4.75	278	1441	1153(10),865(8),577(30),289(94),287(31)	B-type procyanidin pentamer[1]	3.67±0.02
3	5.15	280	865	739(10),713(28),695(18),577(33),451(90), 425(25),407(92),289(24),287(5)	B-type procyanidin trimer[1]	5.09±0.08
4	5.51	280	867	739(8),713(18),695(19),577(31),451(95), 425(27),407(92),289(25),287(3)	B-type procyanidin trimer[1]	5.28±0.08
5	6.64	280	1153	865(7) 863(18),577(60),575(27),289(41), 287(11)	B-type procyanidin tetramer[1]	7.6±0.17
6	7.67	280	865	739(9),713(12),695(29),577(30),451(91), 425(28),407(97),289(22),287(6)	B-type procyanidin trimer[1]	22.52±0.45
7	8.35	280	865	739(9),713(17),695(15),577(28),451(95), 425(20),407(90),289(24),287(3)	B-type procyanidin trimer[1]	14.13±0.23
8	9.24	280	865	739(12),713(18),695(18),577(30),451(96), 425(37),407(82),289(22),287(6)	B-type procyanidin trimer[1]	12.42±0.82
9	10.83	280	1153	865(7) 863(18),577(60),575(27),289(41), 287(11)	B-type procyanidin tetramer[1]	14.22±0.76
10	12.43	280	1441	1153(10),865(8),577(30),289(94),287(31)	B-type procyanidin pentamer[1]	13.04±0.43
11	13.31	280	865	739(18),713(10),695(15),577(21),451(85), 425(17),407(96),289(24)	B-type procyanidin trimer[1]	7.69±0.02
12	15.17	280	1153	865(6) 863(28),577(61),575(26),289(31), 287(10)	B-type procyanidin tetramer[1]	21.6±0.85
13	16.66	280	1305	1017(51),729(32),577(28),567(19),441(20), 289(2),287(3)	Procyanidin tetramer monogallate[1]	15.42±0.5
14	17.73	280	1593	1441(12),1153(20),1005(13),865(12),577(11), 441(18),289(3),287(4)	Procyanidin pentamer monogallate[1]	7.2±0.04
15	18.98	280	1017	891(21),865(20),847(13),771(3),739(8),695(11), 577(15),407(4),289(13)	Procyanidin trimer monogallate[1]	8.9±0.09
16	21.06	280	1017	891(20),865(23),847(12),771(4),739(5),695(9), 577(18),407 (3),289(11)	Procyanidin trimer monogallate[1]	16.47±0.39
					Total procyanidin trimers	92.5±2
					Total procyanidin tetramers	58.84±2.27
					Total procyanidin pentamers	23.91±0.5
					Total proanthocyanidin hexamers	3.05±0.07
					Total proanthocyanidins	178.31±4.84
VB						
1	8.98	522	465	303(100)	Delphinidin-3-*O*-glucoside[2]	1.15±0.02
2	11.12	514	449	287(100)	Cyanidin-3-*O*-glucoside[2]	0.737±0.002
3	14.73	523	479	317(100)	Petunidin-3-*O*-glucoside[3]	0.97±0.02
4	17.66	514	463	301(100)	Peonidin-3-*O*-glucoside[3]	1.42±0.06
5	20.11	525	493	331(100)	Malvidin-3-*O*-glucoside[3]	2.17±0.04
6	24.11	525	475	271(100)	Pelargonidin-3-*O*-(6”-acetoyl)glucoside[4]	0.236±0.002
7	28.02	525	577	287(100)	Cyanidin-3-*O*-malonyl-acetoyl-glucoside[2]	0.4307±0.0003
8	31.08	525	609	463(30), 301(100)	Peonidin-3-*O*-rutinoside[3]	0.434±0.001
9	36.87	525	535	287(100)	Cyanidin-3-*O*-malonyl-glucoside[2]	0.489±0.002
					Total anthocyanins compounds	8.03±0.14

Calibration curves used for each compound: (1) (+)-catechin (y = 84950x – 23200, R^2 = 0.98; LOD = 0.17 μg mL^{-1}; LOQ = 0.68 μg mL^{-1}), (2) cyanidin-3-*O*-glucoside (y = 134578x − 3E + 06; R^2 = 0.9986; LOD = 0.25 μg mL^{-1}; LOQ = 0.83 μg mL^{-1}); (3) peonidin-3-*O*-glucoside (y = 151438x – 3E + 06, R^2 = 0.9965, LOD = 0.13 μg mL^{-1}; LOQ = 0.40 μg mL^{-1} and (4) pelargonidin-3-*O*-glucoside (y = 61493x − 628875, R^2 = 0.9957, LOD = 0.13 μg mL^{-1}; LOQ = 0.40 μg mL^{-1}

89.94 ± 1.26 mg g^{-1} in LS - likely contribute significantly to the strong antioxidant activity observed in these extracts.

Grape seed extracts are known to contain procyanidins with varying degrees of polymerization [48]. In this study, both LS and VS extracts exhibited polymer chains extending up to six units (Tables 2 and 3). Literature examples include macrocyclic tetramers and pentamers identified in red wines from Bordeaux [49], and cyclic hexamers found in wines from Bolzano, Italy [50]. Additionally, seed extracts from Chardonnay and Pignoletto grape varieties have been reported to contain procyanidin oligomers with polymerization degrees ranging from 6 to 12 [48].

Anthocyanins were the predominant compounds found in the pulp and skin extract of the Vinhão grape, VB, with a total concentration of 8.03 ± 0.14 mg g^{-1} (Table 3). The most abundant anthocyanins included malvidin-3-*O*-glucoside (2.17 ± 0.04 mg g^{-1}), peonidin-3-*O*-glucoside (1.42 ± 0.06 mg g^{-1}), delphinidin-3-*O*-glucoside (1.15 ± 0.02 mg g^{-1}), and petunidin-3-*O*-glucoside (0.97 ± 0.02 mg g^{-1}). These same compounds have also been identified in the skin of Amazon grapes (*Pourouma cecropiifolia* Martius), which share similar physical traits with *V. vinifera* [51]. Red grape varieties are particularly rich in polyphenols, especially anthocyanins, which are typically absent in white grape types [52].

Bioactive properties

Antioxidant activity

The polyphenol and flavonoid content in the extracts suggests potential antioxidant properties, which are expected to be more pronounced in seed extracts compared to those from pulp and skin. To evaluate this, antioxidant activity was assessed using DPPH radical scavenging activity (regression graphic of gallic acid is presented in Figure S3), TBARS assay, and reduction power measurements (Table 4). As anticipated, the Loureiro seed extract, LS, demonstrated significantly stronger radical scavenging ability than the pulp and skin extract, LB, reflected by lower IC_{50} values ($P \leq 0.05$), indicating greater antioxidant potential [36]. A similar pattern was observed in the Vinhão variety, although the difference between VS and VB extracts was not statistically significant ($P > 0.05$), despite being notable.

As shown in Table 4, the LB extract had the highest DPPH IC_{50} value (6.01 ± 2.71 mg mL^{-1}), indicating the weakest radical scavenging activity among all samples. It also recorded the highest TBARS IC_{50} value (0.04 ± 0.003 mg mL^{-1}; $P < 0.05$) and the lowest reducing power (0.003 ± 0.02 mg GAE g^{-1} lyophilized extract; $P < 0.05$). When comparing VB and LB, no significant difference was observed ($P > 0.05$). Among the seed extracts, LS showed the strongest antioxidant performance, with the lowest IC_{50} values for both DPPH (0.24 ± 0.16 mg mL^{-1}) and TBARS (0.004 ± 0.001 mg mL^{-1}), although these were not significantly different from VS ($P > 0.05$). The LS extract also had the highest reducing power (235.2 ± 17.06 mg GAE g^{-1}; $P \leq 0.05$). Gallic acid, GA, used as a reference, demonstrated antioxidant activity comparable to LS and VS ($P > 0.05$). Baron et al. [53] found that polyphenols extracted from Italian red grape skins had 70% greater DPPH activity than crude water extracts. This study also supports the positive correlation between phenolic content and antioxidant capacity, as LS - with the highest phenolic concentration (Tables 1 and 2) - showed antioxidant activity similar to GA in both DPPH and TBARS assays (Table 4).

Table 4 Antioxidant activity measured as 2,2-diphenyl-1-picrylhydrazyl (DPPH), thiobarbituric acid reactive substances (TBARS), and reducing power capacity (RP) of seeds and pulp and skin extracts from Vinhão and Loureiro varieties

Extracts	DPPH IC_{50} (mg mL^{-1})	TBARS IC_{50} (mg mL^{-1})	RP (mg GAE $g^{-1}_{\text{lyophilized extract}}$)
Vinhão pulp and skin (VB)	3.10 ± 0.24^{ab}	0.022 ± 0.01^{ab}	10.96 ± 0.21^{c}
Vinhão seeds (VS)	0.35 ± 0.05^{b}	0.005 ± 0.001^{b}	170.2 ± 8.60^{b}
Loureiro pulp and skin (LB)	6.01 ± 2.71^{a}	0.04 ± 0.003^{a}	0.003 ± 0.02^{c}
Loureiro seeds (LS)	0.24 ± 0.16^{b}	0.004 ± 0.001^{b}	235.2 ± 17.06^{a}
Gallic acid (GA)	0.03 ± 0.01^{b}	0.005 ± 0.001^{b}	-

Results are the mean ± SEM from at least three independent replicas expressed in IC_{50} (DPPH; mg mL^{-1}) and mg GAE.g^{-1}, respectively. One-way analysis of variance was performed by ANOVA, means followed by different lowercase letters in the column differ from each other ($P \leq 0.05$) by the Tukey test

The TBARS assay is a useful *in vitro* technique for evaluating antioxidant potential, particularly in biological and food-related systems. It models lipid oxidation, making it highly relevant for assessing oxidative stability in fat-containing products [54]. In this study, low IC_{50} values observed for LS, VS, and VB extracts (Table 4), comparable to those of GA, suggest strong inhibition of lipid peroxidation. This indicates their potential application as natural preservatives in food. Preventing lipid oxidation is essential to avoid rancidity and undesirable flavors during processing and storage, as highlighted by Luther et al. [55]. These results support the promising use of grape-derived extracts as effective natural additives in the food industry.

In the reducing power assay, antioxidants present in the sample can donate electrons, resulting in the conversion of Fe^{3+} to Fe^{2+} [56]. This mechanism differs from that of the DPPH assay, which involves hydrogen atom transfer to neutralize radicals. The consistency between the results of both assays (Table 4) indicates that the extracts contain compounds capable of functioning as antioxidants through

both electron donation and hydrogen transfer. Notably, LS and VS demonstrated the strongest reducing power via both mechanisms.

Radulescu et al. [57] also demonstrated that grape seed extracts possess stronger antioxidant activity compared to skin extracts across several varieties, including Merlot, Feteasca Neagra, Pinot Noir, and Muscat Hamburg. These seed extracts also contained higher levels of total phenolics and flavonoids. The findings suggest that antioxidant potential is influenced not only by the quantity but also by the specific types of phenolic compounds present. The strong correlation between elevated phenolic and flavonoid concentrations and antioxidant performance indicates that the effectiveness of grape extracts in neutralizing reactive species is closely tied to the presence of these bioactive molecules.

Overall, the data in Table 4 indicate that both grape varieties exhibit strong antioxidant properties, with VS and LS showing the highest activity. This suggests that the seeds are the most bioactive part of the fruit. These findings are consistent with the study by Shen et al. [58], which examined the antioxidant potential of grape cultivars grown in the Chengdu plain of southern China. Using the DPPH assay, their research showed that grape seeds had the greatest antioxidant capacity, followed by the skins, while the pulp had the lowest activity.

Antihaemolytic activity

The OxHLIA assay evaluates antioxidant activity by measuring the ability of compounds to prevent oxidative damage to sheep erythrocyte membranes caused by peroxyl radicals. This method is particularly relevant because it uses biologically meaningful radicals and targets, unlike other *in vitro* assays that rely on synthetic radicals like DPPH [59, 60]. In this study, OxHLIA was used to assess the antihaemolytic potential of the grape extracts (Fig. 1). All extracts, except VB, demonstrated significantly greater antihaemolytic activity than Trolox, indicating their effectiveness in protecting cell membranes from oxidative stress. The LS extract was especially potent, showing the lowest IC_{50} value (4.81 ± 0.22 µg mL^{-1}; $P < 0.05$), suggesting strong free radical neutralization and prolonged erythrocyte survival [61]. These IC_{50} values were notably lower than those

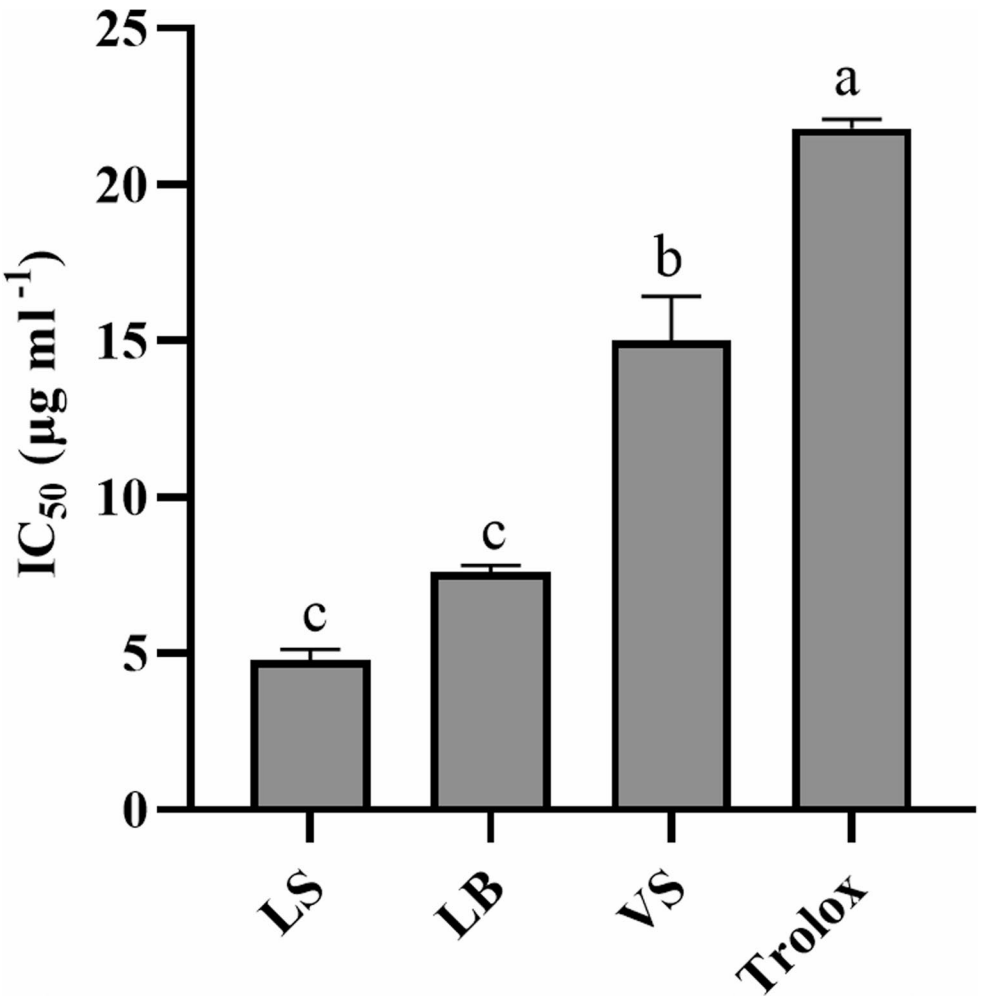

Fig. 1 The antihaemolytic activity of seeds and pulp and skin extracts from Vinhão and Loureiro varieties of *Vitis vinifera* assessed by the OxHLIA assay. The assayed extracts were Loureiro seeds (LS), Loureiro berries and skin (LB), Vinhão seeds (VS), and Trolox as positive control. Results are the mean ± SEM from at least three independent replicas expressed as IC_{50} (µg mL^{-1}). One-way analysis of variance was performed by ANOVA, different letters indicate significant differences ($P \leq 0.05$) by Tukey test

reported for Spanish grape seed extracts from Tempranillo and Garnacha varieties [62], underscoring the exceptional antihaemolytic properties of Loureiro and Vinhão grapes. The phenolic compounds in these extracts are capable of scavenging peroxyl radicals, thereby halting oxidative chain reactions and protecting erythrocyte membranes [63]. This reinforces the link between high phenolic content and antioxidant effectiveness, particularly in the LS extract from the Loureiro variety.

Cytotoxic activity

Table 5 presents the cytotoxicity results of seed and pulp/skin extracts from the Vinhão and Loureiro grape varieties against several cancer cell lines, including AGS, CaCo-2, MCF-7, and NCI-H460. The pulp and skin extracts, VB and LB, showed no cytotoxic effects, with GI_{50} values exceeding 400 μg mL^{-1}, indicating they were ineffective in suppressing tumor cell growth. In contrast, the seed extracts demonstrated notable cytotoxic potential. The LS extract exhibited GI_{50} values ranging from 37.04±2.01 to 274.99±21.95 μg mL^{-1}, while the VS extract showed values between 51.79±2.13 and 62.92±6.04 μg mL^{-1}.

The cytotoxic effects observed in the seed extracts may be linked to their high antioxidant content. Previous studies have shown that antioxidants can promote tumor cell death by influencing protein kinase signaling pathways, which in turn affect gene transcription and the synthesis of proteins essential for cancer cell survival [64]. These changes can disrupt cellular metabolism, hinder cell cycle progression, impair mitochondrial function, reduce ribose synthesis, and suppress glycolysis in tumor cells, ultimately leading to apoptosis. Moreover, some antioxidant compounds may also exhibit prooxidant behavior, further contributing to reduced cell viability [65].

Interestingly, LS showed the lowest cytotoxicity ($P < 0.05$) against MCF-7 cells, with a GI_{50} of 274.99 ± 21.95 μg mL^{-1} (Table 5), while VS exhibited significantly higher toxicity ($P < 0.05$), with a GI_{50} of 62.92 ± 6.04 μg mL^{-1}. For the remaining cell lines tested, LS and VS displayed comparable cytotoxic effects ($P > 0.05$). These results are consistent with previous in vitro and in vivo research demonstrating the anti-cancer potential of grape seed polyphenols in various human cancers, including breast, lung, and gastric adenocarcinoma [66–68].

Table 5 Cytotoxic activity of seeds and pulp and skin extracts from Vinhão and Loureiro varieties to tumor cell lines

Cell lines	Cytotoxic activity (GI_{50} μg mL^{-1})				
	Loureiro		Vinhão		Control
	Seeds (LS)	Pulp and skin (LB)	Seeds (VS)	Pulp and skin (VB)	Elipticin
AGS	37.04 ± 2.01^{bA}	>400	51.79 ± 2.13^{aA}	>400	1.23 ± 0.03^{aB}
CaCo-2	38.02 ± 4.01^{bA}	>400	52.07 ± 4.21^{aA}	>400	1.21 ± 0.02^{aB}
MCF-7	274.99 ± 21.95^{aA}	>400	62.92 ± 6.04^{aB}	>400	1.02 ± 0.02^{aC}
NCI-H460	41.89 ± 3.01^{bA}	>400	53.52 ± 1.24^{aA}	>400	1.01 ± 0.01^{aB}

Results are the mean±SEM from at least three independent replicas and expressed in GI_{50} (μg mL^{-1}). Two-way analysis of variance was performed by ANOVA, different lowercase letters in the same column indicate significant differences ($P \leq 0.05$) in each extract and different capital letters indicate significant differences ($P \leq 0.05$) at each variety to tumor cell lines by Tukey test

Anti-inflammatory activity

The anti-inflammatory activity of extracts from the seeds and from the pulp and skin of Vinhão and Loureiro grapes was assessed by measuring their ability to suppress NO production in LPS-stimulated RAW 264.7 macrophages. None of the tested samples showed significant inhibition at concentrations up to 400 μg mL^{-1} (data not shown). While some grape extracts have demonstrated anti-inflammatory potential - such as n-hexane seed extracts from Pecorello grapes [69], muscadine (*Vitis rotundifolia*) skin and seed extracts [70], aqueous extracts from Italian red grape skins [53], Uruguayan red grape pomace [71], and hydroethanolic peel extracts from *V. labrusca* [72] - the current study did not observe similar effects. Interestingly, Zhang et al. [73] reported that procyanidin dimer B2 could suppress LPS-induced inflammation in human THP-1 monocytes. Despite the high levels of procyanidins in the extracts analyzed here, no anti-inflammatory activity was detected. This discrepancy may be due to differences in grape origin, extraction techniques, or the specific in vitro model used, all of which can influence the concentration and composition of bioactive compounds and their biological effects.

Conclusion

This research explores the chemical makeup and biological effects of grape extracts from Vinhão and Loureiro, two widely cultivated *V. vinifera* varieties in Portugal's Vinho Verde region. The findings highlight notable differences in polyphenol and flavonoid levels between seed extracts and those from pulp and skin (Table 1), with seeds consistently containing higher concentrations of these bioactive compounds. UPLC analysis revealed a diverse range of phenolics contributing to the antioxidant properties of the extracts (Tables 2 and 3). The antioxidant potential was further

confirmed through DPPH, TBARS, and reducing power assays (Table 4), with seed extracts showing particularly strong activity. Additionally, cellular assays demonstrated protective effects against oxidative haemolysis (Fig. 1). The cytotoxicity results (Table 5) suggest that grape seed extracts may have therapeutic potential in cancer prevention or treatment, although further investigation is needed to understand their mechanisms and clinical relevance.

By examining the bioactive potential of extracts from various grape components - namely seeds and pulp and skin - this study emphasizes the distinctive qualities of the Portuguese Vinhão and Loureiro varieties. It also highlights their promising applications in fields such as pharmaceuticals, cosmetics, and nutraceuticals. Additionally, the rich presence of bioactive compounds in winemaking residues supports the idea of repurposing grape waste, offering benefits across economic, environmental, and social dimensions. Ultimately, this research opens new avenues for incorporating Portuguese grape extracts into diverse industries, promoting sustainable agricultural practices and fostering innovation in product development.

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Declarations

Conflict of interest The authors declare no conflicts of interest.

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