Article

Exploring the Bioactive Properties of Hydroethanolic Cork Extracts of *Quercus cerris* and *Quercus suber*

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Abstract: The bioactive properties of underutilized corks such as *Quercus cerris* cork and planted *Quercus suber* cork in the Eastern Mediterranean are not well-known but are crucial in developing lignocellulosic biorefineries. To assess their biological potential, hydroethanolic cork extracts of *Quercus cerris* and *Quercus suber* were analyzed for phenolic composition, antioxidant, antiproliferative, antimicrobial activities, and hepatoxicity, as well as NO-production inhibition. Here, we show that a mild hydroethanolic extraction of *Q. cerris* and *Q. suber* corks yielded 3% phenolic extracts. The phenolic composition was similar in both cork extracts, with phenolic acids and ellagitannins as the primary compounds. The bioactivity of hydroethanolic cork extracts from *Q. cerris* surpassed that of *Q. suber* and showed effectiveness against all cancer cell lines tested. This first comprehensive study on the bioactivities of different corks involves detailed characterizations of phenolic compounds of cork extracts using UPLC-DAD-ESI/MSn, evaluations of the antioxidant properties with TBARS and OxHLIA methods, evaluation of antiproliferative activity against gastric (AGS), lung (NCI-H460), colon (CaCo2), and breast cancer (MCF7) cell lines, as well as evaluations of hepatotoxicity and NO-production inhibition. The findings from this study will help bolster the potential of using underutilized cork-rich barks as a valuable resource in bark-based biorefineries.

Keywords: cork; Quercus; antioxidant; antiproliferative activity; antimicrobial activity

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1. Introduction

Cork, also known as phellem in plant anatomy, is a component of periderms in tree barks [1]. In most tree species, the proportion of cork in the bark is small, but a few species have a high proportion of cork, resulting in cork-rich barks. In some species, the bark has a single periderm with a substantial cork layer. *Quercus suber*, commonly known as cork oak, is an example of such a tree species that develops a thick layer of cork. This cork layer can be sustainably harvested through periodic peelings, typically carried out at 9-year intervals in the most important production regions. The production of cork is primarily concentrated in the western Mediterranean region, with Portugal being the world's leading cork producer, followed by Spain [2]. In other species, multiple periderms are formed which, together with the phloem layers that separate them, constitute the bark rhytidome. An example of a species with a cork-rich rhytidome is *Quercus cerris*, commonly known as Turkey oak, in which the successive periderms incorporate significant cork layers. Using cork from *Q. cerris* requires the separation of cork from the phloem fractions that compose the rhytidome, which can be achieved through trituration and granulometric and

densimetric fractionation, thereby producing pure cork as well as several residual cork and phloem fractions [3].

Cork contains a significant proportion of extractives of both lipophilic and polar nature. The lipophilic extractives of corks from both species have a similar chemical composition, comprising alkanes, fatty acids, other aliphatic acids, fatty alcohols, and the pentacyclic triterpenoid friedelin [3]. Friedelin has been reported to have promising properties, such as antioxidant, anti-inflammatory, analgesic, and antipyretic [4,5]. The cork of *Q. cerris* contains a noteworthy amount of polar extractives (5.6% of cork dry weight), but its phenolic composition is currently unknown. Given the chemical similarity of *Q. suber* and *Q. cerris* corks, a similar phenolic profile may be expected [3]. The phenolic composition of *Q. suber* cork polar extracts includes at least five different groups of compounds such as the following: (i) derivatives of benzoic and cinnamic acids, including gallic, protocatechuic, vanillic, caffeic, ferulic, and ellagic acids; (ii) protocatechuic aldehyde, vanillin, coniferyl aldehyde, and sinapic aldehyde; (iii) coumarin derivatives, including aesculetin and scopoletin; (iv) catechin and epicatechin; and (v) flavonoid glycosides [6–11]. The phenolic compounds of cork extracts from *Q. suber* from Portugal have shown strong antioxidant activity [12]. However, the phenolic composition of cork extracts from *Q. suber* trees grown outside of the species' natural distribution area is unknown, and may differ, as the phenolic composition and antioxidant activities of plants' secondary metabolites show geographical variation [13,14].

Cork extractives have been receiving increasing attention due to their chemical proprieties, particularly their bioactivity, which holds potential application in pharmaceutical, healthcare, cosmetics, and food industry [15–17]. Biochemical parameters, such as antioxidant activity, antimicrobial activity, and cytotoxicity, are crucial for evaluating the bioactive potential of biomass extracts, as they can provide benefits to human health when consumed or applied. Therefore, information on the phenolic composition is essential for screening bioactive properties from underutilized or undervalued species like *Q. cerris*.

This work aims to fill the knowledge gap regarding the bioactive properties and the phenolic composition of hydroethanolic extracts of *Q. cerris* cork, as well as of *Q. suber* cork grown outside of its natural distribution area, from a biorefinery perspective. Since biorefineries are applied to produce value-added materials and chemicals, the aim is to contribute to the valorization of underutilized cork-rich species through the characterization of their biologically relevant extracts. In this way, hydroethanolic cork extracts from *Q. cerris* and *Q. suber* trees grown in Turkey were examined, and their antioxidant, antiproliferative, NO-production inhibition, and antimicrobial properties were determined.

2. Materials and Methods

2.1. Materials

Raw bark chunks from mature *Quercus cerris* L. trees were collected in Kahramanmaras, Turkey in 2015, while *Quercus suber* planks were obtained from a cork oak plantation in Izmir, Turkey in 2015. The bark samples were kept in laboratory conditions (20 °C) until granulate preparation.

2.2. Granulate Preparation

Good-quality grade *Q. cerris* cork granulates with a diameter of 5–7 mm, characterized by absence of phloem, were obtained through a pilot-scale grinding and fractionation of the bark. These granulates were subsequently re-granulated and sieved to produce cork particles ranging from 40 to 60 mesh (250–420 µm). For *Q. suber*, planks of virgin cork were stripped from cork oak trees, mechanically reduced to 1–2 mm particles, and further granulated to the same 40–60 mesh size. Each of the cork granulates were air-dried for a minimum of 7 days at room temperature (20 °C), and stored in sealed bags.

2.3. Extract Preparation

Approximately 3 g cork samples were extracted with hydroethanolic solutions by stirring (150 rpm) in 50 mL of ethanol/water (80:20, v/v) at 25 °C for 60 min, and subsequently filtering the mixture through Whatman® No. 4 filter paper. After the initial extraction, the samples underwent a second 60 min extraction with an additional 50 mL of the hydroethanolic solution, as previously described. The combined extracts were concentrated under reduced pressure at 40 °C and 150 mbar using a Büchi R-210 rotary evaporator (BÜCHI Labortechnik AG, Flawil, Switzerland) and then lyophilized using a FreeZone 4.5 (Labconco, Kansas City, MO, USA).

2.4. LC-DAD-ESI-MSn Analysis of Phenolic Compounds

The hydroethanolic extracts prepared above were dissolved in ethanol/water (20:80, v/v) to a final concentration of 10 mg/mL, and then filtered through 0.22 μm disposable filter disks. The analysis was carried out using a Dionex Ultimate 3000 UPLC (Thermo Scientific, San Jose, CA, USA), supplied with a DAD detector (280, 330, and 370 nm as the chosen wavelength) and combined with an electrospray ionization mass detector. A detailed description of the chromatographic and mass spectrometry conditions can be consulted in a previous study [18].

The identification of each phenolic compound was focused on the chromatographic data obtained (retention time, UV-Vis spectra, and mass spectra), and compared with existing standard compounds or earlier-defined data in the literature, using the Xcalibur® software (ThermoFinnigan, San Jose, CA, USA). The quantification of phenolic compounds was carried out using calibration curves constructed from standards, which were based on their UV signals. When a standard compound was not available, the most structurally similar compound from the literature was used for quantification [18]. The area of the peaks was obtained through manual integration using the baseline-to-valley information mode with baseline projection. The results were expressed in mg per g of the extract.

2.5. Antioxidant Activity

The antioxidant activity in the hydroethanolic extracts was assessed through two biochemical-based assays: the thiobarbituric acid reactive substances (TBARS) assay and the oxidative hemolysis (OxHLIA) assay.

For the TBARS assay, the extracts were dissolved in water and diluted to concentrations ranging from 10 mg/mL to 0.3125 mg/mL. The inhibition of lipid peroxidation in porcine (Sus scrofa) brain cell homogenates was determined by measuring the decrease in TBARS and the color intensity of the malondialdehyde–thiobarbituric acid (MDA–TBA) complex, with absorbance measured at 532 nm.

The lipid peroxidation inhibition ratio (%) was calculated using the following formula (Equation (1)):

$$\frac{\mathrm{A}-\mathrm{B}}{\mathrm{A}} \times 100 \quad (1)$$

where A and B represent the absorbance of the blank and extract samples, respectively. The results are presented as the extract concentration (mg/mL) required for 50% inhibition (EC_{50}), representing the sample concentration providing 50% antioxidant activity. EC_{50} values were calculated from the graph of percentage inhibition plotted against the extract concentration. Trolox (Sigma-Aldrich, St. Louis, MO, USA) was used as a positive control.

The anti-hemolytic activity of the extracts was assessed using the OxHLIA assay with red blood cells (RBCs) isolated from healthy sheep. A solution of erythrocytes (2.8%, v/v; 200 μL) was mixed with 400 μL of either extract solution (0.0938–3 mg/mL) in PBS (phosphate-buffered saline: 150 mM NaCl, 8.1 mM Na_2HPO_4, and 1.9 mM NaH_2PO_4, pH 7.4), or with water for complete hemolysis. After pre-incubation at 37 ºC for 10 min with shaking, AAPH (200 L, 160 mM in PBS, from Sigma-Aldrich, St. Louis, MO, USA) was added, and the optical density was measured at 690 nm every 10 min in a microplate reader (Bio-Tek Instruments, ELX800, Santa Clara, CA, USA) until complete hemolysis.

The results were expressed as IC_{50} values (μg/mL) at Δt of 60, representing the extract concentration required to protect 50% of the erythrocyte population from hemolysis. Trolox was also used as a positive control.

2.6. Antiproliferative and NO-Production Inhibition

The antiproliferative activity of the hydroethanolic extracts was assessed within a range of concentrations from 400 to 6.25 μg/mL, as previously described [19], using the sulforhodamine B (Sigma-Aldrich, St. Louis, MO, USA) colorimetric assay against three human tumor cell lines: gastric adenocarcinoma (AGS), colorectal adenocarcinoma (CaCo-2), lung carcinoma (NCI-H460) and breast carcinoma (MCF-7). The cell lines AGS (Catalogue No. 89090402) and CaCo-2 (Catalogue No. 860102022) were commercially acquired from the European Collection of Authenticated Cell Cultures—ECACC; in turn, NCI-H460 (ACC 737) and MCF-7 (ACC 115) were acquired from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH. The cell lines were maintained in accordance with the manufacturer's recommendations and used only up to a maximum of passage 50, to ensure their viability and quality control. A primary culture obtained from pig liver (PLP2) was also tested. Ellipticine was applied as a positive control. The results were expressed as GI_{50} values (μg/mL), which represent the extract concentration accountable for 50% inhibition of cell proliferation.

The capacity of the extracts to inhibit the nitric oxide (NO) production within a range of concentrations (from 400 to 6.25 μg/mL) was studied using a lipopolysaccharide (LPS)-stimulated murine macrophage cell line (RAW 264.7, Catalogue No. 91062702)). This cell line was commercially obtained from the European Collection of Authenticated Cell Cultures—ECACC. NO production was quantified based on the nitrite concentration utilizing the Griess Reagent System kit and based on previously described protocols [20]. Dexamethasone (50 mM) was used as a positive control, and cells with and without LPS were tested as the negative control. The results were expressed as EC_{50} values (μg/mL), representing the extract concentration responsible for 50% of NO production inhibition.

2.7. Antimicrobial Activity

Five Gram-negative bacteria (*Enterobacter cloacae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enterica*, and *Yersinia enterocolitica*), as well as three Gram-positive bacteria (*Bacillus cereus*, *Listeria monocytogenes*, and *Staphylococcus aureus*), isolated from food, were selected to test the antibacterial activity of the hydroethanolic extracts.

Five Gram-negative bacteria (*E. coli*, *Klebsiella pneumoniae*, *Morganella morganii*, *Proteus mirabilis*, and *P. aeruginosa*), along with three Gram-positive bacteria (*Enterococcus faecalis*, *L. monocytogenes*, and Methicillin-resistant *Staphylococcus aureus* (MRSA)) were chosen as clinical isolates for evaluating the antibacterial activity of the hydroethanolic extracts.

For antifungal activity, two micromycetes were used, namely, *Aspergillus brasiliensis* and *A. fumigatus*. The microdilution method was performed to determine the minimum inhibitory, bactericidal and fungicidal concentrations (MICs, MBCs, and MFCs, expressed in mg per mL). The positive controls used were streptomycin, methicillin, ampicillin and ketoconazole (Sigma-Aldrich, St. Louis, MO, USA), whereas the negative control was 5% dimethyl sulfoxide (DMSO).

2.8. Statistical Analysis

All the experiments and measurements were carried out in triplicate and the results were expressed as mean ± standard deviation (except for the antimicrobial analysis). The SPSS Statistics Software (IBM SPSS Statistics for Windows, Version 22.0., IBM Corp., Armonk, NY, USA) was used to assess significant differences among the two samples by applying a two-tailed paired Student's *t*-test.

3. Results and Discussion

3.1. Phenolic Composition

The assignment of phenolic compounds in the *Q. cerris* and *Q. suber* cork samples is shown in Table 1, and the corresponding quantification is given in Table 2. Compounds were tentatively identified based on chromatographic behavior (Supplementary Figures S1 and S2), UV-Vis absorption spectra, and fragmentation pattern. Protocatechuic acid (peak 3), caffeic acid (peak 4), epicatechin (peak 5), ferulic acid (peak 6), and ellagic (peak 7) were identified by comparison with authentic standards.

Peaks 1, 2, and 8 presented deprotonated molecules at m/z 477, 345, and 187, respectively. They showed characteristic fragments of gallic acid in MS^2 (m/z 169 and m/z 125), suggesting they are gallic acid derivatives. These compounds were tentatively assigned as methyl gallate-pentosyl-hexoside and methyl gallate hexoside, due to the neutral losses of 162 and 132 Da (corresponding to hexose and pentose moieties) and 15 Da (attributed to a methyl group), and gallic acid monohydrate.

Peak 9 gave molecular ion at m/z 461, and fragments at m/z 315 (M-H-146)- and 300 (M-H-146-15)-. Although UV-Vis spectra were poorly informative, it was possible to observe the presence of a λmax around 365 nm, suggesting that peak 8 was an ellagic-acid derivative. Based on this data, this compound was identified as methyl ellagic acid rhamnoside [21,22]. Interestingly, derivatives of cinnamic acid and coumarins were not found in either cork extract, possibly due to the applied mild-maceration methods which resulted in lower phenolic content than the extended-time extraction methods such as the Soxhlet method. *Q. cerris* cork showed a phenolic profile akin to that of *Q. suber* cork, but with a higher phenolic content. Additionally, *Q. cerris* cork contained flavonoids, such as epicatechin, which were absent in *Q. suber* cork. Previous reports already indicated the presence of proanthocyanidins in *Q. suber* cork [6]. Ellagic acid emerged as the primary phenolic compound detected in both extracts, with protocatechuic acid, ferulic acid, and gallic acid derivatives following suit, aligning with earlier findings [6,7].

Table 1. Chromatographic and spectral data and suggested assignment of phenolic compounds.

Peak	Rt (min)	λ Max (nm)	$[M-H]^-$ (m/z)	MS^2	MS^3	MS^4	Assignment
1	5.17	264	477	183	169, 125	-	Methyl gallate-pentosyl-hexoside
2	5.39	-	345	183	169, 125	-	Methyl gallate hexoside
3	5.78	5.78	260, 292	153	-	-	Protocatechuic acid
4	10.45	323	179	-	-	-	Caffeic acid
5	10.45	-	289	-	-	-	Epicatechin
6	18.06	321	193	-	-	-	Ferulic acid
7	19.08	366	301	-	-	-	Ellagic acid
8	21.8	-	187	169, 125	-	-	Gallic acid monohydrate
9	25.21	363	461	315	300	-	Methyl ellagic acid rhamnoside
10	27.92	-	551	343	328	313	5,6-Dihydroxy-7,3′,4′-Trimethoxyflavone hexoside

Table 2. Quantification of the phenolic compounds present in Q. cerris and Q. suber cork hydroethanolic extracts.

Compound	Quantification (mg/g of Extract)		Student's *t*-Test *p*-Value
	***Q. cerris* Cork**	***Q. suber* Cork**	
Methyl gallate-pentosyl-hexoside	1.5 ± 0.2	-	-
Methyl gallate hexoside	0.16 ± 0.01	-	-
Protocatechuic acid	2.5 ± 0.10	0.94 ± 0.02	<0.001
Caffeic acid	0.6 ± 0.10	0.83 ± 0.01	<0.001
Epicatechin	2.5 ± 0.50	-	-

Table 2. *Cont.*

Compound	Quantification (mg/g of Extract)		Student's *t*-Test *p*-Value
	Q. cerris Cork	*Q. suber* Cork	
Ferulic acid	0.66 ± 0.01	1.05 ± 0.004	<0.001
Ellagic acid	2.7 ± 0.30	1.21 ± 0.01	<0.001
Gallic acid monohydrate	-	tr	-
Methyl ellagic acid rhamnoside	1.40 ± 0.02	1.25 ± 0.01	<0.001
5,6-Dihydroxy-7,3′,4′-Trimethoxyflavone hexoside	-	0.49 ± 0.01	-

-: not analyzed; tr: traces; calibration curves used are the following: gallic acid: $y = 131{,}538x + 292{,}163$; caffeic acid: $y = 533{,}585x + 10^6$; epicatechin: $y = 10{,}314x + 147{,}331$; ellagic acid: $y = 26{,}719x - 317{,}255$; potocatechuic acid: $y = 214{,}168x + 27{,}102$; quercetin-3-*O*-glucoside: $y = 34{,}843x - 160{,}173$. Significant differences ($p < 0.001$) between extracts were assessed by a Student's *t*-test.

The antioxidant and antimicrobial properties of plant extracts are closely tied to the content and composition of phenolic compounds, with phenolic acids and ellagitannins being the primary contributors to antioxidant activity [23]. Furthermore, various Gram-positive and Gram-negative bacteria showed varying sensitivities to phenolic compounds [24]. To assess these activities, bioactive assays were conducted on the hydroethanolic extracts of *Q. cerris* and *Q. suber* cork, as discussed below.

3.2. Extraction Yield and Antioxidant Activity

The hydroethanolic maceration extraction produced approximately 3% yield from both cork samples, as shown in Table 3. The mild conditions employed during this extraction method resulted in lower extract yields compared with those obtained through prolonged extraction with higher temperatures. Maceration is the preferred method when targeting bioactive compounds, as it limits their degradation under the applied room temperature and short-extraction-time conditions. This was particularly relevant in this study, which is centered on exploring the bioactive properties of the cork extracts. Nevertheless, it should be noted that the extraction yield is a significant parameter for assessing biomass feedstocks in the context of biorefineries, and although the focus of this study was not to optimize extraction yields, this should be addressed in further studies.

The antioxidant activity of *Q. cerris* and *Q. suber* corks is presented in Table 3. Both corks showed antioxidant activity, which was confirmed by two different and physiologically relevant antioxidant assays. *Q. cerris* cork hydroethanolic extract showed higher antioxidant activity when comparing the values of effective concentration (EC_{50}, 96 µg/mL) and inhibitory concentration (IC_{50}, 35 µg/mL) to those of *Q. suber* cork extract (759 and 49 µg/mL, respectively). These findings align with previous studies on *Q. suber* cork extracts [12,25,26]. It is important to note that the results from the current study represent the first assessment of antioxidant activity in cork hydroethanolic extracts using physiologically relevant methods, in contrast to the commonly employed in vitro methods such as DPPH, to which they cannot be directly compared.

The polar extracts of *Q. suber* corks from Portugal and Algeria showed antioxidant activity, as determined by their DPPH scavenging ability, with IC_{50} values of 2.12 µg/mL and 5.69 µg/mL, respectively [25,26].

Table 3. Extraction yield, antioxidant activity, antiproliferative, and NO-production inhibition activities of the *Q. cerris* and *Q. suber* cork hydroethanolic extracts.

	Q. cerris Cork	*Q. suber* Cork	Student's *t*-Test *p*-Value
Extraction yield (%)	3.0	2.8	0.226
Antioxidant activity			
TBARS (EC_{50}; µg/mL) [a]	86 ± 1	759 ± 9	<0.001
OxHLIA Δt = 60 min (EC_{50}; µg/mL) [b]	35 ± 2	49 ± 3	<0.001

Table 3. *Cont.*

	Q. cerris Cork	*Q. suber* Cork	Student's *t*-Test *p*-Value
Antiproliferative activity (GI_{50} μg/mL) [c]			
AGS	144 ± 10	211 ± 15	<0.001
CaCo-2	208 ± 1	>400	-
NCI-H460	208 ± 2	>400	-
MCF-7	210 ± 14	>400	-
Hepatotoxicity (GI_{50} μg/mL) [c]			
PLP2	195 ± 5	>400	-
NO-production inhibition (EC_{50} μg/mL) [d]			
RAW 264.7	>400	>400	-

-: not analyzed; [a] EC_{50}: extract concentration corresponding to 50% of antioxidant activity (TBARS) or [b] extract concentration required to keep 50% of the erythrocyte population intact for 60 min (OxHLIA assay); [c] GI_{50}: extract concentration responsible for 50% inhibition of cell proliferation; [d] EC_{50}: extract concentration responsible for achieving 50% of the inhibition of NO-production. Positive controls—Trolox EC_{50} values: 5.4 ± 0.3 μg/mL (TBARS), 19.7 ± 0.4 μg/mL (OxHLIA, Δt 60 min); Ellipticine GI_{50} values: 1.23 ± 0.03 μg/mL (AGS), 1.21 ± 0.02 μg/mL (CaCo-2), 1.0 ± 0.1 μg/mL (NCI-H460), 1.02 ± 0.02 μg/mL (MCF-7) and 1.4 ± 0.1 μg/mL (PLP2); Dexamethasone EC_{50} values: 6.3 ± 0.4 μg/mL (RAW 264.7). Significant differences ($p < 0.001$) between the two samples were assessed by a Student's *t*-test. AGS—gastric adenocarcinoma; CaCo-2—colorectal adenocarcinoma; NCI-H460—lung carcinoma; MCF-7—breast carcinoma; PLP2—primary culture of pig liver cells; RAW 264.7—murine macrophage cell line.

3.3. Antiproliferative and NO-Production Inhibition Activities

Certain plants show anti-proliferative effects on cancer cell lines, although they can also have cytotoxic effects on healthy cell lines. This duality has prompted the screening of plant extracts for their antiproliferative potential [27–30].

Therefore, both the antiproliferative and the NO-production inhibition activities are crucial parameters for characterizing the bioactivity of cork extracts. Only a limited number of authors have studied these properties on cork extracts, with one report available for *Q. suber* [12]. To the best of our knowledge, this study represents the first report of these properties for *Q. cerris* cork hydroethanolic extracts.

The obtained results are presented in Table 3, and suggest that *Q. cerris* cork extract exhibits the capacity to interfere in the normal proliferation of several studied cancer cell lines, including against AGS, CaCo-2, NCI-H460, and MCF-7 (GI_{50} between 144 and 210 μg/mL). *Q. suber* cork extract exhibits antiproliferative capacity only for the AGS cell line (GI_{50} = 211 μg/mL); moreover, it does not exhibit hepatotoxicity for the PLP2 cell line. These results are consistent with a prior report on *Q. suber* cork extracts and their effects on colon cancer and breast cancer cell lines [12]. Interestingly *Q. cerris* cork had a stronger growth-inhibitory activity than *Q. suber* cork.

The NO-production inhibition capacity was also studied, and the obtained results demonstrated that the extracts did not exhibit the ability to inhibit the production of the pro-inflammatory mediator nitric oxide at the concentrations tested [31]. The anti-inflammatory activity of *Q. suber* cork extract was previously reported [15], whereas the anti-inflammatory activity of *Q. cerris* cork extract is reported for the first time in this study.

3.4. Antimicrobial Activity

Plant extracts can have antimicrobial activity against bacteria and fungi, which varies depending on the type of microorganism [24]. For instance, it may involve the disruption of the cell membrane of both Gram-positive and Gram-negative bacteria [32], interfering with protein synthesis, DNA replication, intermediary metabolism, and other mechanisms [33]. Similar mechanisms were reported for antifungal agents [34].

Antibacterial and antifungal activities of *Q. suber* and *Q. cerris* cork extracts were previously reported in various studies [15,17,35–38]. Both corks showed antibacterial activity against both Gram-positive and Gram-negative bacteria (Table 4). In general,

Q. cerris cork showed lower inhibition concentrations for bacteria of relevance to food, compared with those for *Q. suber*, while the opposite occurred for clinically isolated bacteria.

The antibacterial activity of cork extracts against Gram-negative bacteria is particularly noteworthy. For example, *Q. cerris* cork showed an MIC value of 2.5 mg/mL for *E. coli*, a spoiling bacterium in food products, and *Y. enterocolitica,* which is pathogenic. These results suggest a higher antimicrobial effectiveness of *Q. cerris* cork extracts, given that Gram-negative bacteria possess an additional lipopolysaccharide cell wall, making them more resistant to antimicrobial agents. For Gram-positive bacteria, both extracts stood out against *S. aureus* (MIC value of 2.5 mg/mL), another important pathogenic bacterium causing a foodborne disease.

Another important result is the wide range of antibacterial activities demonstrated by the hydroethanolic cork extracts against several bacteria, including those isolated from food sources and clinical specimens. Notably, these extracts had significant activity against methicillin-resistant bacteria (MRSA), which is a human pathogen responsible for a variety of infections, including pneumonia, skin infections [39], and bloodstream infections [40], and notorious for its resistance against penicillin antibiotics. Thus, the hydroethanolic cork extracts show promising potential for commercial screening using a wide range of bacteria. Unconventional corks, such as *Q. cerris* cork, may prove to be valuable sources for producing cost-effective bioactive compounds that can be employed as food preservatives or in medical applications.

In a previous study on waste *Q. cerris* cork with ethanol, methanol, acetone, and water extracts, using the agar diffusion method, no antibacterial activity against Gram-negative bacteria was observed. This suggests that the choice of the antimicrobial test method significantly affects the results [17]. It appears that the serial dilution method offers higher sensitivity for detecting antibacterial activity.

Cork extracts also displayed antifungal activities, as shown in Table 4. The antifungal activity of the *Q. cerris* cork hydroethanolic extract exceeded that of *Q. suber*, consistent with our previous antifungal test results against *Candida albicans* [17]. It is worth noting that phenolic acids, flavonoids, and tannins have been reported to possess antifungal properties [41]. Therefore, these compounds are likely responsible for the antifungal and antibacterial properties of cork extracts.

Table 4. Antimicrobial activities of *Q. cerris* and *Q. suber* corks hydroethanolic extracts.

Antibacterial Activity	***Q. cerris* Cork**		***Q. suber* Cork**		**Streptomicin * 1 mg/mL**		**Methicilin * 1 mg/mL**		**Ampicillin * 10 mg/mL**	
Food isolates Gram-negative bacteria	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E. cloacae	5	>10	10	>10	0.007	0.007	n.t.	n.t	0.15	0.15
E. coli	2.5	>10	10	>10	0.01	0.01	n.t.	n.t.	0.15	0.15
P. aeruginosa	10	>10	10	>10	0.06	0.06	n.t.	n.t.	0.63	0.63
S. enterica	5	>10	5	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Y. enterocolitica	2.5	>10	5	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Gram-positive bacteria										
B. cereus	10	>10	5	>10	0.007	0.007	n.t.	n.t.	n.t.	n.t.
L. monocytogenes	5	>10	10	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
S. aureus	2.5	>10	2.5	>10	0.007	0.007	0.007	0.007	0.15	0.15
Clinical isolates										
Gram-negative bacteria										
E. coli	5	>10	2.5	>10	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
K. pneumoniae	5	>10	2.5	>10	10	>10	<0.0078	<0.0078	n.t.	n.t.
M. morganii	5	>10	2.5	>10	>10	>10	<0.0078	<0.0078	n.t.	n.t.
P. mirabilis	2.5	>10	5	>10	<015	<0.15	<0.0078	<0.0078	n.t.	n.t.
P. aeruginosa	5	>10	10	>10	>10	>10	0.5	1	n.t.	n.t.

Table 4. *Cont.*

Antibacterial Activity	*Q. cerris* Cork		*Q. suber* Cork		Streptomicin * 1 mg/mL		Methicilin * 1 mg/mL		Ampicillin * 10 mg/mL	
Gram-positive bacteria										
E. faecalis	5	>10	10	>10	<0.15	<0.15	n.t.	n.t.	<0.0078	<0.0078
L. monocytogenes	10	>10	2.5	>10	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
MRSA	5	>10	2.5	>10	<0.15	<0.15	n.t.	n.t.	0.25	0.5
Antifungal activity					Ketoconazole *					
	MIC	MFC	MIC	MFC	MIC	MFC				
A. brasiliensis	5	>10	10	>10	0.06	0.125	-	-	-	-
A. fumigatus	5	>10	10	>10	0.5	1	-	-	-	-

-: not analyzed; * positive controls, MIC—minimum inhibitory concentration, MBC—minimum bactericidal concentration, MFC—minimal fungicidal concentration, n.t.—not tested, MRSA—Methicillin-resistant *Staphylococcus aureus*.

Considering all the bioactivity tests performed, the two cork extracts showed promising potential. *Q. cerris* cork extract demonstrated a higher bioactivity compared with *Q. suber* cork extract, possibly due to its higher phenolic content.

4. Conclusions

The biological potential of two underutilized corks, *Q. cerris* and *Q. suber* cork from Turkey, was evaluated in hydroethanolic extracts obtained after mild maceration. The phenolic composition and bioactive properties of the two different cork extracts, which included antioxidant, anti-proliferative, and anti-inflammatory properties, as well as antimicrobial activity, were reported here for the first time.

The following specific conclusions can be drawn from this study:

1. The phenolic composition of hydroethanolic extracts of *Q. cerris* and *Q. suber* corks is similar, with phenolic acids and ellagitannins as the principal compounds.
2. Hydroethanolic cork extracts of *Q. cerris* and *Q. suber* have significant antioxidant, antimicrobial, and anti-proliferative properties.
3. The hydroethanolic cork extracts appear to be effective against gastric-, lung-, colon- and breast-cancer cell lines.

Complementary extract characterization with screening of biologically relevant compounds, the application of different extraction methods and binary extraction systems, as well as the testing of corks from different species, are required to further explore the bioactive potential of underutilized corks.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr12081579/s1, Figure S1: Phenolic profile of *Quercus cerris* cork. The peaks are identified in Table 1; Figure S2: Phenolic profile of *Quercus suber* cork. The peaks are identified in Table 1.

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References

1. Angyalossy, V.; Pace, M.R.; Evert, R.F.; Marcati, C.R.; Oskolski, A.A.; Terrazas, T.; Kotina, E.; Lens, F.P.; Mazzoni-Viveiros, S.C.; Angeles, G. IAWA List of Microscopic Bark Features. *IAWA J.* **2016**, *37*, 517–615. [CrossRef]
2. Pereira, H. *Cork: Biology, Production and Uses*; Elsevier: Amsterdam, The Netherlands, 2007; ISBN 978-0-444-52967-1.
3. Şen, A.; Miranda, I.; Santos, S.; Graça, J.; Pereira, H. The Chemical Composition of Cork and Phloem in the Rhytidome of Quercus Cerris Bark. *Ind. Crops Prod.* **2010**, *31*, 417–422. [CrossRef]
4. Sunil, C.; Duraipandiyan, V.; Ignacimuthu, S.; Al-Dhabi, N.A. Antioxidant, Free Radical Scavenging and Liver Protective Effects of Friedelin Isolated from *Azima tetracantha* Lam. Leaves. *Food Chem.* **2013**, *139*, 860–865. [CrossRef] [PubMed]
5. Antonisamy, P.; Duraipandiyan, V.; Ignacimuthu, S. Anti-Inflammatory, Analgesic and Antipyretic Effects of Friedelin Isolated from *Azima tetracantha* Lam. in Mouse and Rat Models. *J. Pharm. Pharmacol.* **2011**, *63*, 1070–1077. [CrossRef] [PubMed]
6. Conde, E.; Cadahía, E.; García-Vallejo, M.C.; Fernández de Simón, B. Polyphenolic Composition of Quercus Suber Cork from Different Spanish Provenances. *J. Agric. Food Chem.* **1998**, *46*, 3166–3171. [CrossRef]
7. Conde, E.; Cadahía, E.; García-Vallejo, M.C.; Fernández de Simón, B.; González Adrados, J.R. Low Molecular Weight Polyphenols in Cork of Quercus Suber. *J. Agric. Food Chem.* **1997**, *45*, 2695–2700. [CrossRef]
8. Santos, S.A.O.; Villaverde, J.J.; Sousa, A.F.; Coelho, J.F.J.; Neto, C.P.; Silvestre, A.J.D. Phenolic Composition and Antioxidant Activity of Industrial Cork By-Products. *Ind. Crops Prod.* **2013**, *47*, 262–269. [CrossRef]
9. Mazzoleni, V.; Caldentey, P.; Silva, A. Phenolic Compounds in Cork Used for Production of Wine Stoppers as Affected by Storage and Boiling of Cork Slabs. *Am. J. Enol. Vitic.* **1998**, *49*, 6–10. [CrossRef]
10. Mislata, A.M.; Puxeu, M.; Ferrer-Gallego, R. Aromatic Potential and Bioactivity of Cork Stoppers and Cork By-Products. *Foods* **2020**, *9*, 133. [CrossRef]
11. Conde, E.; Cadahía, E.; Garcia-Vallejo, M.C.; Gonźalez-Adrados, J.R. Chemical Characterization of Reproduction Cork from Spanish Quercus Suber. *J. Wood Chem. Technol.* **1998**, *18*, 447–469. [CrossRef]
12. Fernandes, A.; Fernandes, I.; Cruz, L.; Mateus, N.; Cabral, M.; de Freitas, V. Antioxidant and Biological Properties of Bioactive Phenolic Compounds from *Quercus suber* L. *J. Agric. Food Chem.* **2009**, *57*, 11154–11160. [CrossRef] [PubMed]
13. Liu, Y.; Chen, P.; Zhou, M.; Wang, T.; Fang, S.; Shang, X.; Fu, X. Geographic Variation in the Chemical Composition and Antioxidant Properties of Phenolic Compounds from *Cyclocarya paliurus* (Batal) Iljinskaja Leaves. *Molecules* **2018**, *23*, 2440. [CrossRef] [PubMed]
14. Liu, Y.; Fang, S.; Zhou, M.; Shang, X.; Yang, W.; Fu, X. Geographic Variation in Water-Soluble Polysaccharide Content and Antioxidant Activities of *Cyclocarya paliurus* Leaves. *Ind. Crops Prod.* **2018**, *121*, 180–186. [CrossRef]
15. Carriço, C.; Ribeiro, H.M.; Marto, J. Converting Cork By-Products to Ecofriendly Cork Bioactive Ingredients: Novel Pharmaceutical and Cosmetics Applications. *Ind. Crops Prod.* **2018**, *125*, 72–84. [CrossRef]
16. Pinto, C.; Cravo, S.; Mota, S.; Rego, L.; e Silva, J.R.; Almeida, A.; Afonso, C.M.; Tiritan, M.E.; Cidade, H.; Almeida, I.F. Cork By-Products as a Sustainable Source of Potential Antioxidants. *Sustain. Chem. Pharm.* **2023**, *36*, 101252. [CrossRef]
17. Şen, U.; Viegas, C.; Duarte, M.P.; Maurício, E.M.; Nobre, C.; Correia, R.; Pereira, H.; Gonçalves, M. Maceration of Waste Cork in Binary Hydrophilic Solvents for the Production of Functional Extracts. *Environments* **2023**, *10*, 142. [CrossRef]
18. Bessada, S.M.F.; Barreira, J.C.M.; Barros, L.; Ferreira, I.C.F.R.; Oliveira, M.B.P.P. Phenolic Profile and Antioxidant Activity of *Coleostephus myconis* (L.) Rchb.f.: An Underexploited and Highly Disseminated Species. *Ind. Crops Prod.* **2016**, *89*, 45–51. [CrossRef]
19. Rodrigues, M.J.; Gangadhar, K.N.; Vizetto-Duarte, C.; Wubshet, S.G.; Nyberg, N.T.; Barreira, L.; Varela, J.; Custódio, L. Maritime Halophyte Species from Southern Portugal as Sources of Bioactive Molecules. *Mar. Drugs* **2014**, *12*, 2228–2244. [CrossRef]
20. Taofiq, O.; Calhelha, R.C.; Heleno, S.; Barros, L.; Martins, A.; Santos-Buelga, C.; Queiroz, M.J.R.P.; Ferreira, I.C.F.R. The Contribution of Phenolic Acids to the Anti-Inflammatory Activity of Mushrooms: Screening in Phenolic Extracts, Individual Parent Molecules and Synthesized Glucuronated and Methylated Derivatives. *Food Res. Int.* **2015**, *76*, 821–827. [CrossRef]
21. Wyrepkowski, C.C.; Gomes da Costa, D.L.M.; Sinhorin, A.P.; Vilegas, W.; De Grandis, R.A.; Resende, F.A.; Varanda, E.A.; Dos Santos, L.C. Characterization and Quantification of the Compounds of the Ethanolic Extract from Caesalpinia Ferrea Stem Bark and Evaluation of Their Mutagenic Activity. *Molecules* **2014**, *19*, 16039–16057. [CrossRef]
22. Kim, J.-P.; Lee, I.-K.; Yun, B.-S.; Chung, S.-H.; Shim, G.-S.; Koshino, H.; Yoo, I.-D. Ellagic Acid Rhamnosides from the Stem Bark of Eucalyptus Globulus. *Phytochemistry* **2001**, *57*, 587–591. [CrossRef] [PubMed]
23. Alañón, M.E.; Castro-Vázquez, L.; Díaz-Maroto, M.C.; Hermosín-Gutiérrez, I.; Gordon, M.H.; Pérez-Coello, M.S. Antioxidant Capacity and Phenolic Composition of Different Woods Used in Cooperage. *Food Chem.* **2011**, *129*, 1584–1590. [CrossRef]
24. Puupponen-Pimiä, R.; Nohynek, L.; Meier, C.; Kähkönen, M.; Heinonen, M.; Hopia, A.; Oksman-Caldentey, K. Antimicrobial Properties of Phenolic Compounds from Berries. *J. Appl. Microbiol.* **2001**, *90*, 494–507. [CrossRef] [PubMed]
25. Santos, S.A.O.; Pinto, P.C.R.O.; Silvestre, A.J.D.; Neto, C.P. Chemical Composition and Antioxidant Activity of Phenolic Extracts of Cork from *Quercus suber* L. *Ind. Crops Prod.* **2010**, *31*, 521–526. [CrossRef]
26. Touati, R.; Santos, S.A.O.; Rocha, S.M.; Belhamel, K.; Silvestre, A.J.D. The Potential of Cork from *Quercus suber* L. Grown in Algeria as a Source of Bioactive Lipophilic and Phenolic Compounds. *Ind. Crops Prod.* **2015**, *76*, 936–945. [CrossRef]
27. Steenkamp, V.; Gouws, M.C. Cytotoxicity of Six South African Medicinal Plant Extracts Used in the Treatment of Cancer. *S. Afr. J. Bot.* **2006**, *72*, 630–633. [CrossRef]

28. Brankiewicz, A.; Trzos, S.; Mrożek, M.; Opydo, M.; Szostak, E.; Dziurka, M.; Tuleja, M.; Łoboda, A.; Pocheć, E. Cytotoxic and Antioxidant Activity of *Hypericum perforatum* L. Extracts against Human Melanoma Cells from Different Stages of Cancer Progression, Cultured under Normoxia and Hypoxia. *Molecules* **2023**, *28*, 1509. [CrossRef]
29. Al-Nemari, R.; Bacha, A.B.; Al-Senaidy, A.; Almutairi, M.H.; Arafah, M.; Al-Saran, H.; Abutaha, N.; Semlali, A. Cytotoxic Effects of Annona Squamosa Leaves against Breast Cancer Cells via Apoptotic Signaling Proteins. *J. King Saud. Univ.-Sci.* **2022**, *34*, 102013. [CrossRef]
30. Mbaveng, A.T.; Damen, F.; Çelik, İ.; Tane, P.; Kuete, V.; Efferth, T. Cytotoxicity of the Crude Extract and Constituents of the Bark of Fagara Tessmannii towards Multi-Factorial Drug Resistant Cancer Cells. *J. Ethnopharmacol.* **2019**, *235*, 28–37. [CrossRef]
31. Maroon, J.C.; Bost, J.W.; Maroon, A. Natural Anti-Inflammatory Agents for Pain Relief. *Surg. Neurol. Int.* **2010**, *1*, 80. [CrossRef]
32. Gonelimali, F.D.; Lin, J.; Miao, W.; Xuan, J.; Charles, F.; Chen, M.; Hatab, S.R. Antimicrobial Properties and Mechanism of Action of Some Plant Extracts against Food Pathogens and Spoilage Microorganisms. *Front. Microbiol.* **2018**, *9*, 1639. [CrossRef] [PubMed]
33. Radulovic, N.S.; Blagojevic, P.D.; Stojanovic-Radic, Z.Z.; Stojanovic, N.M. Antimicrobial Plant Metabolites: Structural Diversity and Mechanism of Action. *Curr. Med. Chem.* **2013**, *20*, 932–952. [CrossRef] [PubMed]
34. Odds, F.C.; Brown, A.J.P.; Gow, N.A.R. Antifungal Agents: Mechanisms of Action. *Trends Microbiol.* **2003**, *11*, 272–279. [CrossRef] [PubMed]
35. Sánchez-Hernández, E.; González-García, V.; Casanova-Gascón, J.; Barriuso-Vargas, J.J.; Balduque-Gil, J.; Lorenzo-Vidal, B.; Martín-Gil, J.; Martín-Ramos, P. Valorization of *Quercus suber* L. Bark as a Source of Phytochemicals with Antimicrobial Activity against Apple Tree Diseases. *Plants* **2022**, *11*, 3415. [CrossRef]
36. Mota, S.; Pinto, C.; Cravo, S.; Rocha e Silva, J.; Afonso, C.; Sousa Lobo, J.M.; Tiritan, M.E.; Cidade, H.; Almeida, I.F. Quercus Suber: A Promising Sustainable Raw Material for Cosmetic Application. *Appl. Sci.* **2022**, *12*, 4604. [CrossRef]
37. Gonçalves, F.; Correia, P.; Silva, S.P.; Almeida-Aguiar, C. Evaluation of Antimicrobial Properties of Cork. *FEMS Microbiol. Lett.* **2016**, *363*, fnv231. [CrossRef]
38. Hassikou, R.; Oulladi, H.; Arahou, M. Antifungal Activity of Quercus Suber Extracts on Trichophyton Rubrum and Candida Albicans. *Phytothérapie* **2014**, *12*, 206–212. [CrossRef]
39. Pantosti, A.; Venditti, M. What Is MRSA? *Eur. Respir. J.* **2009**, *34*, 1190–1196. [CrossRef]
40. Kourtis, A.P.; Hatfield, K.; Baggs, J.; Mu, Y.; See, I.; Epson, E.; Nadle, J.; Kainer, M.A.; Dumyati, G.; Petit, S. Vital Signs: Epidemiology and Recent Trends in Methicillin-Resistant and in Methicillin-Susceptible *Staphylococcus aureus* Bloodstream Infections—United States. *Morb. Mortal. Wkly. Rep.* **2019**, *68*, 214. [CrossRef]
41. Hsu, H.; Sheth, C.C.; Veses, V. Herbal Extracts with Antifungal Activity against Candida Albicans: A Systematic Review. *Mini Rev. Med. Chem.* **2021**, *21*, 90–117. [CrossRef]

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