

Article

Unveiling the Potential of Unexplored Winery By-Products from the Dão Region: Phenolic Composition, Antioxidants, and Antimicrobial Properties

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Abstract: The winery industry generates significant amounts of organic waste, such as pruning firewood, stems, and wine lees, which can cause environmental issues and affect the economic sustainability and competitiveness of the industry. Given the known antioxidant and antimicrobial properties of phenolic compounds, we analyzed these by-products to quantify their phenolic composition and evaluate the effectiveness of pruning firewood and stem extracts from six autochthonous varieties of the Dão Region in inhibiting the growth of bacteria from diabetic foot wounds isolated from hospital patients. The study employed colorimetric methods to measure total phenols, *ortho*-diphenols, and flavonoids in the phenolic composition. The ABTS, DPPH, and FRAP methods were applied to assess the antioxidant capacity, and the disk diffusion method was applied to determine the antimicrobial activity of Gram-positive and Gram-negative bacteria. The results showed that Jaen had the most *ortho*-diphenols and flavonoids in pruning firewood, and the highest levels of these compounds were located in stem extracts. Jaen also had the highest antioxidant capacity in both pruning firewood and stems across all methods used. Notably, red wine lees displayed the highest biological and antioxidant activities. Moreover, pruning firewood extract displayed great efficacy in inhibiting the growth of Gram-positive bacteria, making it a promising candidate as a natural alternative against antibiotic resistance, which is a global public health concern.

Keywords: sustainable circular economy; valuable by-product resources; phenolic compounds potential; robust antioxidant properties; potential antimicrobial compounds



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1. Introduction

The agri-food sector is heavily reliant on grape production as a significant economic activity worldwide. In 2020, over 83 million tons of grapes were produced, with a significant proportion of these grapes used for wine production. However, the high volume of grape by-products results in considerable economic and environmental costs [1].

To address this issue and mitigate excessive seasonal by-product production, the circular economy concept proposes reducing the food waste by repurposing it as feedstock for the creation of new products and applications [2]. Moreover, this approach supports alternative recycling flows in the food system [3]. The circular economy is associated with social tendencies towards developing a restorative/regenerative structure that promotes innovation, adding value to by-products that were previously considered end-of-life materials once the compounds that hindered their initial reuse have been removed [4].

The generation of these residues is significantly higher during the harvest compared to post-grape harvesting, with production being concentrated within a short time frame [5,6]. Some of these same wine residues, such as stems [7–9], grape seeds, skins, and pulps [10–12], have been studied regarding their phenolic composition and biological activity.

However, there is a notable scarcity of research on the phenolic composition and biological activity of pruning firewood and lees, despite their significance as important by-products within the agricultural sector. Interestingly, the Dão Region, despite being the country's oldest demarcated wine region, remains relatively unexplored and understudied concerning the by-products of the wine industry. In 2021/2022, the year of the present study, 286,821 hL of wine has been produced in the Region (<https://www.ivv.gov.pt/np4/home.html>, accessed on 2 September 2023)

Given this backdrop, the primary objective of our work is to quantify the phenolic compounds present in the aforementioned by-products, assess the antioxidant activity of each, and evaluate the antibacterial effects of the extracts derived from pruning firewood and stems across the three studied varieties. Specifically, the study focused on their impact on relevant pathogenic bacteria species associated with diabetic foot infections. Remarkably, the global prevalence of diabetes has been steadily increasing over the years. In fact, according to the International Diabetes Federation, approximately 537 million people had diabetes in 2021, and this number is projected to rise to 643 million by 2030 (<https://diabetesatlas.org/>, accessed on 2 September 2023).

Our research aimed to shed light on a relatively uncharted area, contributing valuable insights to this underrepresented facet of the wine industry, particularly in the historically significant but underexplored Dão Region.

This preliminary study opens the door to an array of exciting research opportunities that span pharmaceuticals, food science, cosmetics, and sustainability. It underscores the importance of harnessing the inherent value of by-products for innovative solutions to pressing global challenges. We look forward to seeing how future research endeavors will build upon these foundations and drive advancements in multiple fields.

2. Materials and Methods

2.1. Chemical Products

The compounds Folin-Ciocalteu's reagent, 3,4,5-trihydroxybenzoic acid (gallic acid), acetic acid, both extra pure (>99%), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS+), 6-hydroxy-2,5,7,8-tetra-methylchromone-2-carboxylic acid (Trolox), potassium persulfate, 2,4,6-Tripyridyl-s-Triazine (TPTZ), ferric chloride, catechin, sodium carbonate, methanol, sodium molybdate, sodium acetate, and iron chloride were purchased from Sigma-Aldrich (Steinheim, Germany). Saline water (0.9% NaCl), aluminum chloride, sodium nitrite and sodium hydroxide were acquired from Merck (Darmstadt, Germany). Hydrochloridric acid was acquired from Fluka Chemika (Neu-Ulm, Switzerland). All culture media and antibiotics used in the study of antimicrobial activity were purchased from Oxoid (Thermo Fisher Scientific Inc., Lisbon, Portugal). Ultrapure water was obtained using a Millipore water purification system (Merck, Darmstadt, Germany).

2.2. Sampling

For the comprehensive analysis of phenolic compounds and antioxidant activity, our sampling strategy encompassed three crucial by-products: pruning firewood, stems, and lees. These by-products were meticulously gathered from a selection of six autochthonous *Vitis vinifera* varieties indigenous to the Demarcated Region of Dão. Among these varieties, four were of the red wine category, namely Touriga Nacional, Tinta Roriz, Alfrocheiro, and Jaen, while the remaining two were distinct white varieties, Borrado das Moscas and Encruzado. As for the assessment of antimicrobial activity, we narrowed our focus to the by-products of two of the red varieties (Alfrocheiro and Jaen) and the white variety Borrado das Moscas.

In this study, the by-products used were obtained from Quinta São Simão da Aguireira, graciously provided by Sociedade dos Vinhos Borges. To prepare these samples for analysis, we processed the pruning firewood and stem samples by grinding them into fine powder. The wine lees, on the other hand, underwent a lyophilization process. All the samples,

encompassing the three by-products, have been securely stored at room temperature and diligently shielded from light. These measures ensured the preservation of the samples until the time of their future analysis.

2.3. Extracts Preparation for Phenolic Content Determination and for Antioxidant Capacity Evaluation

To extract the phenolic compounds, we initiated the process by mixing samples weighing 40 mg with a solution consisting of 1.5 mL of methanol/distilled water (70:30, *v/v*). The mixture was carefully vortexed and agitated for a duration of 30 min, all performed at room temperature. After this phase, the mixture underwent centrifugation at 5000 rpm using a Sigma centrifuge (Steinheim, Germany) for 15 min at 4 °C, effectively segregating the supernatants from the solid residue. These supernatants, containing the valuable extracted compounds, were carefully preserved in a 5 mL volumetric flask for further analysis. This extraction process was repeated three times, with the supernatants from each successive extraction being combined. The cumulative volume was then adjusted to reach a final volume of 5 mL, maintaining the previously mentioned solvent composition [13,14].

Subsequently, the methanolic extracts were filtered through 0.2 µm PVDF filters (Millex HV13, Millipore, Bedford, MA, USA) and were carefully stored under controlled conditions at 4 °C, ensuring their preservation until the time of spectrophotometric analyses. For the assessment of antioxidant activity, all the extracts previously prepared were diluted to a concentration ratio of 1:10 [15], as seen in Figure 1.

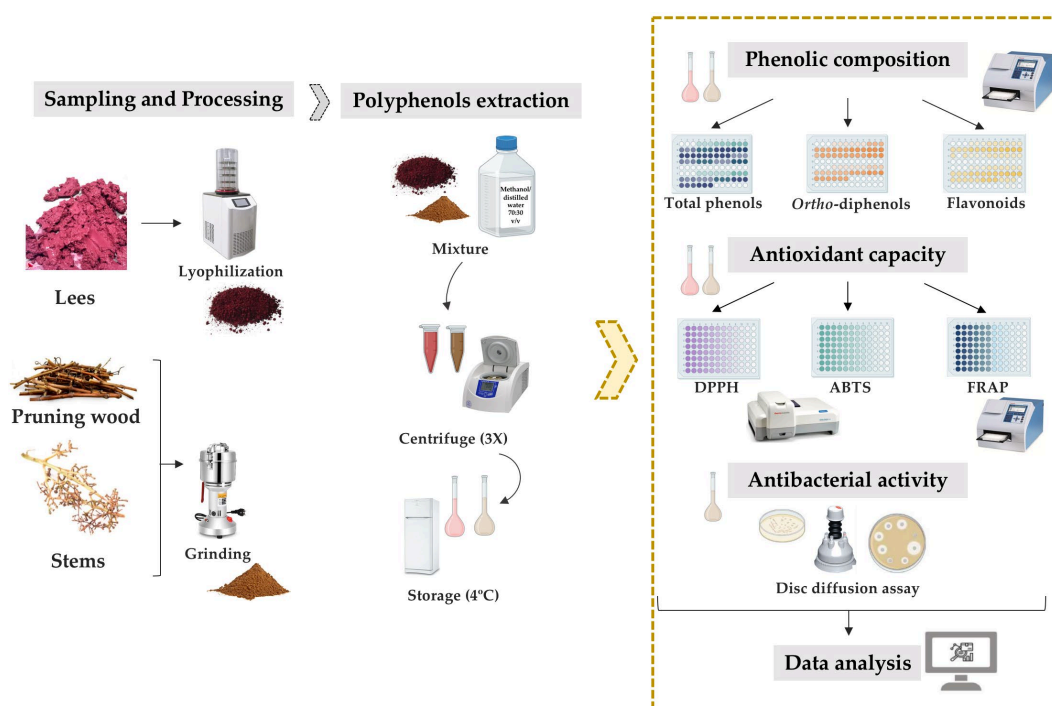


Figure 1. Schematic representation of the methodology used in this study. ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; DPPH, 2,2-Diphenyl-1-picrylhydrazyl; FRAP, ferric reducing antioxidant power.

2.4. Extracts Preparation for Antimicrobial Activity Analysis

For antimicrobial activity analysis, a more extensive extraction procedure was employed [16]. In this case, the samples designated for this specific analysis, each weighing 400 mg, were mixed with 3 mL of the methanol/distilled water solution (70:30, *v/v*). The mixture was subjected to vortexing and agitation for 30 min at room temperature. Following this, centrifugation was carried out at 3700 rpm for 12 min at 4 °C. The resulting supernatants were carefully collected in a 5 mL volumetric flask. To further enhance the

extraction, an additional step was performed. Specifically, 1.5 mL of methanol/distilled water solution (70:30, *v/v*) was added to the mixture, followed by the same process of shaking and centrifugation as before. The cumulative final volume reached 5 mL, and this entire volume was subsequently filtered through 0.2 μm PVDF filters (Millex HV13, Millipore, Bedford, MA, USA). To facilitate handling, the filtered solution was divided into five Eppendorf tubes, desiccated under a nitrogen stream, and eventually reconstituted with 10% DMSO, allowing for the dilution and preparation of the compounds for analysis. The resulting solution was stored at 4 °C, awaiting future analysis as seen in Figure 1.

2.5. Determination of Phenolic Content

The quantification of total phenols, flavonoids, and ortho-diphenols followed established spectrophotometric methods, as detailed by Machado and Domínguez-Perles in 2017 [17], with minor adjustments to suit our study. To assess the content of total phenols in pruning firewood, stems, and lees, we employed the Folin—Ciocalteu spectrophotometric method, referencing the methodology outlined by Dias et al. in 2015 [16], utilizing gallic acid as a standard for calibration.

In summary, the procedure involved adding 20 μL of the sample, 100 μL of Folin—Ciocalteu reagent (diluted at a ratio of 1:10 with H_2O), and 80 μL of Na_2CO_3 . The reaction mixture was then incubated in a light-protected oven at a controlled temperature of 40–45 °C for a period of 30 min. After the incubation, the absorbance of the reaction was measured at a wavelength of 750 nm.

The outcomes of this analysis were expressed as milligrams of gallic acid per gram of dry weight ($\text{mg GA g}^{-1} \text{DW}$), providing a quantitative measure of the total phenol content in the respective samples. This well-established methodology, coupled with the small modifications introduced, enabled us to accurately assess the phenolic composition in the pruning firewood, stems, and lees, further enhancing our understanding of these valuable by-products. In addition, to assess the flavonoid content present in the pruning firewood, stem, and lees samples, we implemented a precise and well-established procedure. Specifically, 24 μL of the respective samples was meticulously mixed with 28 μL of NaNO_2 (sodium nitrite) solution at a concentration of 50 g L^{-1} . After precisely 5 min, 28 μL of AlCl_3 (aluminum chloride) solution at a concentration of 100 g L^{-1} was also introduced into the mixture, allowing the reaction to proceed for a further 6 min. Following this, 120 μL of NaOH (sodium hydroxide) solution at a concentration of 1 M was added to the reaction mixture. To quantify the flavonoid content, the absorbance of the reaction was promptly recorded at a wavelength of 510 nm. For this quantification, catechin, a well-established standard, was employed. The results of this analysis were expressed in terms of milligrams of catechin per gram of dry weight ($\text{mg CAT g}^{-1} \text{DW}$), providing a clear and standardized measure of the flavonoid content in the respective samples [15].

The analyses were carried out utilizing 96-well microplates manufactured by Frilabo (Milheirós, Portugal) and were read using a microplate reader from Thermo Fisher Scientific (Lisbon, Portugal). It is important to note that, to ensure the reliability of our findings, each sample underwent three replicate evaluations ($n = 3$) for all the conducted analyses [18]. This rigorous approach ensured robust and consistent results, enhancing the validity of our assessment of the flavonoid content in these valuable by-products.

2.6. Determination of Antioxidant Capacity

The assessment of free radical scavenging activity involved a rigorous and standardized approach, employing spectrophotometric methods on a micro scale. These methods included the DPPH and ABTS assays, with adaptations based on the procedure outlined by Queiroz et al. in 2017 [19]. Additionally, the FRAP method as described by Vuolo, Lima, and Junior in 2019 was employed to further evaluate antioxidant potential.

For the DPPH method, the absorbance at 520 nm was meticulously measured after 15 min of reaction between the phenolic compounds and the DPPH \bullet radical. This involved adding 190 μL of the adjusted DPPH solution to 10 μL of the sample. Similarly,

for the ABTS^{•+} method, the absorbance at 734 nm was read after 30 min of reaction between the phenolic compounds and the ABTS^{•+} radical. This was achieved by combining 188 µL of ABTS solution with 12 µL of the sample, following the procedure established by Queiroz et al. in 2017 [19].

In the case of the FRAP assay, the absorbance at 593 nm was measured after a 30-min incubation at 37 °C, ensuring protection from light. This incubation involved 280 µL of the FRAP solution, combined with 10 µL of the sample. The analyses were executed using 96-well microplates provided by Frilabo (Milheirós, Portugal) and were read using a microplate reader from Thermo Fisher Scientific (Lisbon, Portugal), ensuring a high degree of precision and consistency. To ensure the robustness of our findings, each sample underwent three replicate evaluations (n = 3) for all conducted analyses. This rigorous approach guaranteed the reliability and validity of our assessment of free radical scavenging activity in these valuable by-products.

2.7. Bacterial Isolates

Pathogenic bacterial isolates, classified as Gram-positive (Gram +) and Gram-negative (Gram −), are recognized by the World Health Organization (WHO) as threats to human health. The pathogenic bacteria associated with diabetic foot were recovered from patients and provided by the *Centro Hospitalar de Trás-os-Montes e Alto Douro* (CHTMAD) in the northern region of Portugal. This collaboration has been governed by a research protocol established in 2004 in conjunction with the University of Trás-os-Montes e Alto Douro (UTAD).

The specific recovered strains are currently housed within the Microbiology Laboratory of the Department of Veterinary Sciences at UTAD and are part of the MJMC collection. Those strains included the Gram-*Acinetobacter baumannii* MJMC 525, *A. baumannii* MJMC 561, *Pseudomonas aeruginosa* MJMC 526, *P. aeruginosa* MJMC 553, *P. aeruginosa* MJMC 5593, and the Gram + Methicillin-resistant *Staphylococcus aureus* (MRSA) MJMC 534-B, and MRSA MJMC 583. In addition to these clinical isolates, we employed reference strains from the American Type Culture Collection (ATCC) for our research purposes. Specifically, we used *Escherichia coli* ATCC 25922 and *S. aureus* ATCC 25923 (Table 1). This comprehensive selection of bacterial strains allows for a thorough investigation of the antimicrobial potential of the compounds under study, and can be seen in Table 1.

Table 1. Bacterial isolates tested.

Bacterial Species	Strains Designation	Source
<i>Escherichia coli</i>	ATCC 25922	American Type Culture Collection (control)
<i>Acinetobacter baumannii</i>	MJMC 525	Trochanteric ulcer
<i>Acinetobacter baumannii</i>	MJMC 561	Abscess
<i>Pseudomonas aeruginosa</i>	MJMC 526	Sacred ulcer
<i>Pseudomonas aeruginosa</i>	MJMC 553	Calcaneal ulcer
<i>Pseudomonas aeruginosa</i>	MJMC 559	Chronic left leg ulcer
<i>Staphylococcus aureus</i>	ATCC 25923	American Type Culture Collection (control)
MRSA	MJMC 534-B	Sacred ulcer
MRSA	MJMC 583	Ulcer

MRSA, Methicillin-resistant *Staphylococcus aureus*; ATCC, American Type Culture Collection.

2.8. Antimicrobial Activity

The antimicrobial potential of the phenolic extracts, derived from the pruning firewood and stem samples of the three chosen varieties, was assessed using the disk diffusion

method originally outlined by Bauer et al. in 1966, with certain adaptations incorporated [20].

In summary, the process began with the preparation of a bacterial inoculum. This was achieved by introducing a colony isolated from pure strains into a 0.9% NaCl solution, with the turbidity meticulously adjusted to match the 0.5 McFarland standard units. This prepared inoculum was then carefully spread across Petri dishes with a diameter of 90 mm, each containing 20 mL of Mueller Hinton Agar.

Following this, sterile paper discs measuring six mm in diameter were uniformly placed on the surface of the agar plate that had been previously seeded with the bacterial inoculum. These paper discs served as carriers to absorb 15 µL of the polyphenolic extract, which was prepared in a 10% Dimethylsulphoxide (DMSO) solution. This methodology allowed us to effectively assess the impact of the polyphenolic extracts on the growth of the bacterial strains under investigation, providing valuable insights into the potential antimicrobial properties of the extracts derived from the pruning firewood and stem samples.

After the overnight incubation at 37 °C, the next step involved measuring the diameter of the clear inhibitory zones that had formed around the discs impregnated with the polyphenolic extracts. These measurements were conducted in mm. Each experiment included essential controls for comparison: a negative control consisting of 10 µL of DMSO, and a positive control employing a standard commercial antibiotic, specifically gentamicin (10 µg).

The evaluation of antibacterial activity was carried out using the following equation:

$$\% \text{ RIZD} = ((\text{IZD sample} - \text{IZD negative control}) / \text{IZD antibiotic standard}) \times 100\%.$$

In this equation:

‘% RIZD’ represents the percentage of the relative diameter of the inhibition zone, measured in mm.

‘IZD sample’ corresponds to the inhibition zone diameter for the tested sample.

‘IZD negative control’ signifies the inhibition zone diameter for the negative control (10 µL DMSO).

‘IZD antibiotic standard’ represents the inhibition zone diameter for the positive control (standard commercial antibiotic gentamicin).

This analytical approach, based on the equation, has been previously established in studies by Leal et al. in 2020 [9]. Importantly, the equation considers and compensates for potential effects of the solvent (DMSO) other than water, ensuring that the assessment of antibacterial activity is accurate and unbiased.

2.9. Statistical Analysis

Data from each determination described above were submitted to 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 of < 0.05. All sample results are presented as mean values ± standard deviation (*n* = 3).

3. Results and Discussion

3.1. Total Phenols, Ortho-Diphenols, Flavonoids Content and Antioxidant Capacity

As evident from the data presented in Table 2, each variety within each sub-product exhibits distinct values. This variability can be attributed to a myriad of factors, encompassing the genetic and physiological traits inherent to each variety, the specific geographic location of cultivation, soil composition, fertilization practices, and the prevailing environmental conditions [8,16,21]. Notably, it is important to acknowledge that all the varieties under investigation were collected from the same geographic location.

Table 2. Results of the Phenolic Composition Evaluation of Pruning Firewood and Stems.

Samples	Pruning Firewood			Stems		
	Total Phenols (mg GA g ⁻¹ DW)	Ortho-Diphenols (mg GA g ⁻¹ DW)	Flavonoids (mg CAT g ⁻¹ DW)	Total Phenols (mg GA g ⁻¹ DW)	Ortho-Diphenols (mg GA g ⁻¹ DW)	Flavonoids (mg CAT g ⁻¹ DW)
Touriga Nacional	18.57 ± 0.97 ^a	19.72 ± 1.28 ^{bc}	14.92 ± 0.60 ^a	7.42 ± 0.14 ^{ab}	5.95 ± 0.55 ^a	4.86 ± 0.29 ^a
Tinta Roriz	20.77 ± 0.27 ^{ab}	20.35 ± 0.85 ^c	16.10 ± 0.16 ^a	17.02 ± 0.40 ^c	15.02 ± 0.68 ^c	13.29 ± 0.50 ^c
Alfrocheiro	19.21 ± 1.29 ^a	17.62 ± 0.77 ^{ab}	16.42 ± 1.46 ^a	11.46 ± 0.70 ^b	9.77 ± 0.31 ^b	7.99 ± 0.34 ^b
Jaen	23.62 ± 2.48 ^b	23.73 ± 1.31 ^d	21.98 ± 0.85 ^b	39.12 ± 2.87 ^d	20.65 ± 0.55 ^d	21.97 ± 1.98 ^d
Borrado das Moscas	18.40 ± 0.43 ^a	15.90 ± 0.59 ^a	14.40 ± 0.62 ^a	6.38 ± 0.36 ^a	5.34 ± 0.10 ^a	4.37 ± 0.06 ^a
Encruzado	24.01 ± 1.34 ^b	20.24 ± 0.94 ^{bc}	20.12 ± 1.77 ^b	-	-	-
<i>p</i> -value	***	***	***	***	***	***

Values are presented as mean ± standard deviation (n = 3). Different letters indicate significantly different results (ANOVA, $p < 0.05$). Significance, NS: not significant ($p > 0.05$), * significant at $p < 0.05$, ** significant at $p < 0.01$, *** significant at $p < 0.001$.

Given this common origin, the observed differences can be attributed primarily to the physiological characteristics unique to each variety. Upon closer examination of the results, particularly regarding pruning firewood, the total phenol contents demonstrated a range from 18.40 ± 0.43 to 24.01 ± 1.34 mg GA g⁻¹ DW, with the Borrado das Moscas and Encruzado varieties exhibiting the respective endpoints of this spectrum. The ortho-diphenol content ranged from 15.90 ± 0.59 mg GA g⁻¹ DW for Borrado das Moscas to 23.73 ± 1.31 mg GA g⁻¹ DW for the Jaen variety. In terms of flavonoids, the values varied from 14.40 ± 0.62 mg CAT g⁻¹ DW for Borrado das Moscas to 21.98 ± 0.85 mg CAT g⁻¹ DW for the Jaen variety.

These findings underscore the importance of considering the inherent characteristics of each grape variety when interpreting the results. While external factors do contribute to variability, the distinctive attributes of each variety play a significant role in shaping the observed differences in phenolic content within the pruning firewood sub-product.

The data reveals a consistent trend: Jaen consistently demonstrates the highest values across the studied phytochemical parameters, standing out as significantly different from the other varieties. Conversely, Borrado das Moscas consistently exhibits the lowest values, also significantly distinct from the other varieties.

This trend persists when examining the stem samples. The Jaen variety consistently displays the highest values for all assessed phytochemical parameters, indicating significant differences when compared to the other varieties. Conversely, Borrado das Moscas consistently exhibits the lowest values, often showing significant differences when compared to the other studied varieties.

Shifting focus to the values obtained for the lees, a noteworthy finding emerges. Among the by-products, red wine lees showcase the highest concentration of phenolic compounds, including total phenols, ortho-diphenols, and flavonoids, as shown in Table 3. This observation highlights the considerable richness of these compounds within red wine lees, showcasing their significant potential.

Table 3. Result of the Phenolic Composition Evaluation of Lees.

Samples	Total Phenols (mg GA g ⁻¹ DW)	Ortho-Diphenols (mg GA g ⁻¹ DW)	Flavonoids (mg CAT g ⁻¹ DW)
White Wine Lees	5.72 ± 0.39 ^a	6.34 ± 0.25 ^a	3.31 ± 0.041 ^a
Red Wine Lees	35.17 ± 1.68 ^b	30.04 ± 0.33 ^b	22.87 ± 2.70 ^b

Values are presented as mean ± standard deviation (n = 3). Different letters indicate significantly different results (ANOVA, $p < 0.05$). Significance, NS: not significant ($p > 0.05$), * significant at $p < 0.05$, ** significant at $p < 0.01$, *** significant at $p < 0.001$.

The relationship between antioxidant activity and phenolic composition is evident from the findings presented in Table 4. Once again, the Jaen variety stands out with the highest and most significantly distinct values compared to the other studied varieties, irrespective of the sub-products being analyzed. As previously highlighted, the red wine lees, as seen in Table 3, contain the most substantial concentration of phenolic compounds, encompassing total phenols, ortho-diphenols, and flavonoids. Remarkably, this elevated phenolic content translates into a correspondingly higher antioxidant activity, as illustrated in Table 5.

Table 4. Results of the antioxidant capacity of pruning firewood and stems (mmol Trolox g^{−1} DW).

Samples	Pruning Firewood			Stems		
	ABTS+•	DPPH•	FRAP	ABTS+•	DPPH•	FRAP
Touriga Nacional	0.23 ± 0.01 ^{ab}	0.14 ± 0.01 ^{ab}	0.01 ± 0.00 ^{ab}	0.04 ± 0.00 ^a	0.04 ± 0.00 ^{ab}	0.04 ± 0.00 ^a
Tinta Roriz	0.22 ± 0.02 ^{ab}	0.15 ± 0.01 ^{bc}	0.02 ± 0.00 ^{bc}	0.11 ± 0.01 ^c	0.09 ± 0.00 ^c	0.09 ± 0.00 ^c
Alfrocheiro	0.19 ± 0.02 ^a	0.12 ± 0.01 ^{ab}	0.01 ± 0.00 ^a	0.07 ± 0.01 ^b	0.05 ± 0.00 ^b	0.05 ± 0.00 ^b
Jaen	0.25 ± 0.02 ^b	0.18 ± 0.01 ^c	0.02 ± 0.00 ^c	0.16 ± 0.01 ^d	0.18 ± 0.01 ^d	0.18 ± 0.01 ^d
Borrado das Moscas	0.20 ± 0.00 ^{ab}	0.11 ± 0.00 ^a	0.01 ± 0.00 ^a	0.04 ± 0.01 ^a	0.03 ± 0.00 ^a	0.03 ± 0.00 ^a
Encruzado	0.24 ± 0.02 ^b	0.15 ± 0.01 ^{bc}	0.02 ± 0.00 ^{ab}	-	-	-
<i>p</i> -value	*	**	***	***	***	***

Values are presented as mean ± standard deviation (n = 3). Different letters indicate significantly different results (ANOVA, $p < 0.05$). Significance, NS: not significant ($p > 0.05$), * significant at $p < 0.05$, ** significant at $p < 0.01$, *** significant at $p < 0.001$.

Table 5. Results of the antioxidant capacity of lees (mmol Trolox g^{−1} DW).

Samples	ABTS+•	DPPH•	FRAP
White Wine Lees	0.05 ± 0.00 ^a	0.05 ± 0.00 ^a	0.03 ± 0.00 ^a
Red Wine Lees	0.25 ± 0.01 ^b	0.23 ± 0.00 ^b	0.23 ± 0.01 ^b

Values are presented as mean ± standard deviation (n = 3). Different letters indicate significantly different results (ANOVA, $p < 0.05$). Significance, NS: not significant ($p > 0.05$), * significant at $p < 0.05$, ** significant at $p < 0.01$, *** significant at $p < 0.001$.

From this comprehensive analysis, we can infer that, in general, pruning firewood boasts a higher phenolic compound content, thereby leading to superior antioxidant activity. Furthermore, it was observed that this by-product possesses an extremely fibrous nature, suggesting the necessity of considering fiber analysis to optimize its utilization in the development of novel products.

It is worth noting that in the existing literature, Leal et al., 2020 [9] conducted a similar analysis, but focused on stems from the Touriga Nacional and Tinta Roriz grape varieties in the Douro region, which has distinct geographical and environmental characteristics compared to the Dão region studied here. Despite these differences, their study reported substantially higher values for total phenols, ortho-diphenols, and flavonoids in both varieties. For Touriga Nacional, the values were (96.12 ± 8.14 mg GA g^{−1} DW), (77.26 ± 5.31 mg GA g^{−1} DW), and (62.61 ± 4.42 mg CAT g^{−1} DW) respectively, while for Tinta Roriz, they were (79.29 ± 2.28 mg GA g^{−1} DW), (62.22 ± 1.36 mg GA g^{−1} DW), and (61.47 ± 4.43 mg CAT g^{−1} DW), respectively [9].

3.2. Antimicrobial Activity

The antimicrobial activity results for the pruning firewood and stem extracts, as determined using the disc diffusion method, are presented in Table 6. Specifically, for Gram + bacteria, the observed inhibition halos ranged from undetected to 13 mm for the pruning firewood extracts, while for the tested stem extracts, the range was from undetected to 11 mm.

Table 6. Halos of inhibition (mm) obtained for extracts of pruning firewood, stems, and negative and positive controls tested.

Bacterial Isolates	ALF PF	BM PF	Jaen PF	ALF S	BM S	Jaen S	DMSO 10%	CN 10 µg
<i>Escherichia coli</i> ATCC	0	0	0	0	0	0	0	16
<i>Acinetobacter baumannii</i> MJMC 525	0	0	0	0	0	0	0	10
<i>Acinetobacter baumannii</i> MJMC 561	7	0	7	0	0	0	0	0
<i>Pseudomonas aeruginosa</i> MJMC 526	0	0	0	0	0	0	0	25
<i>Pseudomonas aeruginosa</i> MJMC 553	11	10	12	10	0	0	0	25
<i>Pseudomonas aeruginosa</i> MJMC 559	12	10	13	11	0	0	0	26
<i>Staphylococcus aureus</i> ATCC	12	10	12	10	7	9	0	21
MRSA MJMC 534-B	12	10	12	7	0	11	0	23
MRSA MJMC 583	13	10	12	0	0	9	0	13

ALF, Alfrocheiro; BM, Borrado das Moscas; PF, Pruning Firewood; S, Stems; DMSO, dimethylsulfoxide; CN, gentamicin; MRSA, Methicillin-resistant *Staphylococcus aureus*; ATCC, American Type Culture Collection.

In the context of pruning wood, it is noteworthy that all evaluated varieties exhibited inhibition of bacterial growth against Gram + bacteria. Meanwhile, for the stem samples, there was also inhibition of bacterial growth across all varieties, albeit with smaller halos compared to the halos observed in the same varieties' pruning firewood by-product.

Switching focus to Gram-bacteria, the inhibition halos varied from non-detected to 13 mm for the pruning firewood extracts, and from non-detect to 11 mm for the stem extracts. Interestingly, in the case of Gram-bacteria, both the pruning firewood and stem extracts demonstrated no inhibition of bacterial growth in *E. coli* and *A. baumannii* MJMC 525 strains. Furthermore, for the *A. baumannii* MJMC 561 strain, the stem extracts of all three varieties similarly showed no inhibition of bacterial growth. In contrast, the tested antibiotic, gentamicin (10 µg), successfully prevented growth for most isolates, except for the *A. baumannii* MJMC 561 strain. This comprehensive analysis underscores the varying degrees of antimicrobial activity exhibited by the extracts against different bacterial strains, with notable differences between the pruning firewood and stem extracts, as well as the impact of the tested antibiotic on bacterial growth inhibition.

The Table 7 provides a comprehensive overview of the RIZD (Relative Inhibition Zone Diameter) percentages for each variety based on the extracts derived from pruning firewood and stems. These results reveal instances where the percentages equate to 100, indicating that certain extracts possess comparable efficacy in inhibiting bacterial growth to the antibiotic utilized in the analysis. A notable example is observed for the MRSA MJMC 583 strain, with the extract from the pruning firewood of the Alfrocheiro variety displaying a %RIZD of 100.

For the *A. baumannii* MJMC 561 isolate, no results could be obtained due to the unique circumstances of the pruning firewood extracts (Alfrocheiro and Jaen) inhibiting bacterial growth while the antibiotic (gentamicin) exhibited no halos, rendering the calculation of %RIZD impossible. Extracts that did not exhibit inhibition of bacterial growth demonstrated a %RIZD value of 0.

In the context of pruning firewood, the scarcity of relevant literature hinders direct comparison with these findings, as this wine by-product has not been previously analyzed for antimicrobial activity. This study, being preliminary, holds the potential to pave the way for innovative applications of this wine by-product as a natural alternative strategy to combat clinically relevant multi-resistant bacteria, thereby reducing reliance on conventional antibiotics. Indeed, the escalation of bacterial resistance rates has significantly compromised the efficacy of existing antibiotics. Considering this pressing global public health challenge, research into natural alternative compounds and complementary therapies has become paramount in addressing the mounting threat of antibiotic-resistant bacteria [22]. This imperative approach ensures a sustainable trajectory for healthcare and disease management [23]. Particularly, in human health, reducing the likelihood of resistance development guarantees the availability of effective treatment options for infec-

tions, thereby upholding public health. Additionally, the adoption of natural compounds can yield favorable environmental and economic outcomes. From an environmental perspective, natural compounds often exhibit biodegradability and a reduced potential to foster antibiotic-resistant genes in the ecosystem [24,25]. This aligns with efforts to mitigate environmental impacts. Furthermore, there are noteworthy economic implications. The pursuit of alternative antibacterial agents offers the potential to alleviate the economic burdens associated with antibiotic resistance [26,27]. This, in turn, can curtail escalating healthcare expenditures and bolster overall productivity.

Table 7. Percentage of inhibition zone diameter (%RIZD) in relation to the tested antibiotic.

Antibiotic Bacterial Strains	CN 10 µg					
	ALF PF	BM PF	Jaen PF	ALF S	BM S	Jaen S
<i>Escherichia coli</i> ATCC	0	0	0	0	0	0
<i>Acinetobacter baumannii</i> MJMC 525	0	0	0	0	0	0
<i>Acinetobacter baumannii</i> MJMC 561	0	0	0	0	0	0
<i>Pseudomonas aeruginosa</i> MJMC 526	-	-	-	-	0	0
<i>Pseudomonas aeruginosa</i> MJMC 553	44	40	48	40	0	0
<i>Pseudomonas aeruginosa</i> MJMC 559	46	38	50	42	0	0
<i>Staphylococcus aureus</i> ATCC	57	48	57	48	33	43
MRSA MJMC 534-B	52	43	52	30	0	48
MRSA MJMC 583	100	77	92	-	0	69

CN, gentamicin; ALF, Alfrocheiro; BM, Borrado das Moscas; PF, Pruning Firewood; S, Stems; MRSA, Methicillin-resistant *Staphylococcus aureus*; ATCC, American Type Culture Collection; -, not performed.

On the other hand, regarding the stem, previous studies by Gyawali and Ibrahim, 2014 [28] and Mattos et al., 2017 [29] have demonstrated the inhibitory effect of this by-product on bacterial growth. This effect is attributed to the accumulation of phenolic compounds, such as caffeic acid and p-coumaric acid, which contain hydroxyl (OH) groups in their structure. These compounds interact with the bacterial cell membrane, destabilizing its structure, leading to lysis and the release of cellular components [28,29]. The hydroxyl (OH) groups promote electron shifts with proton exchange, disrupting the cytoplasmic membrane gradient in bacterial cells, ultimately depleting ATP levels and potentially leading to cell death [28].

Furthermore, it is evident that stem extracts exhibit greater effectiveness in inhibiting the growth of gram-positive isolates compared to gram-negative ones. This disparity can be attributed to the lipopolysaccharide layer present on the outer membrane of gram-negative bacteria, acting as a physical barrier that impedes the penetration of phenolic compounds [30].

In comparison to other by-products, a study by Corrales et al., 2010 [31] focused on evaluating the antimicrobial activity of extracts from Riesling grape skins, specifically targeting bacterial isolates of *E. coli* and *S. aureus*, employing a distinct methodology. The results of their study revealed inhibition halos ranging from 1 to 6 mm for the *S. aureus* bacteria. It is notable that these values, compared to the results presented in Table 6 of our research, were comparatively lower. For the *E. coli* bacterial isolate, Corrales et al.'s study [31] showed no inhibition of bacterial growth, mirroring the outcome of our investigation.

However, the findings from Butkhup et al., 2010 [32] stand in contrast to Corrales et al., 2010 [31]. Butkhup et al.'s study demonstrated inhibition halos for the *E. coli* bacterial isolate with extracts from skins and seeds of the Shiraz (Syrah) grape variety, indicating inhibition values of 4 ± 0.06 mm (excluding the 6 mm disc diameter used in the study) for the film extract and 7 ± 0.12 mm (excluding the 6 mm disc diameter used in the

study) for the seed extract. In contrast, for the bacterium *S. aureus*, Butkhup et al.'s study yielded inhibition halos of 11 ± 0.23 and 12 ± 0.10 mm (excluding the 6 mm disc diameter used in the study), which were higher than the values obtained in our study, considering other extracts of by-products and the varieties under investigation.

These findings highlight the variability in antimicrobial activity observed across different studies, emphasizing the influence of the extraction method, grape variety, and specific bacterial isolates being tested. While Corrales et al.'s results showed modest inhibition, Butkhup et al.'s study indicated more pronounced inhibition, particularly for the *S. aureus* bacterial isolate, surpassing the values obtained in our study for other extracts from various by-products and the selected grape varieties.

In the study conducted by Zambrano et al., 2019 [33], the antimicrobial activity of grape pulp, specifically the Othello variety, was evaluated alongside other extracts and against various bacterial isolates, including *E. coli* and *S. aureus*. When comparing their results with the present study, notable similarities and differences emerge. For the *S. aureus* bacterial isolate, Zambrano et al. (2019) achieved inhibition halos ranging from 8 to 16 mm (excluding the 5 mm disc diameter used in their study), surpassing the values presented in Table 6 of our research. This indicates that grape pulp exhibits greater inhibitions on bacterial growth than the stem and pruning firewood extracts. Conversely, for the *E. coli* isolate, Zambrano et al. (2019) achieved halos of inhibition with values between 2 to 10 mm (excluding the 5 mm disc diameter used in their study), which represent better results compared to our study, where no inhibition of bacterial growth was observed for the same isolate.

Historically, many studies have portrayed the stem, in comparison with other by-products, as possessing the strongest antimicrobial potency against Gram + bacteria, making it a potential option for combatting such pathogens. However, the present study, which also examined pruning firewood, reveals that the pruning firewood exhibits bacterial growth inhibition values superior to those obtained for the stem, even for the same isolates. This suggests that pruning firewood is also a viable option for combating these pathogens, thereby expanding the spectrum of biological alternatives for replacing antibiotics that have lost their efficacy.

Nevertheless, it is crucial to acknowledge that the antimicrobial potential of each extract can be influenced by the extraction method used and the specific microorganism tested, as highlighted by Zambrano et al. [33]. Additionally, the polyphenolic content of the extracts plays a pivotal role. For instance, compounds belonging to the flavonols, flavanols, and tannins class exhibit higher microbial activity in comparison to other polyphenols, as documented by Baenas et al., 2018 [34]. This underscores the multifaceted nature of antimicrobial activity, where factors such as extraction methodology and polyphenolic composition contribute to the observed outcomes.

4. Conclusions

In an era where consumers increasingly seek natural products with discernible health benefits, this preliminary study, conducted in a region with limited existing research, has unveiled the significant bioactive compound content within wine by-products. Pruning firewood, stems, and even lees have exhibited robust antioxidant and antibacterial activities. This discovery, aligned with the principles of circular economy and industrial symbiosis, signifies a promising avenue for the pharmaceutical, food, and cosmetic industries.

What is particularly noteworthy is that pruning firewood exhibited superior antioxidant potential compared to the other wine industry sub-products studied, surpassing the previously documented prowess of stems in this aspect. The stem, long recognized for its substantial potential, now faces a compelling challenger in pruning firewood. This study's findings advocate for the practical applicability of pruning firewood in diverse realms.

The remarkable efficacy of pruning firewood against Gram + bacteria, notably the MRSA MJMC 534-B, and MRSA MJMC 583 strains recovered from patients with diabetic foot, positions it as a promising candidate for addressing antibiotic resistance. This dis-

covery underscores the urgency and potential of utilizing such by-products to combat the growing threat of multi-resistant bacterial strains.

The results obtained from this study provide compelling evidence that various by-products possess inherent value for human applications. Specifically, the exceptional attributes of pruning firewood merit further detailed exploration for potential integration across various sectors, possibly as antibiotic adjuvants, given the alarming rise of multi-resistant bacteria. This study's outcomes reinforce the idea that innovative uses of by-products hold significant promise in addressing pressing challenges in healthcare and sustainability.

Building upon the promising findings of this preliminary study, several exciting avenues for future research emerge. Firstly, a deeper exploration of the specific bioactive compounds within pruning firewood responsible for its remarkable antioxidant and antibacterial properties is essential. The isolation and identification of these compounds could pave the way for innovative pharmaceuticals, functional food ingredients, or cosmetics with enhanced health benefits. Furthermore, as our study underscores the principles of the circular economy and industrial symbiosis, we encourage further investigations into similar synergistic relationships among different industries and their by-products, potentially revealing hidden potentials for transforming waste into valuable resources.

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