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Potential application of grape (*Vitis vinifera* L.) stem extracts in the cosmetic and pharmaceutical industries: Valorization of a by-product

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ABSTRACT

Wastes from the wine industry can cause sustainably problems, thus requiring their reuse. Thus, new products can be generated through these wastes, giving environmental, social, and economic advantages. In this sense, the objective of this work was to determine the phenolic composition of six varieties of grape stem extracts by High Performance Liquid Chromatography and to evaluate their biological activities (antioxidant, antimicrobial, anti-inflammatory, and anti-aging). The results showed that grape stem extracts confirm to be a potential source of phenolic compounds, with catechin being the most abundant compound in all varieties (0.44 ± 0.02–2.03 ± 0.08 mg/g dry weight). The grape stem extracts also presented antioxidant activity, reaching values up to 0.84 ± 0.06, 0.64 ± 0.05, and 1.03 ± 0.06 mmol Trolox/g dry weight for ABTS, DPPH, and FRAP assays, respectively. Concerning antimicrobial activity, the extracts presented high efficacy against foot wound ulcers Gram-positive bacteria. In addition, the extracts presented anti-inflammatory capacity exhibiting inhibitions of Nitric Oxid production, by lipopolysaccharide-stimulated macrophages, up to 35.25 %, and revealed, for the first time, anti-aging effect by inhibiting anti-tyrosinase (~54 %) and anti-elastase (~98 %) activities. Therefore, grape stem has demonstrated its biological potential, being of interest for cosmetic, pharmaceutical, and food industries.

1. Introduction

Efficient use of resources through minimization of waste, long-term value retention, reduction of primary resources, and closed cycles of products within the limits of environmental protection and socio-economic advantages can be defined as Circular Economy. This economic model enhances sustainable development while decreasing the negative consequences of lack of resources and environmental degradation (Morseletto, 2020). Various activities in the agricultural sector generate large amounts of by-products and wastes, where these wastes can cause sustainability problems due to the large quantities produced in a limited time period and due to the organic matter content (Coderoni and Perito, 2019). One of these activities is the grape production, reaching 73.3 million tons in 2017. It is estimated that 52 % of this production goes to the wine industry (OIV, 2018), where large quantities of by-products are generated, such as grape pomace, seeds, pulp, skins, leaves, stems, and wine lees (Gouvinhas et al., 2019). The recovery of these by-products/wastes has become increasingly important, providing multiple environmental, economic and social benefits, since their reuse generates the production of new added products, as a consequence of this circular approach (Coderoni and Perito, 2019).

Grape stem, which accounts for 25 % of total by-products, is the less characterized and valued of all by-products generated (Barros et al., 2014). This by-product is a rich source of phenolic compounds, celluloses, hemicelluloses and lignins (Gouvinhas et al., 2019). However, is it usually destined to the production of spirits, dietary fiber, vegetable protein concentrates, fertilizers (Domínguez-Perles et al., 2014), and animal feed (Sahpazidou et al., 2014).

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In the last years, bioactive compounds present in by-products have attracted attention due to their health benefits, leading to a growing interest of the scientific community for the exploration of these residues (Gouvinhas et al., 2018). Phenolic compounds are secondary metabolites produced by plants that demonstrated to have several health benefits, acting as antioxidants, antimicrobials, anticarcinogens, antidiabetics, among others (Gouvinhas et al., 2019). Regarding to grape stems polyphenolic composition, several phenolic compounds were identified, such as flavonols (*e.g.* quercetin-3-*O*-rutinoside, kaempferol-3-*O*-glucoside), flavanols (*e.g.* catechin, epicatechin), anthocyanins (*e.g.* malvidin-3-*O*-glucoside, malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside), phenolic acids (*e.g.* gallic, caffeic and caftaric acids), stilbenes (*e. g.* trans-resveratrol, Ɛ-viniferin) and procyanidins (e.*g.* procyanidin B2 and B3) (Anastasiadi et al., 2012; Barros et al., 2014; Dias et al., 2015; Domínguez-Perles et al., 2016; Gouvinhas et al., 2018; Sahpazidou et al., 2014).

The human body, when exposed to external conditions (for example pollutants, radiation and pathogens) produces a large quantity of reactive oxygen species (ROS), which causes oxidative stress, and may also induce inflammation (Zhang and Tsao, 2016). Phenolic compounds prevent or inhibit the production of ROS, suspending oxidation processes and repairing tissues deficiencies (Baenas et al., 2018). Moreover, while non-steroidal anti-inflammatory drugs are the most widely used in the world to fight inflammation, they have undesirable side-effects, for example on the kidneys and cardiovascular system, limiting their use. Thus, new studies are being performed to identify natural compounds that can be applied as anti-inflammatory agents or in the development of new drugs (Aouey et al., 2016). In addition, oxidative stress and inflammation are also related to aging processes (Martín-Ortega and Campos, 2019), where changes in the biosynthetic activity of skin cells, namely some constitutive and extracellular matrix proteins (*e.g.* collagen and elastin) arise, as well as in a simultaneous activity modulation of several aging-related enzymes (*e.g.* elastase, tyrosinase) (Aguilar-Toalá et al., 2019; Taghouti et al., 2018). The enzyme tyrosinase (also called monophenol monooxygenases), in humans, is responsible for melanin synthesis in melanocytes, but when overproduction of melanin occurs in the skin, pigmentation disorder, such as hyperpigmentation, over-tanning, age spots and melasma occur (Liyanaarachchi et al., 2018). Elastase is an enzyme that hydrolyses elastin, influencing the mechanical properties of connective tissues, thus when a high activity of this enzyme is observed, or less elastin is produced, the skin loses firmness and elasticity (Aguilar-Toalá et al., 2019).

Antibiotic resistance is a public health problem worldwide, having impact in morbidity and/or high mortality rates, especially in developing countries (Lima et al., 2019). Specific microorganisms such as *Listeria monocytogenes* and *Escherichia coli* are present frequently in processed food, constituting a health risk (Gutiérrez-del-Río et al., 2018). In addition, one of the main causes of hospitalization worldwide are diabetic foot ulcer infections, mostly caused by *Staphylococcus aureus* (Zenão et al., 2017). To combat bacterial resistance, alternative antibiotics should be searched and developed to overcome the effectiveness of those currently available (Lima et al., 2019).

Thus, in order to replace synthetic drugs/antibiotics with more effective natural compounds, there is a growing demand to find natural compounds that can be used in the development of new products. Grapes stem may be integrated into this demand due to its polyphenolic content, thus enhancing sustainable development, with advantages for both the environment and the corporate economy. In this way, the aim of this study was to evaluate the scavenging activity and the antimicrobial activity of grape stem extracts, against gastrointestinal and diabetic foot wound bacteria. Furthermore, the anti-inflammatory and the anti-aging activities, allowing to prove if this by-product will be a good bet to produce new formulations. This investigation allowed to determine, for the first time, not only anti-tyrosinase and anti-elastase activities of grape stems, but also their capacity to inhibit the growth of foot wound ulcers bacteria.

2. Material and methods

2.1. Chemicals

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

2.2. Sampling

For the accomplishment of this work the sampling was constituted by six grape (*Vitis vinifera* L.) stems varieties: Tinta Roriz, Touriga Nacional, Castelão, Syrah (red varieties), Arinto, and Fernão Pires (white varieties). These varieties were collected during the 2018 season, in Quinta do Pinto, Alenquer (Lisbon). After harvesting, the grape stems were washed, and posteriorly were dried in oven (Memmert, Schwabach, Germany) for 72 h at 40 °C. Then, the samples were ground and stored at room temperature protected from light until the analysis.

2.3. Grape stem extracts preparation

For the preparation of the extract, 40 mg of the previously milled grape stem was weighed, and 1.5 mL of MeOH/H_2O (70:30, v/v) mixture were added. Then, samples were vortexed and agitated for 30 min at room temperature, for the extraction of the phenolic compounds, and after centrifuged at 10,000 *rpm* for 15 min, at 4 °C (Sigma, Steinheim, Germany), collecting the supernatant (Leal et al., 2020). This procedure was repeated 3 times for each sample of grape stem. Supernatants were filtered through a 0.45-µm PVDF filter (Millex HV13, Millipore, Bedford, MA, USA) and stored at 4 °C until analysis.

2.4. Identification and quantification of phenolic compounds by HPLC

The phenolic profile of grape stems samples was assessed by Reverse Phase - High Performance Liquid Chromatography - Diode Array Detector (RP-HPLC-DAD), with a C18 column (250 × 4.6 mm, 5 µm particle size; ACE, Aberdeen, Scotland), as the method exposed by Queiroz et al. (2017). In this work, the reverse phase HPLC method is based on a polar mobile phase with the mixture of solvent A: H_2O/HCOOH (99.9:0.1, v/v), and solvent B: CH_3CN/HCOOH (99.9:0.1, v/v), and a stationary phase with non-polar characteristics. The following linear gradient scheme was used (t in min; %B): (0; 5%), (15; 15 %), (30; 30 %), (40; 50 %), (45; 95 %), (50; 95 %) and (55; 5%). At 55 min, return to 5% of B to stabilize and prepare the column for the next sample. The analysis was performed at room temperature, 25 °C, with a flow rate of 1.0 mL/min and a sample injection volume of 20 µL. All samples were injected in triplicate. The equipment consisted of a LC pump (SRVYR-LPUMP), an auto-sampler (SRVYR-AS), and a photodiode array detector (SRVYR-PDA5) in series (Thermo Fisher Scientific, Inc., Waltham, USA). For the compounds identified at 280 nm, standards of epicatechin, caftaric acid and ellagic acid were used to quantify flavanols, hydroxycinnamic acids, and hydroxybenzoic acids, respectively. For the compounds recognized at 330 nm, standards of

caftaric acid, quercetin-3-*O*-rutinoside and resveratrol were used to quantify, respectively, hydroxycinnamic acids, flavonols, and stilbenes. Finally, for the quantification of anthocyanins at 520 nm a malvidin-3-*O*-galactoside standard was used.

2.5. Antioxidant activity

The antioxidant activity was determined by $ABTS^{\bullet+}$, $DPPH^{\bullet}$ and FRAP methods. The $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ assays were adapted to a micro-scale according to Mena et al. (2011). In $ABTS^{\bullet+}$ assay the antioxidant activity was evaluated by measuring the variation in absorbance at 734 nm after 30 min of reaction, while in the DPPH assay this activity was determined by measuring the variation in absorbance at 520 nm after 15 min of reaction. The FRAP assay was adapted to a micro-scale and performed according to Bolanos de la Torre et al. (2015). The reaction was incubated at 37 °C protected from light for 30 min and the absorbance was read at 593 nm. The assays were performed using a Multiscan FC microplate reader (Thermo-Fisher Scientific, Oporto, Portugal) and the results were expressed as millimoles of Trolox per gram of dry weight (mmol T/g dw).

2.6. Bacterial isolates

Clinical isolates were collected from biological samples of the gastrointestinal segment of humans and diabetic foot ulcers, provided by the Hospital Centre of Trás-os-Montes and Alto Douro (CHTMAD), under the protocol established since 2004, belonging to the MJS, MJMC and MJH collections. Gram-positive bacteria *Staphylococcus aureus* MJS241 and *Enterococcus faecalis* MJS257, and Gram-negative bacteria *Escherichia coli* MJS260 and *Klebsiella pneumoniae* MJS281 were collected from clinical-human gastrointestinal segment, while Gram-positive isolates *Staphylococcus aureus* MJMC109 and *Staphylococcus aureus* MJMC534B, and Gram-negative isolates *Klebsiella pneumoniae* MJH602 and *Enterobacter aerogenes* MJMC534A were collected from diabetic foot ulcers. The bacterial isolates were previously identified by biochemical methods (API 20E, API 20NE, API Staphy (BioMérieux)) and by molecular methods, namely, the partial sequencing of the 16S rRNA gene. Reference bacteria obtained from American Type Culture Collection (ATCC) were also used, specifically *Listeria monocytogenes* ATCC 15313 (Gram-positive) and *Pseudomonas aeruginosa* ATCC 10145 (Gram-negative), and from Spanish Type Culture Collection (CECT), namely *Staphylococcus aureus* CECT 976 (Gram-positive). Prior to antimicrobial activity tests, isolates were cultivated on BHI agar for 24 h at 37 °C (Gouvinhas et al., 2020).

2.7. Antimicrobial activity

2.7.1. Disc diffusion assay

The disc diffusion method was performed as described by Gouvinhas et al. (2018). In this method the suspensions were carried out from a pure culture in 0.9 % NaCl, and the turbidity was adjusted to the value of 0.5 McFarland. Then, suspensions of the bacterial isolates were cultivated with a swab in Petri dishes (90 mm diameter) containing 20 mL of Muller-Hinton agar. Afterwards, sterile white disks (6 mm diameter) were placed on the plates using a disc dispenser (OXOID) and impregnated with 10 µL of the different samples (dissolved in 10 % DMSO) at the same concentration (200 mg/mL). In the performed tests, commercial antibiotics, namely, Gentamicin 10 µg, Gentamicin 30 µg, and Ciprofloxacin 10 µg, were used as positive controls and 10 % DMSO as a negative control. After 24 h of incubation at 37 °C, the microbial growth inhibition halos were measured around the disks impregnated with the samples and antibiotics.

The antimicrobial activity was calculated according to the following equation: '$\%\ RIZD = (IZD_{sample} - IZD_{negative\ control}) / IZD_{antibiotic}\ x\ 100\ \%$', where IZD corresponds to inhibition halos (mm) and RIZD to the percentage of the relative diameter of the inhibition halo (Gouvinhas et al., 2018). This equation considers, and compensates, the possible effect of solvent (blank) other than water. Additionally, the antimicrobial activity of each extract was also classified according to the following: No effect (0): inhibition halo = 0; Moderate efficacy (0–100): 0 < inhibition halo < antibiotic inhibition halo; Good efficacy (> 100): antibiotic inhibition halo < inhibition halo < 2 x antibiotic inhibition halo; High efficacy (▲): inhibition halo > 2 x antibiotic inhibition halo.

2.7.2. Minimum inhibitory concentration (MIC)

The Minimum Inhibitory Concentration (MIC) method was performed, with some modifications as reported by Dias et al. (2015). The bacterial cultures were passed into flasks with Muller-Hinton broth and incubated at 37 °C for 12 − 18 h. Then, the absorbance of bacterial suspension was adjusted at 0.100 with a wavelength of 625 nm, to obtain a concentration of 1×10^8 cfu/mL. In the first row of the 96-well microplates (Frilabo, Milheirós, Portugal), 200 µL of each sample (30 mg/L), previously dissolved in 10 % DMSO, and the positive and negative controls were added. In the remaining wells, 100 µL of Muller-Hinton broth were added, and ½ serial dilutions were performed for all samples and controls. Muller-Hinton broth and DMSO solution were used as negative controls, and the gentamicin and ciprofloxacin antibiotics were used as positive controls, having the same concentration of samples. Then, 20 µL of bacterial suspension were added. Finally, 20 µL of resazurin indicator solution, prepared by dissolving 270 mg tablet in 40 mL of sterile water, were added to each well. The microplates were incubated at 37 °C for 18, and then the color change was evaluated visually. The lowest concentration where there was no color change (purple) was considered the minimum inhibitory concentration (MIC).

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

2.8. Cell culture and cell viability assay

The Raw 264.7 cell line (Mouse macrophages, Abelson murine leukemia virus-induced tumor, CLS, Germany) was cultured in flasks (Orange Scientific, Frilabo, Portugal) with DMEM culture medium, supplemented with 10 % FBS (v/v), and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) at 37 °C in an atmosphere of 5% CO_2/95 % air with controlled humidity and handled as described by Queiroz et al. (2017).

For the cell viability assay, cells were incubated in fresh FBS-free culture medium (control) and in fresh FBS-free culture medium containing the grape stem extracts (previously resuspended in phosphate-buffered saline solution (20 mg/mL)) with final concentrations ranging from 1.00–50.00 µg/mL for Touriga Nacional, Syrah and Fernão Pires extracts, 5.00–75.00 µg/mL for Tinta Roriz and Castelão extracts, and 25.00–150.00 µg/mL for Arinto extract. The cells were exposed to extracts for 24 h and 48 h, and after the exposure time, the culture media was replaced with FBS-free medium supplemented with 10 % (v/v) Alamar Blue (AB). After 4 h, the absorbance was read at 570 and 620 nm using a Multiskan EX microplate reader (MTX Labsystems, USA). The cell viability was calculated as reported in Andreani et al. (2014) and the results were expressed as percentage of control (non-exposed cells).

2.9. Anti-inflammatory activity

The anti-inflammatory activity of grape stem extracts was performed as report by Queiroz et al. (2017). The cells were incubated,

during 24 h, in fresh FBS-free medium containing grape stem extracts, in the absence and in the presence of 1 μg/mL LPS (lipopolysaccharide), to induce the nitrite (NO) production. In each condition a control (cells not exposed cells to extracts) was made. Grape stem extracts were applied at different concentrations, namely, 5.00, 12.50 and 25.00 μg/mL for Tinta Roriz, Touriga Nacional, Castelão, Syrah, and Fernão Pires extracts, and 12.50, 25.00, 50.00 and 75.00 μg/mL for Arinto extract.

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

2.10. Anti-aging activity

2.10.1. Tyrosinase inhibition assay

In this assay, reported by Taghouti et al. (2018), 25 μL of each sample (1 mg/mL), plus 80 μL of phosphate buffer (50 mM, pH 6.8) and 40 μL L-DOPA (2.50 mM) were added, and the plate was incubated for 2 min at 37 °C. Then, 40 μL of tyrosinase (40 U/mL, in phosphate buffer (50 mM, pH 6.5)) were added to initiate the reaction. The reaction was incubated for 10 min at 37 °C, and after this time, the absorbance was read at 492 nm, in the microplate reader (Thermo-Fisher Scientific, Oporto, Portugal). Methanol was used as positive control. The tyrosinase inhibition was calculated according to the following equation: '*% Inhibition = (Abs$_{control}$ – Abs$_{sample}$) / Abs$_{control}$l x* 100 %'.

2.10.2. Elastase inhibition assay

For elastase inhibition, the Taghouti et al. (2018) method was used. Initially, 50 μL of each sample (1 mg/mL), plus 160 μL of Tris−HCl buffer (0.20 mM, pH 8) and 20 μL of N-(methoxysuccinyl)-ala-ala-proval-4-nitroanilide (0.80 mM, in buffer) were added, and incubated for 10 min at room temperature. Then, 20 μL of elastase (0.40 U/mL, in Tris−HCl buffer) were added to initiate the reaction, this was incubated for 20 min at room temperature. After, the absorbance was read at 410 nm using a microplate reader (Thermo-Fisher Scientific, Oporto, Portugal). Methanol was used as positive control. The elastase inhibition was calculated according to the previous mentioned equation (section 2.9.1).

2.11. Statistical analysis

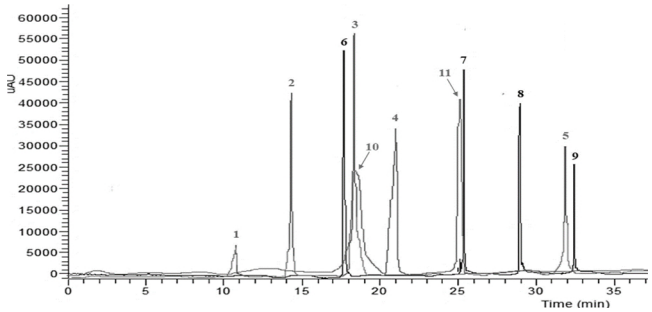
The phenolic composition and, antioxidant and anti-aging activities data were subjected to the IBM SPSS 22.0 statistical software (SPSS Inc., Chicago, IL, USA), using analysis of variance (ANOVA) and a multiple range test (Tukey's test), for a p-value < 0.05. The results of the samples are presented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

3. Results and discussion

3.1. Phenolic compounds identified and quantified in grape stem extracts

The identification of phenolic compounds by RP-HPLC-DAD was realized by comparison with pure standards of DAD spectra, retention times, and literature data (Anastasiadi et al., 2009; Gouvinhas et al., 2018; Queiroz et al., 2017). In grape stem extracts, eleven phenolic compounds were identified and quantified, belonging to different classes, namely hydroxybenzoic and hydroxycinnamic acids, flavanols, flavonols, stilbenes, and anthocyanins (Table 1). When analyzing the

Table 1
Phenolic compounds identified in grape stem extracts and their quantification.



Identified compounds	λ	R_t	Tinta Roriz	Touriga Nacional	Castelão	Syrah	Arinto	Fernão Pires	p-value
(1) Gallic acid	280 nm	10.93	ND	ND	ND	[x]0.04 ± 0.00^{b}	[x]0.04 ± 0.00^{a}	[x]0.05 ± 0.00^{c}	**
(2) Protocatechuic acid	280 nm	14.57	ND	[x]0.40 ± 0.02^{c}	[x]0.12 ± 0.00^{a}	ND	0.24 ± 0.01^{b}	0.26 ± 0.00^{b}	***
(3) Catechin	280 nm	18.62	[x]1.62 ± 0.11^{d}	2.03 ± 0.08^{e}	0.44 ± 0.02^{a}	1.33 ± 0.02^{c}	0.66 ± 0.04^{b}	1.27 ± 0.03^{c}	***
(4) Epicatechin	280 nm	21.24	0.09 ± 0.00^{b}	0.18 ± 0.01^{d}	0.04 ± 0.00^{a}	0.11 ± 0.00^{c}	0.03 ± 0.00^{a}	0.17 ± 0.00^{d}	***
(5) *Trans*-cinnamic acid	280 nm	31.99	0.08 ± 0.00^{a}	0.16 ± 0.01^{c}	0.09 ± 0.01^{a}	0.08 ± 0.00^{a}	0.14 ± 0.00^{bc}	0.13 ± 0.01^{b}	***
(6) Caftaric acid	330 nm	17.69	0.43 ± 0.01^{b}	0.98 ± 0.08^{d}	0.20 ± 0.00^{a}	0.40 ± 0.01^{b}	0.22 ± 0.00^{a}	0.68 ± 0.04^{c}	***
(7) Q-3-*O*-Rut	330 nm	25.39	0.37 ± 0.01^{c}	0.44 ± 0.00^{d}	0.24 ± 0.01^{b}	0.41 ± 0.01^{cd}	0.15 ± 0.00^{a}	0.44 ± 0.03^{d}	***
(8) Resveratrol	330 nm	29.02	0.07 ± 0.00^{ab}	0.14 ± 0.00^{d}	0.07 ± 0.00^{b}	0.06 ± 0.00^{a}	0.08 ± 0.00^{c}	0.09 ± 0.00^{c}	***
(9) Ɛ-viniferin	330 nm	32.50	0.07 ± 0.00^{a}	0.11 ± 0.01^{c}	0.07 ± 0.00^{a}	0.07 ± 0.00^{a}	0.07 ± 0.00^{a}	0.08 ± 0.00^{b}	***
(10) Mv-3-*O*-Glt	520 nm	18.65	0.37 ± 0.02	ND	ND	ND	–	–	–
(11) Mv-3-*O*-Glc	520 nm	25.38	0.36 ± 0.01^{d}	0.10 ± 0.01^{b}	0.06 ± 0.00^{a}	0.19 ± 0.01^{c}	–	–	***

Q: Quercetin; Rut: Rutinoside; Mv: Malvidin; Glt: Galactoside; Glc: Glucoside; λ: wavelength; R_t: retention time (min); ND: Not detected.
The values are presented as mean ± standard deviation (n = 3). Different letters indicate significantly different results (ANOVA, p < 0.05).
Significance: non-significant, N.S. (p > 0.05); * significant at p < 0.05; ** significant at p < 0.01; *** significant at p < 0.001.
[x] The results are expressed as mg/g dw.

Table 2
Scavenging capacity of grape stem extracts by ABTS, DPPH, and FRAP methods.

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

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

concentrations obtained, significant differences were found between all varieties. In all varieties, catechin revealed to be the major compound, with concentration ranging from 0.44 ± 0.02–2.03 ± 0.08 mg/g dw. Usually, the differences observed between varieties can be explained by several factors, namely, genetic and physiological characteristics of each cultivar, agroclimatic conditions (geographic location, soil composition, fertilization, and environmental conditions) and ripening stage (Dias et al., 2015; Garrido and Borges, 2013), but in this case, since all varieties were harvested from the same location, the differences in phenolic content are mainly due to genetic and physiological characteristics.

Regarding to other studies on phenolic compounds identified and quantified in grape stem extracts, in the work of Dias et al. (2015), fifteen compounds were detected and quantified in red varieties (Sousão, Touriga Nacional, Tinta Barroca, Tinta Amarela), while in white varieties (Fernão Pires, Viosinho, Rabigato) thirteen compounds were quantified. In common with the present study, five compounds were found, namely, epicatechin, caftaric acid, quercetin-3-*O*-rutinoside, Ɛ-viniferin, and malvidin-3-*O*-glucoside. Concerning to epicatechin, higher concentrations (2199–3184 mg/100 g dw) were found than those obtained in present work. In contrast, the concentrations of caftaric acid (5.28–13.85 mg/100 g dw), quercetin-3-*O*-rutinoside (0.27–1.22 mg/100 g dw), and Ɛ-viniferin (0.22–2.99 mg/100 g dw) presented lower concentrations than those obtained in the present study, as can be seen in Table 1. Relatively to the presence of anthocyanins, the contents of malvidin-3-*O*-glucoside varied between 2.38–8.02 mg/100 g dw, and when compared with the present work, they were lower than the concentrations obtained for the Tinta Roriz, Touriga Nacional, and Syrah varieties. In addition, Domínguez-Perles et al. (2016) study quantified ten phenolic compounds in the Tinto Cão, Tinta Barroca, Malvasia Fina, and Moscatel Branco cultivars, of which only four were also identified and quantified in the present study, being caftaric acid, quercetin-3-*O*-rutinoside, Ɛ-viniferin, and malvidin-3-*O*-glucoside. The concentrations achieved for caftaric acid (2.18 ± 0.13–19.46 ± 1.37 mg/g dw), quercetin-3-*O*-rutinoside (1.08 ± 0.06–3.42 ± 0.22 mg/g dw), Ɛ-viniferin (2.94 ± 0.16–5.82 ± 0.37 mg/g dw), and malvidin-3-*O*-glucoside (18.17 ± 1.12–28.28 ± 1.80 mg/g dw) were significantly higher when compared with the results presented in Table 1. Additionally, Anastasiadi et al. (2012) work showed the presence of several phenolic compounds in grape stem extracts of six red and white Greek varieties. Comparing with the present study, common components were detected and quantified, namely, gallic acid, catechin, epicatechin, caftaric acid, resveratrol, and Ɛ-viniferin. The results showed that the Greek cultivars analyzed had an average higher content of 79.91 %, 35.11 %, and 74,09 % of gallic acid, resveratrol, and Ɛ-viniferin, respectively, than the average concentrations obtained in the present work. In contrast, the average values achieved for catechin, epicatechin, and caftaric acid were 19.76 %, 34.95 %, and 81.03 % lower, respectively, when compared with the average contents presented for the varieties evaluated in present study. Sahpazidou et al. (2014) also quantified in their work the compounds gallic acid, catechin, epicatechin, resveratrol, and quercetin-3-*O*-rutinoside (or rutin), obtaining average concentrations of 19.24, 9.49, 13.05, 12.99, and 19.56 mg/g dw, being these values higher than those presented in the Table 1.

Several phenolic compounds were identified in different varieties, besides those found in present work. For example, in Portuguese grape stem varieties, different flavonols (quercetin, kaempferol and isorhamnetin) were detected (Dias et al., 2015; Domínguez-Perles et al., 2016; Gouvinhas et al., 2018; Queiroz et al., 2017), while in Greek grape stem varieties different acids were recognized, namely, ferulic, coumaric, caffeic and syringic acids (Anastasiadi et al., 2012, 2009; Sahpazidou et al., 2014).

3.2. Antioxidant activity of grape stem extracts

The results regarding to antioxidant activity by ABTS, DPPH, and FRAP methods are presented in Table 2. Analyzing the results, significant differences between varieties were verified for the three methods. In the three assays, the Touriga Nacional variety demonstrated the highest antioxidant power, reaching values of 0.84 ± 0.06, 0.64 ± 0.05, and 1.03 ± 0.06 mmol T/g dw, for ABTS, DPPH, and FRAP methods, respectively, while the lowest antioxidant power was obtained for Arinto variety, presenting values of 0.35 ± 0.00, 0.15 ± 0.01, and 0.35 ± 0.02 mmol T/g dw for ABTS, DPPH, and FRAP methods, correspondingly. Based on previous studies, significant differences between varieties can be explained by different phenolic contents, since phenolic content may be correlated with antioxidant capacity (Anastasiadi et al., 2012; Barros et al., 2014; Gouvinhas et al., 2018; Katalinić et al., 2010).

In literature data, Domínguez-Perles et al. (2014) evaluated the antioxidant capacity of the Touriga Nacional (red) and Viosinho (white) varieties, by the ABTS method, using different extractions to understand the effects of solvent concentrations, temperature, and extraction time. Domínguez-Perles et al. (2014) study reached maximum scavenging capacity values of 0.18 ± 0.00 and 0.05 ± 0.00 mmol T/g dw for the varieties Touriga Nacional and Viosinho varieties, respectively. These results showed a much lower scavenging capacity than those obtained for the six grape stem varieties (Table 2). In other work, Domínguez-Perles et al. (2016) quantified the antioxidant capacity of grape stem extracts by the ABTS and DPPH assays, using two red (Tinto Cão, Tinta Barroca) and two white (Malvasia Fina, Moscatel) varieties, and reported lower antioxidant activity than the one in the current study (Table 2). In addition, Gouvinhas et al. (2018) study quantified the scavenging capacity of red grape stem extracts of the Syrah, Tinta Barroca, and Sousão varieties by the ABTS and DPPH assays during 64 days of storage. Regarding the ABTS assay, average values of 4.77, 7.76, and 6.44 mmol T/g dw for Sousão, Tinta Barroca, and Syrah varieties, respectively, were obtained (Gouvinhas et al., 2018), which proved to be much higher than those achieved in the present study. In the DPPH assay, Gouvinhas et al. (2018) presented average values of 0.54, 0.90, and 0.84 mmol T/g dw for Sousão, Tinta Barroca, and Syrah varieties, respectively, being the results shown in Table 2 lower that those reached for Tinta Barroca and Syrah varieties. Moreover, Anastasiadi et al. (2012) work also determined the antioxidant activity of grape stem extracts by FRAP assay, using white (Asyrtiko, Athiri, and Aidani) and red (Mandilaria, Mavrotragano, and Voidomatis) cultivars, reporting higher values, on average, 64.92 % for white varieties, when compared with the average value obtained in the present work (0.67 mmol T/g dw). In the case of the red varieties, they presented higher values, on average 59.52 %, when compared with the average value determined for the red varieties tested in present study (0.85 mmol T/g dw).

Other by-products of the wine industry have also been shown to have an antioxidant capacity. However, the grape stem extracts used in

Table 3
Antimicrobial activity of grape stem extracts.

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

TR: Tinta Roriz; TN: Touriga Nacional; CT: Castelão; SH: Syrah; AR: Arinto; FP: Fernão Pires; CN10: gentamicin (10 µg per disc); CN30: gentamicin (30 µg per disc); CIP10: ciprofloxacin (10 µg per disc);▲: extract effective and antibiotic without effect.

the present work, when compared with some studies that determine the antioxidant activity of grape pomace, seeds, and skins, have shown to have an antioxidant capacity equal to or greater than theses by-products, depending on the variety (Jara-Palacios et al., 2014; José Jara-Palacios et al., 2014; Ky et al., 2014; Melo et al., 2015; Poveda et al., 2018; Rockenbach et al., 2011; Teles et al., 2018), thus proving to be a matrix with a high antioxidant power.

3.3. Antimicrobial activity of grape stem extracts

3.3.1. Disc diffusion

The results of antimicrobial activity of grape stem extracts by the disc diffusion assay are presented in Table 3. These results showed that percentages higher than 100, indicate that the extracts have a higher bacterial growth inhibiting power than antibiotics. Analyzing the results, it was also possible to observe that for the *S. aureus* MJS241 and *E. faecalis* MJS257 isolates no value was presented (▲), due to the fact that it was impossible to calculate the %RIZD since antibiotics did not show any effect, however the extracts have demonstrated beneficial effects with the inhibition of the growth of the isolates.

However, the %RIZD for extracts tested in these isolates was significantly higher when compared with the others, demonstrating the potential of this matrix to inhibit bacterial growth. The extracts that did not exhibit any inhibition of bacterial growth presented when a %RIZD of 0 was obtained.

Grape stem extracts showed to inhibit bacterial growth due to the accumulation of phenolic compounds present in grape stems, in the lipid bilayer cell, thus causing changes in the membrane structure and function. Phenolic compounds infiltrate the bacterial cell, applying inhibitory activity in the cellular cytoplasm, leading to lysis and release of intracellular ATP, and may also cause loss of cellular components, increasing the permeability of the cytoplasmic membrane (Mattos et al., 2017). However, grape stem extracts revealed considerable antibacterial activity against all Gram-positive bacteria, which may indicate that phenolic compounds act specifically against the Gram-positive cell wall, since they have a much thicker peptidoglycan layer than Gram-negative bacteria. Lipopolysaccharides from outer Gram-negative bacteria membrane give them extra resistance against antibiotics and other compounds like phenolic compounds (Pisoschi et al., 2017).

The results obtained in the present work are in agreement with those reported by Gouvinhas et al. (2018), where the antibacterial activity of grape stem extracts of Syrah, Tinta Barroca, and Sousão varieties was tested against the same isolates used in the present work. In relation to Gram-positive bacteria, the same behavior was observed for *S. aureus* MJS241 and *E. faecalis* MJS257 isolates, since antibiotics had no effect on bacterial growth inhibition, unlike grape stem extracts. Regarding Gram-negative bacteria, extracts also exhibited an inhibition of bacterial growth of *P. aeruginosa* ATCC 10145 isolate, with a %RIZD that oscillated from 50 to > 100, and for *E. coli* MJS260 and *K. pneumoniae* MJS281 isolates, no inhibition of bacterial growth by the grape stem extracts was detected (Gouvinhas et al., 2018), as also shown in the present work (Table 3).

Other by-products (skins, seeds, pomace) also demonstrated antimicrobial capacity, inhibiting the growth of different *L.monocytogenes*, *S. aureus*, *E. faecalis*, and *E. coli* strains (Butkhup et al., 2010; Corrales et al., 2010; Zambrano et al., 2019). The antimicrobial potential of each extract/by-product may depend on the extraction method, the microorganism tested (Zambrano et al., 2019), and its polyphenolic content (Baenas et al., 2018).

3.3.2. Minimum inhibitory concentration (MIC)

The antimicrobial activity of grape stem extracts was also evaluated by the Minimum Inhibitory Concentration assay, being the results presented in Table 4. Analyzing the results, it is possible to verify that the MIC of grape stem extracts against Gram-positive isolates ranged from 0.47 and 1.88 mg/mL, depending on variety and strain. Concerning to *S. aureus* CECT 976, *S. aureus* MJS241, and *S. aureus* MJMC109 isolates, all the concentrations of grape stem extracts exhibited had bactericidal effect (Table 4). With respect to Gram-negative isolates, the results obtained presented the same behavior observed in the disc diffusion assay, only inhibiting the growth of *P. aeruginosa* ATCC 10145 isolate with MIC ranging from 1.88 to 3.75 mg/mL, depending on variety. The MIC of grape stem extracts in Gram-negative isolates was higher than the achieved for Gram-positive isolates,

Table 4
Minimum Inhibitory Concentration (MIC) of grape stem extracts against bacterial isolates tested.

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

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

[x] The results are expressed as mg/mL.

possibly because Gram-negative bacteria have a lipopolysaccharide layer on the outer membrane that difficult the penetration of the phenolic compounds, as mentioned in the previous point. Nevertheless, for the remaining Gram-negative bacteria the concentrations tested were not sufficient to inhibit their bacterial growth.

There are few data in literature about the antimicrobial activity of grape stems by the MIC method, however Dias et al. (2015) determined the antimicrobial activity of grape stem extracts of seven Portuguese varieties (Sousão, Touriga Nacional, Tinta Barroca, Tinta Amarela, Fernão Pires, Viosinho, and Rabigato), using the same isolates used in the present work, and reported that both Gram-positive (*L. monocytogenes* ATCC 15313, *S. aureus* MJS241, and *E. faecalis* MJS257) and Gram-negative (*P. aeruginosa* ATCC 10145, *E. coli* MJS260, and *K. pneumoniae* MJS281) isolates had MICs ranging from 66.70 to > 134.00 mg/mL, depending on the cultivars tested. These concentrations are very high, compared with those presented in Table 4, but in the case of *E. coli* MJS260, *K. pneumoniae* MJS281, *K. pneumoniae* MJH602, and *E. aerogenes* MJMC534A isolates it may mean that higher concentrations than those tested in the present work may be necessary to inhibit their growth. In addition, other by-products (skins, seeds, pomace) also presented had antimicrobial activity against bacterial isolates of *L. monocytogenes*, *S. aureus*, *E. faecalis*, *P. aeruginosa*, *E. coli*, and *K. pneumoniae* species, with MICs ranging from 0.24 to > 20.00 mg/mL (Katalinić et al., 2010; Oliveira et al., 2013; Peixoto et al., 2018). Once again, these variations between extracts/by-products are mainly due to distinct preparations of the extracts, the performance of the microbiology assays, the distinct bacterial strains, and the differences in phenolic composition of each extract, as mentioned above.

Grape stems have proven to be an effective matrix in inhibiting Gram-positive bacteria, namely, bacterial pathogens of gastrointestinal segment and diabetic foot ulcers, being a by-product that could be applied in the future to replace antibiotics or in synergy with them, fighting against the antibiotic resistance that currently exists, and may also be a matrix used in the development of innovate and efficient product dressing the diabetic foot wounds. Despite grape stems are not effective against Gram-negative bacteria, due to their lipopolysaccharide layer on the outer membrane, literature data show that testing higher concentrations in the future may lead to better inhibition of Gram-negative isolates (Dias et al., 2015; Peixoto et al., 2018).

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

Before performing the anti-inflammatory assay, an effect of grape stem extracts on cell viability/toxicity was performed. The toxicity of the extracts was tested so that the cell viability was not compromised, and therefore cannot interfere with the results regarding the anti-inflammatory activity. In this sense, different concentrations of extracts were tested being the results (expressed as percentage of control) presented in Fig. 1. Observing the results, significant differences were found, principally between the highest concentration tested and controls, and between 24 h and 48 h of exposure at the highest concentration. As expected, the extracts were more toxic at higher concentrations, both 24 h and 48 h exposure, showing a concentration and time-dependent effect. However, for Tinta Roriz variety, we observe a non-linear effect of concentration, which may reflect the fact that, as we are working with extracts (complex mixtures of bioactive compounds), different type of antagonistic and synergistic molecules, on cell viability/proliferation mechanisms, may be present in this extract. This effect was observed at both incubation periods, as the assays were performed separately, it reinforces the theory explained above.

Following testing the cellular toxicity of each variety, concentrations were chosen for the evaluation of anti-inflammatory activity of grape stem, namely concentrations where cell viability was above 90 %. In this way, grape stem extracts were applied at concentrations of 5.00, 12.50, and 25.00 μg/mL, except for Arinto variety extract where concentrations of 12.50, 25.00, 50.00, and 75.00 μg/mL were tested. The data obtained for the anti-inflammatory activity of grape stem extracts are presented in Fig. 2. For all extracts, significant differences were observed when compared with the controls, and between the concentrations tested. Contrary to controls, where the NO production was 100 %, grape stem extracts were able to inhibit LPS-stimulated NO production at all tested concentrations. In the presence of the extracts, the NO production varied between 64.75 % and 83.48 % of control, meaning that they induced NO production inhibitions between 16.52 % and 35.25 %, indicating anti-inflammatory activity. In general, analyzing each variety, it can be verified that NO production inhibition was concentration-dependent, except for Fernão Pires variety, where 25.00 μg/mL produced a NO production (78.29 %) slightly higher than 12.50 μg/mL (75.97 %) (Fig. 2F). The reason for this behavior can be explained, again, by the different compound present in the extract and in their capacity to modulate the LPS-induced NO production pathways.

Concerning to the most effective grape stem extracts, the Arinto variety exhibited a greater capacity in inhibiting NO production, with 35.25 % inhibition (Fig. 2E); however, extracts of this variety were tested at higher concentrations. Therefore, the results obtained for the extracts of the six varieties at 25.00 μg/mL, when compared, showed that at this concentration it is the Syrah variety that presents the greatest capacity of NO production inhibition (32.99 %) (Fig. 2D). The distinct efficiencies in inhibiting NO production may be explain by the differences in phenolic content of the six varieties.

Regarding literature data, as far as we know, only Queiroz et al. (2017) tested the anti-inflammatory activity of grape stem extracts in the Raw 264.7 cell line, using extracts from the Sousão variety, obtaining an inhibition in NO production of 37.20 %. The differences observed between this study and the present work (Fig. 2), may be due to the different concentrations of extracts applied, but mainly to the different phenolic composition in the extracts. With respect to other by-products, in Harbeoui et al. (2019) work studied the NO inhibition capacity of grape seeds of the Syrah, Merlot, and Carignan cultivars (at 5.00 μg/mL) in the Raw 264.7 cell line, presenting inhibition values of 37.05 %, 35.12 %, and 31.32 %, respectively.

In present study, NO production was stimulated by the addition of LPS (lipopolysaccharide), since LPS causes macrophages to activate the production of excessive amounts of pro-inflammatory mediators, such as NO (*via* iNOS (inducible nitric oxide synthase, enzyme responsible for the NO synthesis) modulation) and pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) (Harbeoui et al., 2019). Nevertheless, grape stem extracts were able to inhibit the production of NO due to its polyphenolic content, being attributed to phenolic compounds the capacity to inhibit proinflammatory molecules (NO, TNF-α (Tumor necrosis factor-α), Interleukins (ILs), namely IL-1β, IL-6) production by macrophages, to stimulate the expression of anti-inflammatory markers and inhibit pro-inflammatory enzymes (iNOS and COX-2 (cyclooxygenase-2)), thus causing a decrease in NO production (Zhang et al., 2019).

Environmental, immune and chronic diseases, such as diabetes and cancer all have inflammation in common (Arulselvan et al., 2016). Therefore, the development of drugs more effective in eliminating NO radicals and inhibiting iNOS enzyme activity and/or iNOS gene expression are required (Queiroz et al., 2017). In this sense, grape stem extracts are good candidates for the development of new drugs that can reduce or eliminate tissue inflammation, since they have the capacity to inhibit the production of NO radicals.

3.5. Anti-aging activity of grape stem extracts

In order to understand if grape stems can be used in anti-aging products, the present work evaluated the capacity of grape stem extracts (1 mg/mL) to inhibit the activity of two enzymes, namely tyrosinase and elastase. The results obtained are presented in Table 5. There are significant differences between the extracts of the six varieties. For tyrosinase, the inhibition percentages ranged from 41.47%–53.83%, and for elastase, they ranged from 67.98 % to 98.02

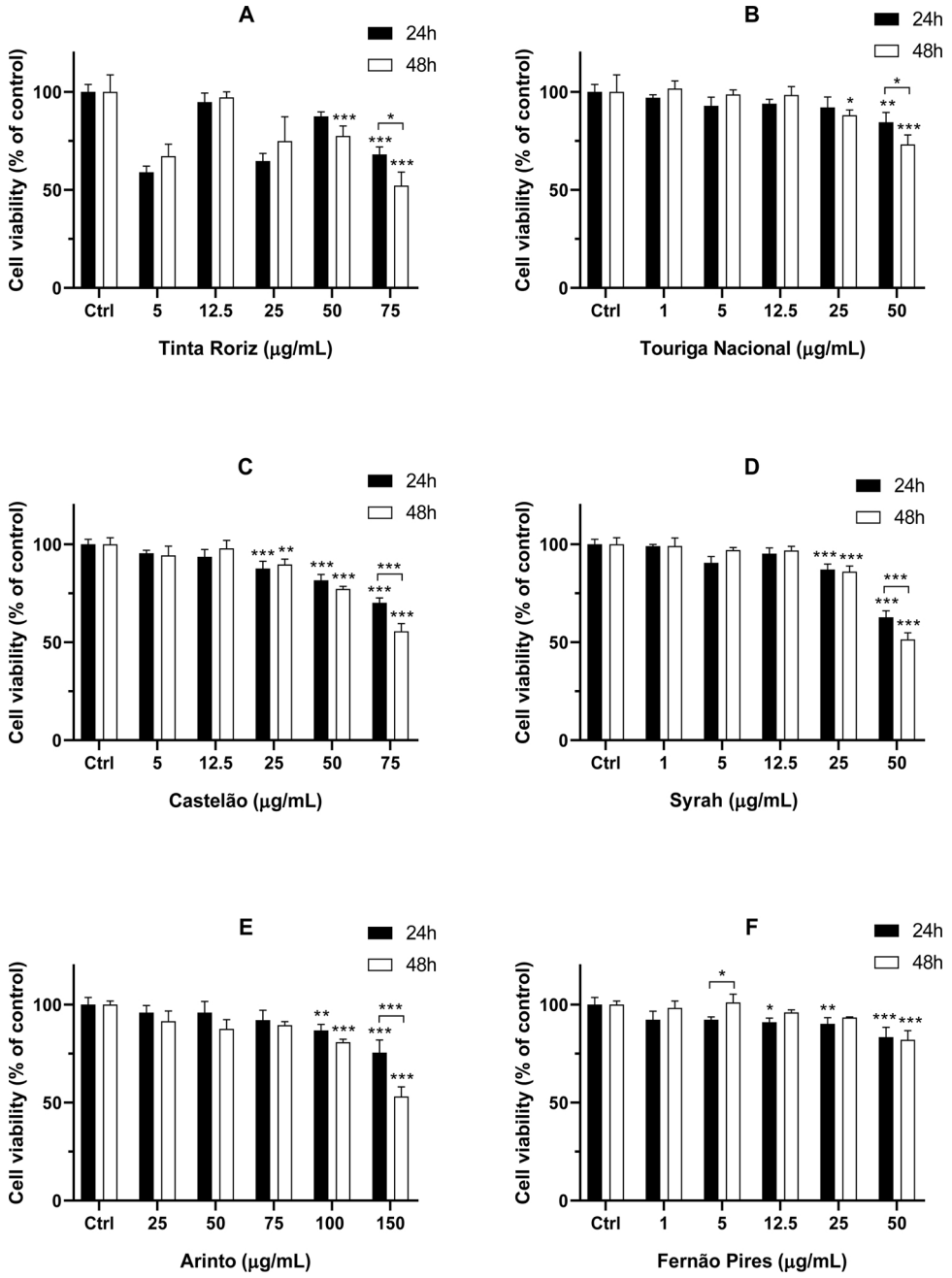


Fig. 1. Effect of grape stem extracts on mouse macrophages (Raw 264.7 cell line) cell viability at 24 h and 48 h of exposure. (A) Tinta Roriz; (B) Touriga Nacional; (C) Castelão; (D) Syrah; (E) Arinto; (F) Fernão Pires. Results are presented as mean ± standard deviation (n = 4). Significance: non-significant, N.S. ($p > 0.05$); * significant at $p < 0.05$; ** significant at $p < 0.01$; *** significant at $p < 0.001$.

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

To the best of our knowledge, to date no studies on anti-tyrosinase and anti-elastase activities of grape stem extracts have been published, being here presented for the first time. Nevertheless, the Wittenauer et al. (2015) study proved that grape pomace extracts (Weisser Riesling cultivar) have anti-elastase activity, presenting 73.00 % inhibition (at 35.3 µg/mL) of this enzyme activity.

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

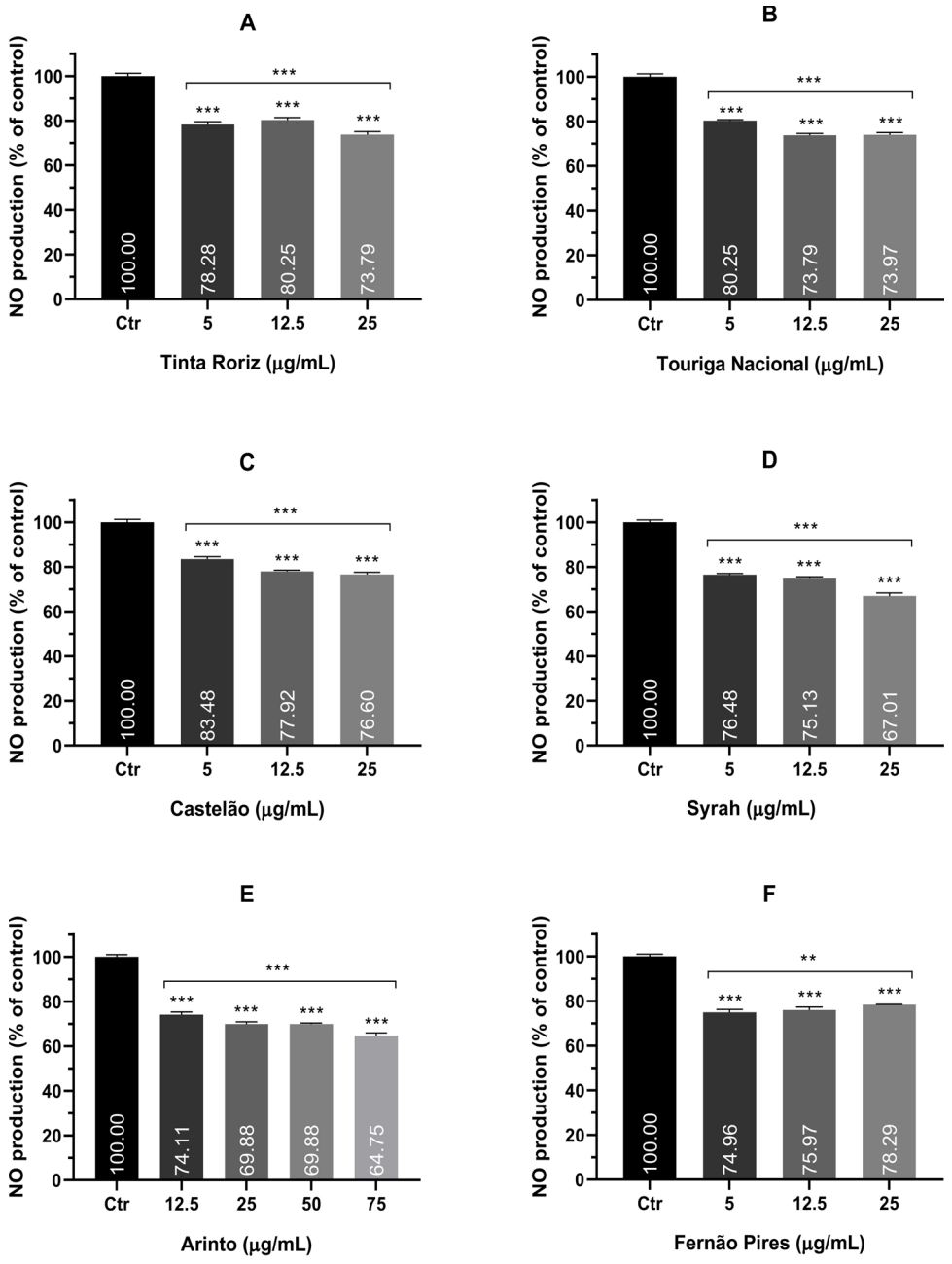


Fig. 2. Anti-inflammatory activity of grape stem extracts on mouse macrophages (Raw 264.7 cell line). (A) Tinta Roriz; (B) Touriga Nacional; (C) Castelão; (D) Syrah; (E) Arinto; (F) Fernão Pires. Results are presented as mean ± standard deviation (n = 4). Significance: non-significant, N.S. ($p > 0.05$); * significant at $p < 0.05$; ** significant at $p < 0.01$; *** significant at $p < 0.001$.

4. Conclusions

The biological potential of grape stems was proven, once again, in this study. This by-product showed antioxidant activity, revealing a good scavenging capacity; antimicrobial activity, which showed high efficacy against Gram-positive bacteria, especially *S. aureus* and *E. faecalis*; anti-inflammatory activity, in which all extracts were shown to inhibit the NO production at non-toxic cellular concentrations. Finally, anti-aging activity was also found, where important results were obtained for the inhibition of the activity of tyrosinase and elastase enzymes. In fact, this study allowed to find out, for the first time, not only anti-aging activities of grape stems, but also their capacity to inhibit the growth of foot wound ulcers bacteria, greatly enhancing the use of this by-product in cosmetic and pharmaceutical industries.

This study proved to be of great importance for the circular economy model to be fulfilled, since, by demonstrating potential applications for grape stem, it presents new purposes for this residue. Also proving that the grape stem can be reused in the cosmetic, pharmaceutical and food industries, avoiding its accumulation and reducing the costs of its treatment. However, further studies are needed to evaluate the toxicity of this by-product, as well as, to analyze the biological properties of each bioactive compounds present in grape stem in

Table 5
Anti-tyrosinase and anti-elastase activities of grape stem extracts.

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

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

order to obtain the highest possible efficacy, so that the extracts can finally be tested in *in vivo* systems.

CRediT authorship contribution statement

Carla Leal: Methodology, Software, Investigation, Writing - original draft. **Irene Gouvinhas:** Conceptualization, Methodology, Investigation, Funding acquisition, Writing - review & editing, Supervision. **Rafaela A. Santos:** Investigation, Methodology. **Eduardo Rosa:** Funding acquisition. **Amélia M. Silva:** Writing - review & editing, Supervision. **Maria José Saavedra:** Supervision. **Ana I.R.N.A. Barros:** Conceptualization, Funding acquisition, Supervision.

Declaration of Competing Interest

None.

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