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Chemical Characterization and Bioactive Properties of Wine Lees and Diatomaceous Earth towards the Valorization of Underexploited Residues as Potential Cosmeceuticals

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Abstract: Annually, wine production is responsible for generating large quantities of residues, which are frequently disposed of and not valorized. So far, different studies have been conducted on grape pomace, yet less attention has been paid to other residues, such as wine lees and diatomaceous earth used in wine filtration. In this context, this study aimed to evaluate and compare the phenolic profile of these underexploited winemaking residues and assess their biological potential based on their antioxidant, antimicrobial, cytotoxic, and anti-aging activities (inhibition of tyrosinase and collagenase). Twenty-nine phenolic compounds, including twelve anthocyanins, were tentatively identified in the residues, with red grape pomace showing the highest diversity of compounds. The diatomaceous earth presented the highest content of non-anthocyanin phenolic compounds, being particularly rich in flavan-3-ols and myricetin-O-hexoside, and also presenting two anthocyanins. This sample also showed a high antioxidant activity, evidencing the best result in the reducing power assay. The red wine lees extract, despite showing a low content of phenolic compounds and less antioxidant activity, presented the highest inhibition capacity of bacteria growth. The extracts did not exhibit cytotoxicity against keratinocyte (up to 400 µg/mL) and fibroblast (up to 100 µg/mL) skin cell lines. However, the capacity of inhibiting tyrosinase and collagenase was low for the lees and diatomaceous earth, contrary to the grape pomace, seeds, and skins extracts that showed promising results, evidencing its potential as a cosmeceutical. Overall, this study highlights for the first time the potential of diatomaceous earth, an underexploited winemaking waste, in the obtention of added-value extracts and/or ingredients for cosmetic industry.

Keywords: winemaking residues; antioxidant activity; skin enzymes; keratinocytes; fibroblasts

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1. Introduction

Polyphenols are secondary metabolites produced by plants that play important roles in plant systems, such as providing them with protection against different types of attacks, including from parasites, insect pests, ultraviolet (UV) radiation, and other environmental threats [1]. Polyphenols may also have beneficial effects on health as they show different biological activities, including antioxidant, anti-inflammatory, antimutagenic, anticarcinogenic, and antiproliferative properties [2]. One of the most interesting characteristics of polyphenols is their antioxidant activity, owing to their reducing properties and their capacity to neutralize free radicals, such as singlet oxygen, which have been related to the ability

to avoid or delay the oxidation of biomolecules (mainly lipids) in biological systems [3,4]. Moreover, the phenolic composition is an important quality parameter in certain foods and drinks, such as wine, contributing to various organoleptic attributes, such as bitterness, astringency, color, flavor, and oxidative stability [5].

Over recent years, different by-products from the food industry have been suggested as potential sources of polyphenols, since a high proportion of interesting bioactive compounds remains in their composition. The winemaking industry is amongst the ones that generate a large number of by-products, mainly grape pomace (consisting of grape skins, seeds, and stems), but also wine lees and other undervalued residues, such as diatomaceous earth used in filtrations. At the end of the winemaking process, residues containing yeasts, bacteria, and organic matter are deposited at the bottom of the wine tanks, corresponding to the lees. To avoid undesirable flavors and aromas, the wine should not remain in contact with these deposits, thus being separated from them [6]. Moreover, to guarantee the desired cleanness and stabilization of the wine, besides transfers, the winemaking industry carries out further operations such as filtration and tartaric stabilization. When properly treated, diatomaceous earth (silica deposits consisting of the accumulation of fossilized shells from microscopic marine algae) yields a material of high porosity non-deformable structure and of a large specific surface. This material is frequently used in wine filtration to eliminate suspended particles, rendering a clear and bright aspect to the wine [7], being an additional residue generated by this industry. While the pomace seeds can be used to produce grapeseed oil and the pomace can further undergo a distillation process or be used as a fertilizer or as animal feed, the stems, lees, and diatomaceous earth are generally discarded and perceived as an environmental problem [8]. However, recent studies suggest that the wine lees can be exploited for their richness in polyphenols, since these compounds can be adsorbed in their colloidal state to the yeast's cell walls as well as being dissolved in the liquid fraction of the wine lees [9,10]. Nevertheless, so far, only a few studies have advanced beyond the evaluation of the total phenolic compounds and carried out the identification of individual phenolics in wine lees. Most of these works concerned red wine lees [11–15], and only limited information is available on white wine lees [16,17]. Likewise, data on the diatomaceous earth from winemaking residues are very scarce. Recently, the feasibility of applying molecularly imprinted polymers to extracts prepared from winemaking diatomaceous earth residues to obtain quercetin-enriched fractions was demonstrated [18]. However, the study mainly focused on some selected compounds without a detailed phytochemical characterization of this by-product being carried out.

In the framework of the United Nations Sustainable Development Goals (SDGs), particularly SDG12 "Ensure sustainable consumption and production patterns", and within a circular economy perspective, there is now a considerable interest in changing the paradigm of wine industry wastes by turning them into by-products that other industries can further use. In this sense, grape pomaces from the wine and juice industries have been widely studied as a source of polyphenols with potential nutraceutical properties to be exploited by the food industry [19–21]. Furthermore, the use of bioactive compounds extracted from these sources is increasingly common in the cosmetic field, mostly based on their antioxidant capacity. In the human body, oxidative stress occurs when the balance between the generation of reactive oxygen species (ROS) and the antioxidant defense systems is compromised. In the skin, environmental stress, such as ultraviolet light, exacerbates ROS production, resulting in cell damage and degradation of the extracellular matrix proteins such as collagen and elastin, ultimately leading to skin aging. The main changes that take place at the dermal connective tissue level, which are essentially translated into the loss of mature collagen and alterations in the elastic network [22], allow uneven pigmentation of the epidermis to occur with aging due to changes in tyrosinase activity in the melanocytes [23]. Distinct phenolic compounds present in grapes, such as anthocyanins, not only possess renowned antioxidant properties, but can also directly inhibit enzymes involved in the skin aging process—namely, tyrosinase and collagenase [24,25]. For this rea-

son, winemaking by-products can be potential sources of innovative functional ingredients for cosmetics, and more studies are required concerning the so far less studied residues.

This work aimed at evaluating several winemaking by-products, with particular focus on underexploited residues for which there is a scarcity of data and knowledge (wine lees and diatomaceous earth), and aimed to compare those with the more widely studied grape pomace, skins, and seeds. The extracts obtained from the residues have been evaluated for their phenolic compounds profile, their antioxidant properties, and their antimicrobial and anti-fungicidal activity. Moreover, to evaluate their potential as cosmeceutical ingredients, the inhibition of tyrosinase and collagenase, as enzymes involved in the skin aging process, as well as the cytotoxicity in fibroblasts (HFF-1) and keratinocytes (HaCaT) cell lines, have also been assessed.

2. Materials and Methods

2.1. Winemaking Industry by-Products

Winemaking by-products were supplied by Caves Campelo S.A. (Portugal) during the harvest season in September 2021. The seeds and skins were manually separated from the red grape pomace. All the other samples were sent individually. The samples of whole pomace, seeds, skins, and stems from red grapes were arranged on separated trays and dried in an oven (Scientific, Series 9000, Scientific Engineering, Johannesburg, South Africa) at 40 °C for 3 days, and then ground into powder in a mill (IKA, A11 basic) to pass a sieve of 20 mesh. The red lees were sent on a plastic bottle and the separation of phases was visible. Thus, they were placed on falcon tubes and centrifuged at 7871× *g* for 5 min. The liquid phase was then pipetted into a flask and separated from the solid phase. After eliminating the ethanol by rotoevaporation (Julabo, TW12), the liquid phase was lyophilized (Telstar, LyoQuest—55 Plus). The separated solid phase from red lees as well as the white lees were also submitted to lyophilization. All the samples were stored at −20 °C inside a container with silica, and were protected from light.

2.2. Extraction of Compounds

Phenolic compounds were extracted using ethanol/water 80:20 (*v*/*v*). A total of 5 grams of powder of each sample was extracted with 100 mL of solvent by sonication (SONO SWISS, sw1) for 5 min followed by stirring (Lbx instruments, S03 series) for 1 h at ambient temperature and protected from light. The samples were centrifuged (Eppendorf, Centrifuge 5810 R) at 7871× *g* for 5 min, and the pellet was re-extracted. The supernatants were collected and joined and filtered, and the ethanol (Fisher Scientific, Loughborough, UK) evaporated under vacuum on a rotary evaporator (Julabo, TW12, Julabo Labortechnik, Seelbach, Germany) at 40 °C. Finally, the extracts were lyophilized, and the obtained dry residues were stored at −20 °C in flasks kept inside a container with silica for further use or weighted and redissolved in ethanol/water 80:20 (*v*/*v*) to a final concentration of 10 mg/mL.

2.3. Total Phenolic Content

The total phenolic compounds content of the hydroethanolic extracts prepared was determined by spectrophotometry in a microplate reader (Epoch 2, Biotek, BioTek Instruments, Inc., Winooski, VT, USA) using the Folin–Ciocalteu reagent. To obtain the calibration curve, an ethanolic solution of gallic acid was used as a standard, within the range of 0.005–0.5 mg/mL. For analysis, different concentrations of sample extracts were prepared from a stock solution (5 mg/mL) prepared from the lyophilizate. In a test tube, 250 µL of extract from each sample or standard were added with 1.25 mL of Folin-Ciocalteu reagent (1:10 *v*/*v* in water) and 1 mL of sodium carbonate (75 g/L). The tubes were vortexed for 15 s and placed to rest in the dark for 30 min at 40 °C for colour development. After this, the tubes were centrifuged at 12,000× *g* rpm for 2 min and the solution transferred to a 96-well microplate, and the absorbance read at 765 nm [26]. Ethanol was used as blank

control. The results were expressed as mg gallic acid equivalents per gram of dry extract (mg GAE/g extract).

2.4. Characterization of Phenolic Compounds by HPLC-DAD-ESI/MS^n

Individual phenolic compounds were separated, identified, and quantified by high-performance liquid chromatography coupled to double diode array and tandem mass spectrophotometry detection (HPLC-DAD-ESI-MS/MS). Dry extracts (40 mg) were redissolved in 2 mL of ethanol/water 80:20 (v/v) and filtered through 0.22 µm disposable filter disks. The analysis was carried out in a Dionex Ultimate 3000 UPLC (Thermo Scientific, San Jose, CA, USA) system equipped with a degasser, a quaternary pump, an auto sampler (kept at 5 °C), an automated thermostatically controlled column compartment, and a diode array detector (DAD) coupled to a mass spectrometer with an electrospray ionization (ESI) source.

Chromatographic separation was achieved with a Waters Spherisorb S3 ODS-2C18 (3 µm, 4.6 mm×150 mm, Waters, Mil-ford, MA, USA) column maintained at 35 °C. The solvents used were: (A) 0.1% formic acid in water, and (B) acetonitrile. The elution gradient established was 15% B (5 min), 15% B to 20% B (5 min), 20–25% B (10 min), 25–35% B (10 min), and 35–50% B (10 min), followed by the re-equilibration of the column in a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 280, 320, 370, and 520 nm as preferred wavelengths and in a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet. MS detection was performed using a Orbitrap Exploris 120 mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with an ESI source operating in negative or positive mode for non-anthocyanin and anthocyanins, respectively. Nitrogen served as the sheath gas (50 psi), and the system was operated 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 kept at a voltage of −70 V. The full MS and MS2 scans covered the mass range from m/z 100–1200. The collision energy used was 35 (arbitrary units). Data acquisition was carried out with Xcalibur® data system (ThermoFinnigan, San Jose, CA, USA).

The phenolic compounds were identified by comparing their retention times, UV-Vis, and mass spectra with those obtained from standard compounds, when available. Otherwise, compounds were tentatively identified comparing the obtained information with available data reported in the literature. For quantitative analysis, a calibration curve for each available phenolic standard was constructed based on the areas of the peaks recorded at 280 nm, except for flavonols (370 nm) and for anthocyanin compounds (520 nm). For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of the most similar available standard. The results were expressed as mg/g of extract (dry weight).

2.5. Antioxidant Activity

Antioxidant activity assays were performed as previously described by Iyda et al. (2019) [26]. The extracts described in 2.1 were re-dissolved in ethanol/water (80:20, v/v) to obtain stock solutions (with various concentrations), which were further diluted to obtain a range of working concentrations to evaluate the antioxidant activity. DPPH radical-scavenging activity was evaluated by using an Epoch 2 microplate reader (BioTek Instruments, Inc., Winooski, VT, USA), and was calculated as a percentage of DPPH discoloration using the formula: $[(A_{DPPH} - AS)/A_{DPPH}] \times 100$, where AS is the absorbance of the sample solution at 515 nm, and A_{DPPH} is the absorbance of the DPPH solution. Reducing power was evaluated by the capacity to convert Fe^{3+} into Fe^{2+} by measuring the absorbance at 690 nm, as described in the literature [27], using the same microplate reader. The lipid peroxidation inhibition was evaluated using porcine brain tissue due to its richness in lipids, and the ability of the assay in generating free radicals produced by oxidative stress. Lipid oxidation originates different products, the most common being malonaldehyde (MDA), which is determined based on the reaction with thiobarbituric acid

(TBA), generating a complex of pink coloration, the absorbance of which was measured at 532 nm. The results were expressed as EC_{50} values, corresponding to the sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay. Trolox was used as positive control.

2.6. Antibacterial Activity

The bacterial strains were clinical isolates obtained from patients hospitalized in various departments at the North-Eastern Local Health Unit (Bragança, Portugal) and Hospital Center of Trás-os-Montes and Alto Douro (Vila Real, Portugal), isolated in a previous work [28]. Five Gram-negative bacteria, namely, *Escherichia coli* (isolated from urine, VRU12881), *Klebsiella pneumoniae* (isolated from urine, VRI17214), *Proteus mirabilis* (isolated from wound exudate, VRI78844), *Pseudomonas aeruginosa* (isolated from expectoration, VRU14123), and *Morganella morganii* (isolated from urine, VRU14272), and three Gram-positive bacteria, namely, *Enterococcus faecalis* (isolated from urine, VRU14041), *Listeria monocytogenes* (isolated from cerebrospinal fluid, VRU17684), and methicillin-resistant *Staphylococcus aureus* (MRSA) (isolated from expectoration, VRI17654), were all tested. These microorganisms were selected based on their antibiotic resistance profile previously described [29]. All microorganisms were incubated at 37 °C in appropriate fresh medium during the 24 h before analysis in order to maintain the exponential growth phase. The minimum inhibitory concentration (MIC) determination was conducted based on the colorimetric broth microdilution assay [30]. The samples were first dissolved in 5% (*v*/*v*) dimethyl sulfoxide (DMSO) and 95% autoclaved distilled water to give a final concentration of 20 mg/mL for the stock solution. Afterwards, the samples were successively diluted with tryptic soy broth (TSB) culture medium to obtain the concentration ranges of 10 to 0.03125 mg/mL. 10 µL of inoculum (standardized at 1.5×10^6 Colony Forming Unit (CFU)/mL) was then added to each well containing 90 µL of sample dilutions, reaching a final concentration of 1.5×10^5 CFU/mL. Two negative controls were prepared, one with TSB and another with the extract only. Two positive controls were also prepared, one with TSB and each inoculum and another with TSB, antibiotics, and bacteria. Ampicillin was used for all the tested bacteria, and imipenem was used against all the strains, except for *E. faecalis* and MRSA, in which vancomycin was used. The microplates were incubated at 37 °C for 24 h and the MIC was determined following the addition (40 µL) of 0.2 mg/mL *p*-iodonitrotetrazolium chloride (INT) and incubation at 37 °C for 30 min. The MIC was defined as the lowest concentration that inhibits the visible bacterial growth determined by color change from yellow to pink if the microorganisms are viable. For the minimum bactericidal concentration (MBC) determination, 10 µL from each well that showed no change in color was plated on Blood agar (7% sheep blood) solid medium and incubated at 37 °C for 24 h. The lowest concentration that yielded no growth was established as the MBC. The MIC and MBC results are expressed in mg/mL.

2.7. Antifungal Activity

The antifungal activity was determined against *Aspergillus fumigatus* (ATCC 204305) and *Aspergillus brasiliensis* (ATCC 16404) according to the methodology described in the literature [31]. The micromycetes were maintained on malt agar and the cultures stored at 4 °C. Before the assay, they were further placed in a new medium and incubated at 25 °C for 72 h. To investigate the antifungal activity, the fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (*v*/*v*). The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0×10^5 in a final volume of 100 µL per well. The extracts were first dissolved in 5% (*v*/*v*) dimethyl sulfoxide (DMSO) and 95% of autoclaved distilled water to give a final concentration of 20 mg/mL for the stock solution. Afterwards, 90 µL of this concentration was added in the first well (96-well microplate) in duplicate with 100 µL of Malt Extract Broth (MEB). The remaining wells were added with 90 µL of MEB, and then the samples were successively diluted to obtain a concentration range from 10 to 0.03125 mg/mL. The MIC was established as the

lowest concentration without visible growth (at the binocular microscope). The minimum fungicidal concentration (MFC) was determined by the sub-cultivation of 2 µL of each well that presented no visible growth into microplates containing 100 µL of MEB per well, and then further incubation 72 h at 26 °C. The lowest concentration with no visible growth was defined as MFC, indicating a 99.5% killing of the original inoculum. Commercial fungicide ketoconazole (Frilabo, Porto, Portugal) was used as positive control. The MIC and MFC were expressed in mg/mL.

2.8. Cytotoxicity Assay in Skin Cell Lines

In vitro spontaneously transformed keratinocytes from histologically normal skin (HaCaT) and fibroblast from human foreskin (HFF-1) cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. The cryopreserved culture of HaCaT was purchased from Cell Line Service (Germany), while HFF-1 was purchased from ATCC. Cells were routinely sub-cultured in 75 cm^2 flasks in humidified 5% CO_2 atmosphere at 37 °C [32]. After cultured cells reached the appropriate confluence, the culture medium was removed from the culture plate, washed twice with Hank's Balanced Salt Solution (HBSS), and then trypsinised using 0.25% trypsin/0.53 mM EDTA, and cells were suspended in a fresh medium and seeded into 96-well plates at 2×10^4 cells/well for 24 h. The cytotoxic effect of the extract was investigated by using Sulforhodamine B (SRB). Briefly, different concentrations of extracts were added to each well, and the control wells were exposed to Triton X-100 at a final concentration of 1% (w/v). After the 48 h incubation period, an ice-cold solution of trichloroacetic acid (TCA) (10%, w/v) was added to each well, followed by incubation for 1 h at 4 °C. Afterwards, the microplates were washed with water and dried at room temperature. A solution of SRB (0.057%, w/v) was then added to each well. Wells were washed three times with an acetic acid solution (1%, v/v) and left to dry at room temperature. Finally, the SRB was solubilised with Tris (10 mM, 200 µL), the absorbance was measured at 540 nm, and the results were expressed as the percentage of cell viability.

2.9. Tyrosinase Inhibitory Activity

The tyrosinase enzyme inhibition activity was evaluated using L-3,4-dihydroxyphenylalanine (L-DOPA) as a substrate with a concentration of 5 mM and 3-methyl-2-benzothiazolinone-hydrazonehydrochloride (MBTH) with a concentration of 20.7 mM as a chromogenic stabilizing agent in 96-well microplates, following the procedure previously described [33,34]. Briefly, test samples (10 µL at 10 mg/mL), phosphate buffer (pH 7.1, 0.1 M), L-DOPA (60 µL), and MBTH (87 µL) were mixed and pre-incubated at 25 °C for 10 min. Subsequently, 6 µL of mushroom tyrosinase enzyme (142 Units of enzyme/mL) were added to each well and the plates were incubated for 30 min at 25 °C. The blank was made with a phosphate buffer (pH 7.1, 0.1 M) and butylresorcinol was used as positive control. The formation of the dopaquinone-MBTH complex was evaluated at 505 nm, using a microplate spectrophotometer (SPECTROstar Nano Multi-Detection Microplate Reader; BMG Labtech, Ortenberg, Germany). The percentage of tyrosinase enzyme inhibition was calculated using the following equation:

$$\% \text{ tyrosinase inhibition} = ((\text{Absorbance Blank} - \text{Absorbance Sample})/(\text{Absorbance Blank})) \times 100$$

2.10. Collagenase Activity Colorimetric Assay

The collagenase inhibition assay was performed using the colorimetric assay kit from Sigma-Aldrich (MAK293) and according to manufacturer's instructions. The eight samples of the extracts from the winemaking residues were prepared with ultrapure water to reach a concentration of 5 mg/mL. A volume of 10 µL of each sample (50 µg of extract) was placed in a 96 well plate in addition with 80 µL of collagenase assay buffer and 10 µL of the provided enzyme (collagenase 0.35 U/mL). For positive control, 10 µL of enzyme only (0.35 U/mL) was used. For inhibitor control, a volume of 10 µL of provided collagenase (0.35 U/mL) and 2 µL of inhibitor (1,10-phenanthroline) was placed into desired wells.

The volume of positive control and inhibitor control wells were adjusted to 100 µL with collagenase assay buffer. The plate was incubated at 37 °C for 10 min. A mixture containing 60 µL of collagenase assay buffer and 40 µL of collagenase substrate FALGPA (N-(3-[2-furyl]acryloyl)-L-leucylglycyl-L-prolyl-L-alanine), was prepared and 100 µL of it was added into the desired wells. Subsequently, the absorbance was read at 345 nm in a microplate reader (Epoch 2, BioTek Instruments, Inc., USA) for 1 h. The percentage of collagenase enzyme inhibition was calculated using the following equation:

$$\% \text{ collagenase inhibition} = ((\text{Activity enzyme} - \text{Activity inhibitor})/(\text{Activity enzyme})) \times 100$$

with the enzyme activity being calculated as indicated by the manufacturer—-namely, by taking the absorbance (A1 and A2) at two time points (T1 and T2) in the linear range with at least two readings in between and at least 1 min apart. Therefore, T1 was considered the first absorbance reading and T2 was established at a 10 min reading.

2.11. Statistical Analysis

For the eight samples evaluated, all the assays were carried out in triplicate and the results were expressed as mean values and standard deviation (SD). Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Tukey's HSD Test with $p = 0.05$. When necessary, a Student's *t*-test was used to determine the significant difference between less than three different samples, with $p = 0.05$. These analyses were carried out using IBM SPSS Statistics for Windows, Version 23.0. (IBM Corp., Armonk, NY, USA).

3. Results and Discussion

3.1. Chemical Characterization

3.1.1. Total Phenolic Compounds

Several works reported on the phenolic composition of different winemaking residues, either regarding their total content in phenolic compounds or by identifying and quantifying the respective individual compounds. However, most focused on grape pomace, particularly from red grape cultivars [35–37]. In this work, besides red wine pomace, other less studied residues were also evaluated—-namely, wine lees and diatomaceous earth from wine filtration. The wine lees from red wine production were supplied by the industry separated into two phases (solid and liquid phases), which were individually assessed. As far as we know, this is the first report on the chemical characterization and bioactive properties of extracts obtained from winemaking diatomaceous earth residue.

Considering the potential interest as a screening assay for the industry, allowing us to estimate which batches of residues can be more promising to be exploited, all extracts were evaluated for its total phenolic content (TPC) based on the reaction with Folin–Ciocalteu. The results for TPC are presented in Figure 1, showing that the highest content was observed for the extracts of grape seeds from red wine pomace, diatomaceous earth, and red wine pomace with values of 146.6 ± 0.6, 111 ± 2, and 126.9 ± 0.9 mg GAE/g extract, respectively. Interestingly, the results suggest that diatomaceous earth is a very promising residue, since it presented a TPC content even higher than the red wine pomace. On the contrary, the extracts from wine lees showed the lowest contents, particularly the one from white wine production (19.7 ± 0.4 mg GAE/g extract).

The obtained results of TPC are consistent with values previously reported for red wine grape pomace by Maicas et al. (between 55.5 to 153.8 mg GAE/g) [38] and by Matos et al. (83.9 ± 2.0 mg GAE/g extract) [39]. Higher values (254 ± 24 mg GAE/g dry extract) were obtained in other studies [40] when evaluating wine lees from red wine production, extracted with a similar solvent as in the current study here (ethanol:water 75:25). However, this can be related to the fact that the lees sample was obtained after the wine had been aged for 12 months in American oak barrels, while the red wine lees herein analyzed were obtained after the fermentation phase (7 to 10 days) of a red wine produced in stainless steel tanks.

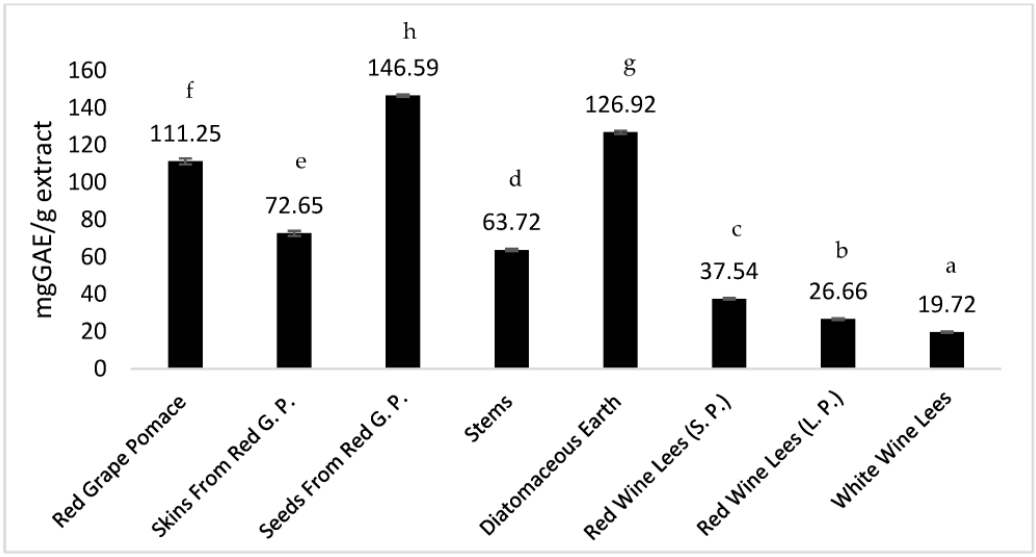

Figure 1. Total phenolic content (TPC) (mg GAE/g dry extract) of hydroethanolic winemaking extracts. (Red Wine Lees (S. P.)—Red Wine Lees Solid Phase; Red Wine Lees L. P.—Red Wine Lees Liquid Phase. Values indicate the means, and error bars represent the standard deviation. In each extract, different letters mean significant differences ($p < 0.05$)).

3.1.2. Phenolic Compounds Profile

Twenty-nine phenolic compounds (non-anthocyanins and anthocyanins) were identified or tentatively identified in the ethanol/water (80:20, *v*/*v*) extracts prepared from the winemaking residues studied in this work—-namely, whole pomace, seeds, skins, stems from red wine production, red and white wine lees, and diatomaceous earth used in red wine production (Table 1). Seventeen non-anthocyanin phenolic compounds were tentatively identified: one phenolic acid (*p*-coumaric acid hexoside), six flavan-3-ols ((epi)catechin derivatives), four flavonols (*O*-glycosylated myricetin, and quercetin derivatives), three dihydroflavonols (taxifolin hexosides), two resveratrol derivatives (resveratrol tetramer and trans-resveratrol), and ethyl-gallate. In addition, twelve anthocyanins were also identified, including *O*-glycosylated and acylated derivatives of delphinidin, cyanidin, malvidin, petunidin, and peonidin.

Most of the detected compounds have been previously described in grape pomace residues from winemaking and other winery byproducts [35,41–44]. Peaks **4** and **9** ($[M-H]^-$ at m/z 289) were identified as (+)-catechin and (-)-epicatechin, respectively, by comparing their retention time and UV spectra with the available standard compounds. Taxifolin and myricetin aglycones were previously reported in red wine samples from China [45], but, in the present work, these two compounds were found to be linked to sugar moieties. Taxifolin derivatives, peaks **6**, **7**, and **8**, all with a deprotonated ion $[M-H]^-$ at m/z 465, presented a unique MS^2 fragment at m/z 303 (taxifolin aglycone), corresponding to the loss of 162 u (hexosyl moiety) and thus being tentatively assigned as taxifolin-*O*-hexosides. Peak **11** ($[M-H]^-$ at m/z 479), with an MS2 fragment at m/z 317 (-162 u; myricetin aglycone), was tentatively identified as myricetin-*O*-hexoside. Peak **15** ($[M-H]^-$ at m/z 521) also released an MS^2 fragment at m/z 317 from the loss of an acetyl-hexoside unit (42 u + 162 u) that allowed its identification as myricetin-*O*-acetyl-hexoside. Peak 12 presented a deprotonated $[M-H]^-$ ion at m/z 197 and MS^2 fragment ions at m/z 169 ($[M-H-28]^-$; gallic acid) and at m/z 125 ($[M-H-28-44]^-$) that corresponded to the successive loss of ethyl (28 u) and CO_2 residues, thus tentatively identified as ethyl gallate. This compound has been previously reported in wine and grape pomace samples [45–47]. In this case, its quantification was not carried out due to a lack of similar standard compounds in our research lab that could be used to construct the calibration curve. Peaks **16** ($[M-H]^-$ at m/z 905) and **17** ($[M-H]^-$ at m/z 227) were tentatively identified as stilbene type-compounds—-namely,

a resveratrol tetramer (cis) and trans-resveratrol, respectively. This tentative identification was performed comparing the obtained results with mass fragmentation data reported in the literature for those compounds [48,49].

Table 1. Retention time (Rt), wavelength at the maximum absorption (λmáx), mass spectra (*m*/*z*), and tentative identification of the non-anthocyanin and anthocyanin phenolic compounds found in winemaking residues from the year 2021.

Peak	Rt (min)	λmáx (nm)	$[M-H]^-$ (*m*/*z*)	MS^2 (*m*/*z*)	Tentative Identification
Non-anthocyanin phenolic compounds					
1	4.58	270	865	739(8), 577(45), 575(8), 425(20), 407(30), 289(11), 287(25)	*β*-type procyanidin trimer
2	4.83	279	577	451(100), 575(39), 425(5), 407(5), 289(5), 287(12)	*β*-type procyanidin dimer
3	5.05	278	1153	865(3), 863(14), 577(7),575(5), 289(14), 287(5)	*β*-type procyanidin tetramer
4	5.45	279	289	245(100), 205(45), 179(13)	(+)-Catechin
5	5.63	279	577	451(100), 575(39), 425(5), 407(5), 289(5), 287(12)	*β*-type procyanidin dimer
6	6.17	298	465	303(100)	Taxifolin-*O*-hexoside
7	6.37	298	465	303(100)	Taxifolin-*O*-hexoside
8	6.7	298	465	303(100)	Taxifolin-*O*-hexoside
9	6.91	282	289	245(100), 205(25), 179(12), 203(13), 231(5), 271(3), 161(3)	(-)-Epicatechin
10	7.16	277	325	163, 145, 119	*p*-Coumaric acid hexoside
11	9.8	274	479	317(100)	Myricetin-*O*-hexoside
12	12.33	277	197	169(100), 124(25)	Ethyl gallate
13	13.78	353	477	301(100)	Quercetin-*O*-hexuronoside
14	14.37	343	463	301(100)	Quercetin-*O*-hexoside
15	18.54	358	521	317(100)	Myricetin-*O*-acetyl-hexoside
16	29.64	284	905	811, 717, 357, 451, 611, 887	Resveratrol tetramer (*cis*)
17	35.24	284	227	186, 159, 143	*trans*-Resveratrol
Peak	Rt (min)	λmax (nm)	$[H]^+$ (*m*/*z*)	MS^2 (*m*/*z*)	Tentative Identification
Anthocyanin phenolic compounds					
18	27.23	523	465	303(100)	Delphidin-3-*O*-glucoside
19	28.64	523	449	287(100)	Cyanidin-3-*O*-glucoside
20	29.61	525	479	317(100)	Petunidin-3-*O*-glucoside
21	31.76	520	463	301(100)	Peonidin-3-*O*-glucoside
22	32.92	526	493	331(100)	Malvidin-3-*O*-glucoside
23	36.78	517	507	303(100)	Dephinidin-3-*O*-acetylglucoside
24	40.13	529	521	317(100)	Petunidin-3-*O*-acetylglucoside
25	43.06	529	535	331(100)	Malvidin-3-*O*-acetylglucoside
26	44.81	531	655	331(100)	Malvidin-3-*O*-caffeoylglucoside
27	45.57	531	625	317(100)	Petunidin-3-*O*-*p*-coumaroylglucoside
28	47.42	526	609	301(100)	Peonidin-3-*O*-*p*-coumaroylglucoside
29	47.78	531	639	331(100)	Malvidin-3-*O*-*p*-coumaroylglucoside

Anthocyanins were identified based on their chromatographic and mass information, in agreement with the data reported in the literature [35,50–54]. The presence of acylated anthocyanins in the winemaking residues is particularly interesting, as they can have greater color stability when exposed to light compared with non-acylated anthocyanins [48,49], thereby making them more suitable to be used as food colorants.

Table 2 presents the quantification (mg/g dry extract) of the non-anthocyanin and anthocyanin phenolic compounds found in all the studied samples. The diatomaceous earth and the red wine pomace presented the highest amounts of non-anthocyanin phenolic compounds, which is in good agreement with the data presented in Figure 1. Moreover, the one of diatomaceous earth was also the richer extract in (-)-epicatechin, showing an amount significantly higher of this flavan-3-ol than the remaining extracts. Only the stems and the seeds from red wine pomace revealed the presence of stilbenes, namely, resveratrol tetramer and trans-resveratrol, respectively, and yet only in trace amounts below the level of quantification. For the white wine lees, only two phenolic compounds were identified, although it was the sample that presented the highest amount of (+)-catechin.

Table 2. Quantification of the non-anthocyanin and anthocyanin phenolic compounds found in the hydroethanolic extracts of winemaking residues from the year 2021 (mean ± standard deviation).

Peak	Red Grape Pomace	Skins	Seeds	Stems	Diatomaceous Earth	Red Wine Lees		White Wine Lees
						(Solid Phase)	(Liquid Phase)	
Non-anthocyanin phenolic compounds (mg/g extract)								
1	0.0686 ± 0.0065 ^{a}	nd	0.0483 ± 0.0028 ^{b}	0.0653 ± 0.0014 ^{a}	nd	nd	nd	nd
2	0.0413 ± 0.0053 ^{e}	nd	0.0637 ± 0.0101 ^{d}	0.167 ± 0.002 ^{a}	0.1554 ± 0.0147 ^{b}	0.0832 ± 0.001 ^{c}	0.1713 ± 0.0163 ^{a}	nd
3	0.0234 ± 0.0047	nd	nd	nd	nd	nd	nd	nd
4	0.0551 ± 0.0066 ^{e}	nd	0.101 ± 0.0191 ^{d}	0.139 ± 0.0047 ^{b}	nd	0.098 ± 0.0293 ^{d}	0.122 ± 0.00 ^{c}	0.1510 ± 0.0236 ^{a}
5	0.0266 ± 0.0015 ^{c}	nd	0.087 ± 0.0048 ^{b}	nd	0.1208 ± 0.0292 ^{a}	nd	nd	nd
6	0.0424 ± 0.0004 ^{d}	0.070 ± 0.0034 ^{a}	nd	nd	nd	0.0536 ± 0.0052 ^{c}	0.057 ± 0.00 ^{b}	nd
7	0.0286 ± 0.0019 ^{d}	nd	0.0335 ± 0.0021 ^{c}	nd	0.0807 ± 0.0171 ^{a}	nd	nd	0.0597 ± 0.0042 ^{b}
8	0.0302 ± 0.0007 ^{c}	nd	0.0525 ± 0.0102 ^{b}	0.0664 ± 0.0091 ^{a}	nd	nd	nd	nd
9	0.0591 ± 0.0024 ^{e}	0.099 ± 0.0141 ^{b}	0.0337 ± 0.0020 ^{f}	0.0753 ± 0.0046 ^{d}	0.254 ± 0.009 ^{a}	0.0729 ± 0.0007 ^{d}	0.0851 ± 0.0138 ^{c}	nd
10	0.0033 ± 0.0002	nd	tr	nd	nd	nd	nd	nd
11	nd	nd	nd	nd	0.532 ± 0.004	nd	nd	nd
12	nd	nd	nd	nd	0.201 ± 0.009	nd	nd	nd
13	0.114 ± 0.0004 ^{e}	0.1140 ± 0.0036 ^{b}	0.0939 ± 0.0001 ^{e}	0.1226 ± 0.0036 ^{a}	0.101 ± 0.0005 ^{d}	0.1051 ± 0.0010 ^{c}	0.0944 ± 0.0001 ^{e}	nd
14	0.0973 ± 0.0014 ^{g}	0.0992 ± 0.0012 ^{d}	0.1065 ± 0.0011 ^{b}	0.097 ± 0.0004 ^{c}	0.108 ± 0.0023 ^{a}	0.0964 ± 0.0011 ^{e}	0.0938 ± 0.0002 ^{f}	nd
15	0.5016 ± 0.0003 ^{a}	nd	nd	nd	nd	nd	nd	nd
16	nd	nd	nd	tr	nd	nd	nd	nd
17	nd	nd	tr	nd	nd	nd	nd	nd
TF3O	0.27 ± 0.0034 ^{a}	0.0998 ± 0.0141 ^{h}	0.334 ± 0.0252 ^{b}	0.443 ± 0.0254 ^{d}	0.730 ± 0.0146 ^{c}	0.254 ± 0.0283 ^{f}	0.3782 ± 0.0025 ^{e}	0.1510 ± 0.0236 ^{g}
TOF	0.81 ± 0.0006 ^{e}	0.2830 ± 0.001 ^{c}	0.286 ± 0.0092 ^{d}	0.2859 ± 0.0058 ^{b}	0.822 ± 0.0159 ^{a}	0.2551 ± 0.0032 ^{d}	0.2460 ± 0.0001 ^{f}	0.0597 ± 0.0042 ^{g}
TPC	1.092 ± 0.004 ^{e}	0.3829 ± 0.015 ^{c}	0.6202 ± 0.0345 ^{d}	0.7322 ± 0.0195 ^{b}	1.553 ± 0.0305 ^{a}	0.509 ± 0.0315 ^{d}	0.6242 ± 0.0025 ^{f}	0.2107 ± 0.0194 ^{g}
Anthocyanin phenolic compounds (mg/g extract)								
18	7.568 ± 0.186 ^{a}	4.134 ± 0.071 ^{b}	2.25 ± 0.003 ^{d}	1.161 ± 0.001 ^{e}	nd	1.075 ± 0.004 ^{f}	2.82 ± 0.043 ^{c}	nd
19	2.886 ± 0.101 ^{a}	1.562 ± 0.021 ^{d}	2.24 ± 0.001 ^{c}	1.166 ± 0.014 ^{e}	nd	1.067 ± 0.005 ^{f}	2.673 ± 0.038 ^{b}	nd
20	7.481 ± 0.174 ^{a}	4.35 ± 0.064 ^{b}	2.009 ± 0.003 ^{d}	1.074 ± 0.004 ^{e}	nd	0.985 ± 0.004 ^{f}	2.446 ± 0.024 ^{c}	nd
21	4.273 ± 0.130 ^{a}	2.182 ± 0.016 ^{c}	1.997 ± 0.001 ^{d}	1.144 ± 0.021 ^{e}	nd	0.964 ± 0.007 ^{f}	2.485 ± 0.051 ^{b}	nd
22	27.142 ± 0.389 ^{a}	16.78 ± 0.469 ^{b}	2.090 ± 0.004 ^{d}	1.614 ± 0.012 ^{e}	nd	1.065 ± 0.004 ^{f}	3.362 ± 0.0359 ^{c}	nd
23	2.743 ± 0.208 ^{a}	1.843 ± 0.04 ^{d}	2.239±0.001 ^{c}	1.071 ± 0.013 ^{f}	nd	1.111 ± 0.003 ^{e}	2.65 ± 0.05 ^{b}	nd
24	3.254 ± 0.043 ^{a}	1.931 ± 0.01 ^{d}	1.993 ± 0.003 ^{c}	0.982 ± 0.006 ^{e}	nd	0.975 ± 0.01 ^{e}	2.379 ± 0.038 ^{b}	nd
25	5.426 ± 0.246 ^{a}	2.779 ± 0.046 ^{b}	2.006 ± 0.0004 ^{d}	1.086 ± 0.009 ^{e}	nd	1.048 ± 0.008 ^{e}	2.619 ± 0.004 ^{c}	nd
26	4.146 ± 0.00 ^{a}	2.262 ± 0.076 ^{c}	2.002 ± 0.003 ^{d}	0.997 ± 0.007 ^{e}	nd	0.983 ± 0.013 ^{e}	2.337 ± 0.036 ^{b}	nd
27	2.861 ± 0.169 ^{a}	2.152 ± 0.038 ^{c}	1.999 ± 0.0057 ^{d}	1.014 ± 0.003 ^{e}	nd	1.031 ± 0.008 ^{e}	2.345 ± 0.047 ^{b}	nd
28	3.157 ± 0.054 ^{b}	1.545 ± 0.104 ^{e}	1.995 ± 0.0005 ^{d}	1.479 ± 0.005 ^{f}	3.401 ± 0.023 ^{a}	0.997 ± 0.009 ^{g}	2.272 ± 0.048 ^{c}	nd
29	9.901 ± 0.06 ^{a}	6.505 ± 0.04 ^{b}	2.033 ± 0.0002 ^{e}	1.274 ± 0.009 ^{f}	3.441 ± 0.012 ^{c}	1.024 ± 0.004 ^{g}	2.371 ± 0.007 ^{d}	nd
TA	80.837 ± 0.861 ^{a}	48.023 ± 0.583 ^{b}	24.852 ± 0.009 ^{d}	14.063 ± 0.013 ^{f}	6.842 ± 0.035 ^{g}	12.324 ± 0.026 ^{e}	30.76 ± 0.368 ^{c}	nd

TF3O—Total flavan-3-ols; TOF—Total Other flavonoids; TPC—Total phenolic compounds; TA—Total anthocyanin. nq—non quantifiable, nd—non detectable, tr—trace amount. Standard calibration curves used for quantification: cyanidin-3-*O*-glucoside (y = 134,578x − 3 × 10^{6}, R^2 = 0.9986, LOD = 0.94 μg/mL; LOQ = 3.13 μg/mL, peaks 19, 20, and 24); (-)—epicatechin (y = 13,304x − 7786.3; R^2 = 0.9998; LOD = 0.07 μg/mL; LOQ = 0.23 μg/mL, peaks 1, 2, 3, 4, 5, and 9); quercetin-3-*O*-glucoside (y = 28,555x + 3032.3, R^2 = 0.9996, LOD = 0.02 μg/mL; LOQ = 0.07 μg/mL, peaks 11 and 16); *p*-coumaric acid (y = 76,029x + 102,258, LOD = 0.71 μg/mL; LOQ = 2.38 μg/mL peak 10); peonidin-3-*O*-glucoside (y = 151,438x − 3 × 10^{6}, R^2 = 0.9965, LOD = 0.13 μg/mL; LOQ = 0.40 μg/mL, peaks 21, 22, 23, 25, 26, 27, 28, 29, and 30); quercetin-3-*O*-glucoside (y = 28,555x + 3032.3, R^2 = 0.9996, LOD = 0.02 μg/mL; LOQ = 0.07 μg/mL, peaks 13 and 14); taxifolin (y = 39,133x − 13,647, R^2 = 0.02; LOD = 0.07 μg/mL; LOQ = 2.02 μg/mL, peaks 6, 7, and 8) and resveratrol (y = 54,835x − 29,986, R^2 = 0.9949; LOD = 0.03 μg/mL; LOQ = 0.11 μg/mL, peaks 17 and 18). ANOVA analysis—In each row different letters mean significant differences ($p < 0.05$).

In general, the non-anthocyanin and anthocyanin phenolic composition was in accordance with what previously reported in red wine pomaces [35,54]. Interestingly, peonidin 3-*O*-*p*-coumarylglucoside and malvidin 3-*O*-*p*-coumarylglucoside were also detected in the diatomaceous earth extract, which was also the sample that presented the highest and third highest level of these two compounds, respectively. It should be noticed that the samples supplied by the Caves Campelo S.A. company were obtained as part of their normal routines and seasonal production. Therefore, the sample of diatomaceous earth supplied corresponded to the final discarded residue, which at industry level corresponds to around four tons that were used to filtrate several batches of wine (obtained from different cultivars, geographical origins, etc., with the requisite of all filtrated wines being red or all being white). For this reason, a direct comparison of the composition of the pomace and that of the diatomaceous earth is not possible with the obtained results. Nevertheless, for the first time, these results highlight this residue as being a potential source of phenolic compounds that otherwise would just be discarded as a waste. The high content of polyphenols verified for the diatomaceous earth sample suggest that the compounds become adsorbed in the silicon atoms during wine filtration, which can afterwards be desorbed with an adequate solvent, allowing extracts to be obtained with a high phenolic concentration.

3.2. Bioactive Properties

3.2.1. Antioxidant Activity

Table 3 shows the results of the antioxidant activity of the extracts obtained from the winemaking residues re-dissolved in ethanol/water (80:20) at different concentrations. Overall, the extracts of grape seeds, red grape pomace, and diatomaceous earth gave the lowest EC_{50} values (meaning the highest antioxidant activity) in all of the assays performed—-namely, DPPH scavenging activity, reducing power, and TBARS inhibition. For the TBARS assay, the sample of skins from red grape pomace also performed very well, showing the best activity with an EC_{50} value of 0.018 mg/mL. The wine lees from red and white wine revealed the highest values (i.e., lowest antioxidant activity), yet still presented an interesting activity since all showed EC_{50} lower than 1 mg/mL. In general, the results were in good agreement with those obtained for the phenolic content, since the wine lees were also the samples evidencing the lowest contents of phenolic compounds (Figure 1, Table 2). Compared to the herein obtained results, Maluf et al. [54] reported a lower EC_{50} (6.9 µg/mL) in the DPPH assay for a grape pomace extract. However, the extract was obtained with a different solvent (75% acetone/water) and from a different grape species (*Vitis lambrusca*), thus resulting in different concentrations of phenolic compounds (69.830 mg EAG/g). Recently, the antioxidant activity of red wine lees extracted with 50% methanol/water was investigated by Lopez-Fernandez et al. [55] that reported an EC_{50} of 0.0129 mg/mL in DPPH assay, but their sample also presented a higher content of total phenolic compounds (148.03 ± 0.48 mg GAE/g). Greater EC_{50} than ours was reported in the literature [30] for the skins obtained from red grape pomace (0.563 mg/mL), while a lower value was observed for the seeds (0.023 mg/mL).

Table 3. Antioxidant activity of extracts obtained from winemaking residues.

Hydroethanolic Extracts	Antioxidant Activity EC_{50} Values (mg/mL)		
	DPPH Scavenging Activity	Reducing Power	TBARS Inhibition
Red Grape Pomace	0.123 ± 0.007	0.17 ± 0.004	0.025 ± 0.0173
Skins From Red Grape Pomace	0.217 ± 0.005	0.24 ± 0.004	0.018 ± 0.0045
Seeds From Red Grape Pomace	0.081 ± 0.005	0.11 ± 0.007	0.021 ± 0.00289
Stems	0.21 ± 0.005	0.41 ± 0.004	0.11 ± 0.00682
Diatomaceous Earth	0.17 ± 0.01	0.11 ± 0.004	0.195 ± 0.0321

Table 3. *Cont.*

Hydroethanolic Extracts	Antioxidant Activity EC_{50} Values (mg/mL)		
	DPPH Scavenging Activity	**Reducing Power**	**TBARS Inhibition**
Red Wine Lees (solid phase)	0.506 ± 0.007	0.68 ± 0.002	0.609 ± 0.00458
Red Wine Lees (liquid phase)	0.578 ± 0.005	0.78 ± 0.001	0.346 ± 0.0185
White Wine Lees	0.93 ± 0.08	0.83 ± 0.09	0.376 ± 0.0224

EC_{50} values correspond to the sample concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. Trolox EC50 values: 0.0536 mg/mL (DDPH), 0.0451 mg/mL (reducing power), and 0.00251 mg/mL (TBARS inhibition).

3.2.2. Antimicrobial Assays

The results of the antibacterial and antifungal activities are presented in Tables 4 and 5, respectively. None of the extracts showed bactericidal activity at the tested concentrations. However, for all the bacteria, bacteriostatic activity was observed for at least one of the assayed samples. In general, the extracts were more effective against Gram-positive than Gram-negative bacteria, with the lowest MIC values being obtained for the red wine lees extracts against MRSA (Methicillin-resistant *Staphylococcus aureus*). Stronger activity against Gram-positive bacteria was also reported [56] when testing extracts obtained from peels, seeds, and stems of two red grape cultivars. Interestingly, the lowest MIC values were generally observed for the extracts of the red wine lees, which together with the extract from white wine lees, were able to inhibit the growth of all tested bacteria. On the other hand, the grape stems presented the weakest activity, since they showed no inhibition of Gram-negative bacteria, despite its activity against Gram-positive strains, particularly *L. monocytogenes* (MIC = 5 mg/mL).

Table 4. Antimicrobial activity of hydroethanolic extracts (mg/mL) obtained from winemaking residues from the harvest of 2021.

	Red Grape Pomace		**Skins**		**Seeds**		**Stems**		**Diatomaceous Earth**		**Red Wine Lees (Solid Phase)**	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Gram-negative bacteria												
Escherichia coli	>10	>10	>10	>10	10	>10	>10	>10	10	>10	10	>10
Klebsiella pneumoniae	>10	>10	>10	>10	>10	>10	>10	>10	10	>10	5	>10
Morganella morganii	5	>10	5	>10	>10	>10	>10	>10	10	>10	5	>10
Proteus mirabilis	10	>10	10	>10	10	>10	>10	>10	10	>10	10	>10
Pseudomonas aeruginosa	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	10	>10
Gram-positive bacteria												
Enterococcus faecalis	10	>10	10	>10	10	>10	10	>10	10	>10	10	>10
Listeria monocytogenes	10	>10	10	>10	5	>10	5	>10	10	>10	5	>10
MRSA	10	>10	10	>10	10	>10	10	>10	5	>10	2.5	>10

	Red Wine Lees (Liquid Phase)		**White Wine Lees**		**Ampicillin (20 mg/mL)**		**Imipenem (1 mg/mL)**		**Vancomycin (1 mg/mL)**	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-negative bacteria										
Escherichia coli	5	>10	10	>10	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
Klebsiella pneumoniae	10	>10	10	>10	10	>10	<0.0078	<0.0078	n.t.	n.t.
Morganella morganii	5	>10	10	>10	>10	>10	<0.0078	<0.0078	n.t.	n.t.
Proteus mirabilis	10	>10	10	>10	<015	<0.15	<0.0078	<0.0078	n.t.	n.t.
Pseudomonas aeruginosa	10	>10	10	>10	>10	>10	0.5	1	n.t.	n.t.
Gram-positive bacteria										
Enterococcus faecalis	5	>10	10	>10	<0.15	<0.15	n.t.	n.t.	<0.0078	<0.0078
Listeria monocytogenes	5	>10	10	>10	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
MRSA	2.5.	>10	5	>10	<0.15	<0.15	n.t.	n.t.	0.25	0.5

MIC values are defined as the lowest concentration of an antimicrobial agent that prevents the visible growth of bacteria. MBC values corresponds to the lowest concentration of an anti-bactericidal agent required to kill the bacteria. MRSA—Methicillin resistant *Staphylococcus aureus*. n.t.—not tested.

Table 5. Antifungal activity of hydroethanolic extracts (mg/mL) obtained from winemaking residues from the harvest of 2021.

	Aspergillus brasiliensis		*Aspergillus fumigatus*	
	MIC	**MIC**	**MFC**	**MFC**
Red Grape Pomace	10	10	>10	>10
Skins from Red Grape Pomace	5	10	>10	>10
Seeds from Red Grape Pomace	>10	10	>10	>10
Stems	10	10	>10	>10
Diatomaceous earth	>10	>10	>10	>10
Red Wine Lees Solid Phase	10	10	>10	>10
Red Wine Lees Liquid Phase	>10	10	>10	>10
White Wine Lees	10	10	>10	>10
Ketoconazole	0.06	0.5	0.125	1

MIC values are defined as the lowest concentration of an antifungal agent that prevents the visible growth of fungus. MFC values correspond to the lowest concentration of an antifungal agent required to kill the fungus.

The results obtained for the antifungal activity were similar to the antibacterial ones, since none of the extracts demonstrated fungicidal potential and most extracts showed only a fungistatic activity at the highest tested concentration (10 mg/mL).

3.2.3. Cytotoxicity Assay in Skin Cell Lines

Due to the possible interest of the studied residues as a source of multifunctional bioactive molecules for cosmetic formulation development, the cell viability effect of the prepared extracts in HaCaT (keratinocytes) and HFF-1 (fibroblast) skin cell lines was also evaluated. As shown in Figure 2, Triton-X was utilised as a positive control to induce a decrease in cell viability. In contrast, cell culture media (DMEM) was used as the negative control, maintaining 100% cell viability. Keratinocytes are a biologically relevant target for skin irritants because they are the first layer of skin cells that come in contact with topically applied compounds. HaCaT cells also present several morphological and functional features typical of normal epidermal keratinocytes, making them a good model for skin toxicity tests [35]. By comprising a mixture of different compounds, the hydroethanolic extracts from winemaking residues may potentiate the development of reactions (allergic or irritant) when applied for dermatologic purposes, thus imposing the need to perform a cytotoxicity evaluation in skin cells and access the minimum concentration, reducing the viability of the tested cell lines. Regarding HaCaT cells, after exposure to the eight extracts (Figure 2), up to 100% cell viability was maintained at 50 µg/mL, while the highest tested concentration (400 µg/mL) was found to significantly inhibit cell viability, particularly for the red wine lees and diatomaceous earth extracts. Concerning HFF-1 cells, the cell viability was maintained up to 100% at 50 µg/mL and more than 100% viability was also observed for all the tested extracts at the highest concentration (400 µg/mL), with one exception (sample C, white wine lees). Therefore, it can be concluded that, in general, all the extracts did not show evidence of affecting the viability of HFF-1 cells. Similar results were observed in previous studies [39] that reported a percentage of cell viability around 100% when HaCaT cells were incubated with 2 mg/mL of Port wine lees extract, and 0.5 mg/mL of red wine lees extract, and when HFF cells were treated with 1 mg/mL and 0.5 mg/mL of the same extracts, respectively.

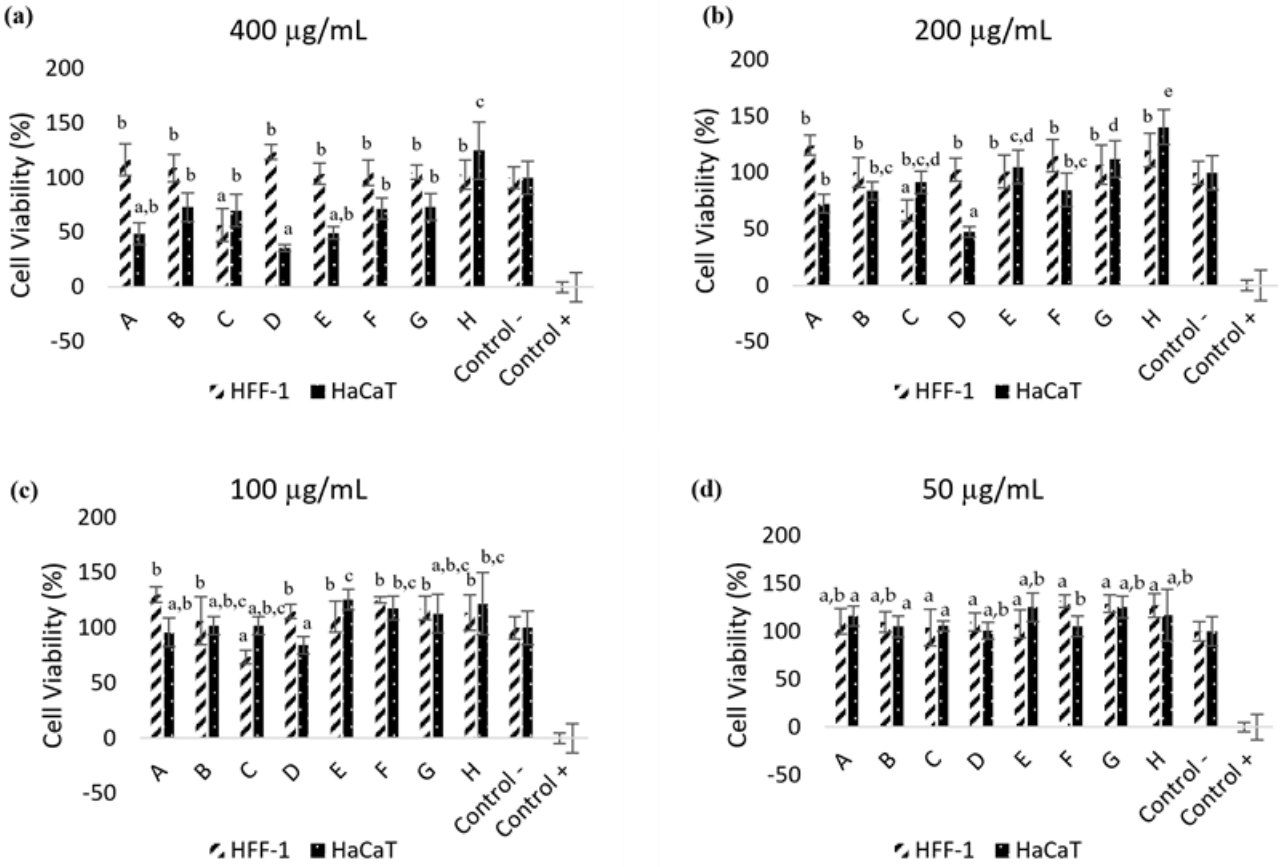

Figure 2. Cell viability effects of hydroethanolic extracts from winemaking residues on HFF-1 and HaCAt cells at different concentrations: (**a**) 400 µg/mL; (**b**) 200 µg/mL; (**c**) 100 µg/mL; and (**d**) 50 µg/mL. Values are expressed as means ± SD. In each extract, different letters mean significant differences between the concentrations ($p < 0.05$). A—Red Wine Lees (Solid Phase); B—Red Grape Pomace; C—White Wine Lees; D—Diatomaceous Earth; E—Red Wine Lees (Liquid Phase); F—Seeds; G—Skins; H—Stems.

3.3. Anti-Tyrosinase and Anti-Ageing Activities

3.3.1. Tyrosinase Inhibition

With aging, pigmentation disorders tend to appear on the skin, which has attracted the attention of cosmetic industries and led to the search for compounds with anti-hyperpigmentation potential. These pigmented lesions are caused by alterations resulting in melanin accumulation. Therefore, inhibiting melanin production is the most explored approach in this field. Because tyrosinase is the enzyme limiting the synthesis of melanin, it is a promising target for the development of skin-whitening cosmetic products. Due to their aromatic structural characteristics, phenolic compounds may have some similarities with tyrosine, the tyrosinase substrate that initiates melanin synthesis. Therefore, phenolic compounds have been described as potential tyrosine analogues that can act as competitive inhibitors of the enzyme [57].

The results obtained for the tyrosinase inhibition assay are presented in Table 6 as the percentage of enzyme inhibition. The extract that showed the most promising result was that prepared from the seeds (45.3%). On the other hand, the red lees showed negligible activity, while the white wine lees and the diatomaceous earth did not show any inhibitory activity against tyrosinase enzyme. The inhibitory action of grape pomace extract on tyrosinase enzyme was also reported [39], which demonstrated that the microwave-treated lees extract presented the best inhibitory effect (50% inhibition at 0.14 mg/mL). Red and white grape stems (1 mg/mL) were also reported to inhibit tyrosinase enzyme, with % inhibition ranging from 41.47% and 53.83% for all grape stem extracts [58]. Phenolic compounds

such as those belonging to chalcones, simple phenols, and hydroxystilbenes families have been referred in the literature as effective tyrosinase inhibitors [59]. However, in this work, it was not always the extracts with the highest content in non-anthocyanin phenolic and anthocyanin phenolic compounds that translated into better tyrosinase inhibition, such as in the case of diatomaceous earth. This indicates that other biomolecules present in the extract might be the most important contributors to this effect. Butylresorcinol is a well-known inhibitor of tyrosinase enzyme that has presented better effectiveness when compared to kojic acid, arbutin, and hydroquinone [60]. In the present work, butylresorcinol presented 100% inhibition of tyrosinase enzyme at 36.52 ± 2.85 µg/mL. Overall, these findings suggest that these winemaking by-products can be suitable raw materials for recovering biomolecules with potent anti-tyrosinase activity.

Table 6. Percentage of tyrosinase and collagenase inhibition towards extracts of winemaking residues.

Extracts	% Collagenase Inhibition	% Tyrosinase Inhibition
Red Wine Lees (Solid Phase)	NA	$1.22 \pm 0.03^{a,b}$
Red Wine Pomace	89 ± 2	$28.2 \pm 0.1^{c,d}$
White Wine Lees	NA	NA
Diatomaceous Earth	18 ± 3	NA
Red Wine Lees (Liquid Phase)	NA	$0.61 \pm 0.02^{a,b}$
Seeds	87 ± 8	45.3 ± 0.1^{e}
Skins	75 ± 7	$34.55 \pm 0.05^{b,c}$
Stems	82 ± 3	$29.93 \pm 0.02^{d,e}$

NA—No Activity. The results identified with different letters in the same column are statistically different (p-value ≤ 0.05). Positive controls: tyrosinase assay (butylresorcinol) presented 100% inhibition at 36.52 ± 2.85 µg/mL; collagenase assay (1,10-phenanthroline) presented 100% inhibition at the conditions suggested by the kit's manufacturer.

3.3.2. Collagenase Inhibition

Collagenase is an enzyme in the matrix metalloproteinase family that breaks down collagen, assisting in the degradation of the extracellular matrix, a key step in the skin aging process. Collagenase has been used to treat Dupuytren's contracture, a disease characterized by the thickening of connective tissue. In this study, a collagenase activity assay kit was used, providing a quick and easy way to measure the inhibition of collagenase activity by the winemaking residues based on the use of a synthetic peptide (FALGPA) that mimics collagen's structure. The obtained results are shown in Table 6. The red grape pomace, the skins, the seeds, and the stems were the extracts that evidenced a higher percentage of collagenase inhibition. Hydroethanolic extract (600 µg/mL) from grape seed, abundant in phenolic compounds, also presented 100% inhibition of collagenase enzyme in a previous study [15,61]. The grape pomace extract with abundant anthocyanin phenolic compounds, such as delphidin-3-*O*-glucoside, malvidin-3-*O*-glucoside, and malvidin-3-*O*-acetylglucoside, presented the best collagenase inhibitory effect. In this assay, the diatomaceous earth also presented some activity, but it showed a much weaker performance compared to the pomace extracts. Both the white and red wine lees did not exhibit any activity. However, a previous study proved that both conventional and microwave-pretreated red wine lees extract presented 50% inhibition of MMP-1 activity [39]. MMP-1 is a collagenase enzyme and its inhibition is vital in preventing extracellular matrix degradation and restoring aging skin. These results showed that these wine by-products could be explored as raw materials to scale up the recovery of structurally diverse biomolecules with a broad spectrum of biological activities relevant to skin care, in contrast to the current disposal approach.

4. Conclusions

Grape pomace has been widely studied concerning its chemical composition and bioactivity, since there is considerable interest from the food, cosmetic, and pharmaceutical industries to turn this by-product rich in bioactive compounds into innovative ingredients. However, other residues from the winemaking process, namely, the wine lees and the diatomaceous earth used for wine filtration, are less studied and are underexploited products. In this study, both of these residues demonstrated that they are potential sources of phenolic compounds, with diatomaceous earth being rich in flavan-3-ols and presenting the highest amount of epicatechin among all of the extracts. This extract also showed the highest amount of non-anthocyanin phenolic compounds, mainly due to its relatively high content in myricetin-*O*-hexoside. As expected, the red grape pomace exhibited the highest amounts of anthocyanins, mainly due to the presence of malvidin derivatives. Interestingly, a few anthocyanins were also found in the diatomaceous earth.

Together with the extracts from the seeds and red pomace, the diatomaceous earth extract also revealed a promising antioxidant activity, since it presented the best results in the reducing power assay. The lees extract, particularly the red ones, showed the highest capacity to inhibit bacterial growth, presenting a bacteriostatic effect against all tested bacteria. In general, the absence of toxicity in HFF-1 cells was observed at the highest tested concentration of extracts. However, some toxicity effects were observed in the HaCaT lines for the highest concentrations of the lees and diatomaceous earth extracts. These extracts also presented the least inhibitory effect against tyrosinase and collagenase enzymes. Therefore, while the extracts from grape pomace, seeds, skins, and stems presented results that suggest their interest as potential ingredients in cosmetic formulations (due to their antioxidant properties and their inhibition of enzymes associated with skin pigmentation and loss of firmness), the extracts of diatomaceous earth and wine lees did not perform as well, since their results suggest a possible cytotoxicity in keratinocytes at high concentrations. Even so, these undervalued residues showed a high potential for producing extracts with high antioxidant properties that can be further exploited by other industries, or for the isolation of added-value compounds, such as anthocyanins, that can be used as natural colorants. For their potential use as cosmetic ingredients, further studies are needed that consider the purification of the extracts in view of decreasing/eliminating the potential cytotoxic effect on keratinocytes.

This work reveals that the wine lees and diatomaceous earth, which are generally discarded and considered by the wine industry as waste, can be valorized as potential sources of bioactive molecules.

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