

Proceeding Paper

Exploring Winemaking By-Products of Tinto Cão Grapes: Antioxidant and Antimicrobial Activity against Multiresistant Bacteria [†]

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Abstract: The growing global concern about antimicrobial resistance necessitates alternative strategies against multidrug-resistant bacteria. Our study explores the antimicrobial potential of phenolic compounds from Tinto Cão grape winemaking by-products. These compounds effectively combat *S. epidermidis*, *K. pneumoniae*, and *L. monocytogenes*, showing promise in addressing antimicrobial resistance. Additionally, we found remarkable antioxidant activity in these compounds. Shoot extracts exhibited the strongest antimicrobial performance, while seed and leaf extracts displayed the highest antioxidant capacity. These findings highlight phenolic compounds as a sustainable solution to multidrug-resistant bacteria, offering an alternative to traditional antibiotics.

Keywords: antimicrobial resistance; antimicrobial activity; antioxidant activity; wine-making by-products; Tinto Cão

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1. Introduction

Antimicrobial resistance (AMR) stands as an urgent and menacing global public health issue, obstructing effective disease prevention and treatment. Despite ongoing efforts, AMR continues to escalate at an alarming rate worldwide. The imprudent use of antibacterial agents in healthcare and agriculture is chiefly responsible for the surge in AMR. Additionally, bacterial evolution, mutations, and the horizontal transfer of

resistance genes further exacerbate the problem (Dadgostar, 2019). AMR encompasses microorganisms' ability to endure antimicrobial agents, including antibiotics, disinfectants, and food preservatives, rendering conventional treatments ineffective. The widespread use of antibiotics fuels the emergence of resistant bacterial strains, significantly impacting patient outcomes and causing a surge in morbidity, mortality, and healthcare expenses (Abushaheen et al., 2020). Antibiotic resistance arises when bacteria develop mechanisms to withstand the drugs designed to combat them, often resulting in the relapse of infections and severe health consequences. Addressing AMR necessitates a holistic "One-Health" approach, recognizing the interconnection between human health, animal welfare, and ecological stability (Christaki et al., 2020).

In light of the adverse effects and the growing resistance to antibiotics, there is an imperative to explore alternative strategies against bacterial infections. The search for novel molecules and approaches to treat infections, while curbing resistance, has led to the investigation of antimicrobial peptides (AMPs) as promising alternatives with a lower risk of resistance development (Arsene et al., 2021). Moreover, a high intake of fruits and vegetables, rich in antioxidant phytochemicals, has demonstrated a correlation with a reduced risk of nontransmissible chronic diseases (NTCDs). Among these phytochemicals, phenolic compounds (PCs) hold a significant role. PCs offer protection against NTCDs through their antioxidant properties and their ability to regulate various cellular processes (de la Rosa et al., 2019). In a broader context, the agro-industrial sector generates substantial organic residues, contributing to both economic and environmental challenges. By reevaluating these by-products, such as grape pomace from the wine industry, as sources of nutritionally valuable compounds, the emerging concept of the circular bioeconomy has the potential to transform food waste into valuable resources (Tapia-Quirós et al., 2020). Addressing AMR calls for novel approaches to combat infections while minimizing the development of resistance. Fruit phenolic-rich extracts and individual PCs are garnering attention for their antibacterial properties, particularly against resistant strains. These compounds present promising alternatives to conventional antibiotics and are aligned with the principles of sustainability and circular bioeconomy (Lima et al., 2019). Therefore, the aim of this study was to extract phenolic compounds from winery by-products (grape skin, seeds, stem, shoot and leaves) from the "Tinto Cão" variety and evaluated their antioxidant activity and antibacterial properties against antibiotic resistant bacterial strains.

2. Materials and Methods

2.1. Extraction of Phenolic Compounds

Phenolic compounds were extracted from grape skins, seeds, stems, leaves and shoot using a 50:50 water/ethanol mixture. Two grams of each sample were mixed with 100 mL of the solvent, followed by 2 h of stirring and 5 min of sonication. After centrifugation at $10,000\times g$ for 15 min, the pellet underwent re-extraction. The resulting supernatants were collected, and the solvents were evaporated under vacuum at 40 °C. The dry residues were weighed and redissolved in dimethyl sulfoxide (DMSO) to a final concentration of 100 µg/mL. Duplicate extractions were performed for each sample.

2.2. Bacterial Strains, Culture Media, and Growth Conditions

The antimicrobial susceptibility tests were conducted on 8 multiresistant bacterial species, including *Enterococcus faecalis* (vanB2-C3735), *Enterococcus faecium* (vanA-C2302), *Escherichia coli* (CTX-M-15), *Klebsiella pneumoniae* (CTX-M-15), *Pseudomonas aeruginosa* (VIM-2), *Staphylococcus aureus* (MRSA CC398), *Staphylococcus epidermidis* (linezo-R), *Salmonella enteritidis*, and two foodborne strains, *Listeria monocytogenes* and *Bacillus cereus*. These strains are part of the University of La Rioja and University of Trás-os-Montes and Alto Douro collections. All bacterial strains were cultured on BHI agar for 24 h at 37 °C.

For the antimicrobial activity assay, Müller-Hinton agar was used under the same conditions.

Antibacterial Susceptibility Test

The Kirby-Bauer disc diffusion method was employed to assess antimicrobial susceptibility. Initial extract solutions at 100 µg/mL were diluted using DMSO to achieve concentrations of 75, 50, 25, and 10 µg/mL. Twenty microliters of these dilutions were loaded onto sterile blank discs (6 mm diameter) and placed on inoculated agar. Positive controls with antibiotic-impregnated discs and negative controls with DMSO-impregnated discs were included. The plates were incubated at 37 °C for 18–24 h, and inhibition zones were measured with a ruler. The test was performed in triplicate.

2.3. Determination of Antioxidant Activity

Antioxidant activity was evaluated through three different methods: DPPH (2,2-diphenyl-1-picrylhydrazyl), FRAP (Ferric Reducing Antioxidant Power) and CUPRAC (Cupric Reducing Antioxidant Capacity). In the DPPH assay, various extract concentrations were tested for their radical scavenging activity. For the reducing power assay, different extract dilutions were examined for their ability to reduce ferricyanide. Trolox was employed as a positive control. The FRAP method involved reducing a ferric complex using antioxidants. A calibration curve was established using iron sulfate standards. Extracts were incubated with the FRAP reagent, and the absorbance was measured at 593 nm. The CUPRAC method quantified the cupric reducing antioxidant capacity. Trolox was used as a standard for creating a calibration curve, and the results were expressed in µM of trolox equivalents per gram of the sample. These procedures were conducted in triplicate for each sample.

3. Results and Discussion

In the pursuit of sustainable practices within the viticultural industry, the valorization of winery byproducts has gained prominence. These byproducts, often considered waste, have been recognized for their rich content of phenolic compounds (Silva et al., 20XX). Phenolic compounds are well-known for their antioxidant properties, which are attributed to their potential to combat oxidative stress and associated health benefits. Moreover, recent research has unveiled the promising antimicrobial activity of these phenolic compounds against multidrug-resistant bacterial strains, highlighting their potential for applications beyond the realm of winemaking. As far as we are aware, this is the first study reporting the antimicrobial activity of extracts of the Tinto Cão variety.

Among the 10 bacteria used, all extracts exhibited the ability to inhibit the growth of *S. epidermidis* (Table 1). In two of our previous studies conducted with phenolic compounds from Touriga Nacional, Preto Martinho, and Sousão varieties by-products, *S. epidermidis* was also the strain that was most inhibited by almost all extracts and the one that required a lower concentration of extract to be inhibited [1,2]. In the same studies, almost all extracts also inhibited the growth of *L. monocytogenes*. With the exception of the skin extract, all extracts showed antimicrobial activity against *L. monocytogenes* and *K. pneumoniae*. Regarding the MIC (Minimum Inhibitory Concentration), the lowest MIC was achieved with the shoot extract against *S. aureus*. The extracts with the highest inhibitory power, meaning those that inhibited the growth of the greatest number of bacteria, were Stem and shoot, each inhibiting 6 bacterial strains. However, none of the extracts had the capacity to inhibit the growth of *E. faecium*, *E. faecalis*, *S. enteritidis*, and *E. coli*. In the study of Xia et al. grape juice and skin extracts from black table grapes strongly inhibited multiple *L. monocytogenes* species but did not inhibit *B. cereus*, *Salmonella*, *E. coli*, *S. aureus*, or *Y. enterocolitica* [3]. In our study, among the Gram-negative bacteria there was no inhibitory effect of neither of the extracts on *S. enteritidis* and *E. coli* at the concentrations tested but both *K. pneumoniae* and *P. aeruginosa* were inhibited. In fact, it has often been

reported that polyphenolic extracts are more efficient against Gram-positive bacteria. Gram-negative bacteria have low susceptibility to polyphenols when compared to Gram-positive bacteria due to the repulsion between these compounds and the lipopolysaccharide present in the surfaces of Gram-negative bacteria [4]. The mechanisms underlying the antibacterial activity of polyphenols are not yet fully understood. Polyphenols are thought to target several bacterial cell constituents (cell wall, cell membrane, bacterial proteins, bacterial adhesion structures), interfere with bacterial metabolite and ion equilibria, impair the proton gradient required for oxidative phosphorylation, inhibit biofilm formation, and interfere with nucleic acid synthesis and with the regulation of gene expression [5–7]. Some have high affinity for bacterial membranes, particularly for those of Gram-positive bacteria, affecting membrane thickness and fluidity, and increasing its permeability [6–8]. In the context of assessing the antimicrobial potential of winery by-product polyphenolic extracts, it becomes apparent that several pivotal factors must be taken into account. These encompass the choice of extraction solvent, the specific extraction techniques employed, the type of pomace fraction utilized, and the grape variety under investigation. Notably, these factors have been identified in previous studies as key determinants affecting the yield, polyphenolic composition, and overall antimicrobial efficacy of such extracts [9,10].

Table 1. Antimicrobial susceptibility (inhibition zones, mm) of multidrug resistant Gram-positive and Gram-negative bacteria.

Bacterial Strain	MIC (mg/mL) (Inhibition Zone (mm))				
	Skin	Seed	Stem	Shoot	Leaf
<i>L. monocytogenes</i>	-	50 (10)	50 (10)	25 (9)	100 (10)
<i>B. cereus</i>	-	-	25 (9)	50 (10)	-
<i>E. faecium</i>	-	-	-	-	-
<i>E. faecalis</i>	-	-	-	-	-
<i>S. aureus</i>	-	75 (10)	25 (8)	10 (8)	-
<i>S. epidermidis</i>	100 (10)	75 (9)	25 (10)	25 (10)	50 (10)
<i>P. aeruginosa</i>	-	-	50 (9)	25 (10)	-
<i>K. pneumoniae</i>	-	50 (10)	25 (9)	25 (9)	75 (10)
<i>S. enteritidis</i>	-	-	-	-	-
<i>E. coli</i>	-	-	-	-	-

In our study, we conducted a comprehensive analysis of the antioxidant activity of various components of the Tinto Cão variety, including the skin, seed, stem, leaf, and shoot. The results, presented as EC50 values in Table 2, provide valuable insights into the relative antioxidant capacity of these components. The seed extracts, followed closely by the leaf extracts, demonstrated the highest antioxidant capacity across all methods employed. Specifically, the seed extract exhibited the most robust antioxidant activity, suggesting that grape seeds are particularly rich in antioxidant compounds. This observation is consistent with previous research highlighting the abundance of antioxidant compounds in grape seeds, including vitamin E, phenolic compounds, phytosterols, fibers, proteins, carbohydrates, minerals, lipids, and melatonin [11,12].

Table 2. Antioxidant activity of Tinto Cão by-products (mean value \pm SD, n = 3).

Tinto Cão Components	Methods		
	DPPH	FRAP	CuPRAC
Skin	1.81 \pm 0.09 ^a	0.573 \pm 0.008 ^b	0.541 \pm 0.002 ^a
Seed	0.63 \pm 0.02 ^b	0.573 \pm 0.002 ^a	0.515 \pm 0.002 ^c
Stem	1.33 \pm 0.04 ^c	0.584 \pm 0.007 ^b	0.536 \pm 0.005 ^a
Shoot	4.16 \pm 0.27 ^d	0.927 \pm 0.003 ^c	0.656 \pm 0.018 ^b

Leaf	0.97 ± 0.03^b	0.548 ± 0.001^a	0.541 ± 0.003^{ac}
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Furthermore, research conducted on Italian Cultivars by Guaita et al. (2023) found that the antiradical capacity was significantly higher in seeds compared to skins. This observation aligns with our results, which also indicated that grape seeds exhibit remarkable antioxidant activity compared to other components of the Tinto Cão variety. Similar trends have been noted in other studies as well.

When comparing our results with studies conducted on Mazuelo-variety stems and Italian Cultivars, some interesting trends emerge. For example, in the study conducted by Quero et al. using Mazuelo-variety stems and the DPPH method, an antioxidant activity of 0.47 ± 0.04 was reported [13]. In contrast, our study on Tinto Cão variety components yielded a higher EC50 value of 1.33 ± 0.04 , indicating lower antioxidant activity in our samples. This variation in results may be attributed to differences in grape varieties, growth conditions, or methodological variations [13]. Furthermore, research conducted on Italian Cultivars by Guaita et al. found that the antiradical capacity was significantly higher in seeds compared to skins. This observation aligns with our results, which also indicated that grape seeds exhibit remarkable antioxidant activity compared to other components of the Tinto Cão variety [14]. Similar trends have been noted in other studies as well. Ky & Teissedre investigated the antioxidant potential of various grape pomace seeds and skins among different varieties and reported that the antioxidant potential was higher in seeds than in skins [15]. It is worth noting that our results also support existing literature highlighting the antioxidant potential of grape stem extracts. De Sá et al. reported the antioxidant activity of Fernão Pires grape stems extracts with an EC50 range of 0.052–0.090 mg mM⁻¹ of DPPH, reinforcing the importance of grape stems as significant sources of phenolic compounds with antioxidant activity [16].

4. Conclusions

This study examined the antioxidant and antimicrobial activity of various parts of the Tinto Cão grape variety, including the skin, seed, stem, leaf, and shoot. The results unveiled significant variations in antioxidant capacity among these vine components, with seeds and stems displaying the highest antioxidant activity. This corroborates prior research emphasizing the richness of antioxidant compounds in grape seeds and stems. Furthermore, our findings contribute to a more comprehensive understanding of the factors influencing the antimicrobial and antioxidant activity of winery by-product extracts. The significance of extraction solvent choices, extraction techniques, pomace fraction, and grape variety was underscored, based on evidence from previous studies. These insights carry substantial implications for the wine industry and underscore the potential for sustainable utilization of vinification by-products.

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