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Quercus rotundifolia Bark as a Source of Polar Extracts: Structural and Chemical Characterization

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Abstract: *Quercus rotundifolia* bark was studied regarding anatomical, chemical, and antioxidant properties from trees in two sites in southern Portugal and are here reported for the first time. The general structure and anatomy of *Q. rotundifolia* bark showed a rhytidome with sequential undulated and anastomosed periderms with a small proportion of cork, while the phloem included broad rays with strong cell sclerification, groups of sclereids with embed large prismatic crystals, and abundant druses in parenchyma cells. The mean chemical composition was 15.5% ash, 1.6% dichloromethane extractives, 6.4% ethanol and 9.3% water extractives, 3.0% suberin, 30.5% total lignin, and 33.8% carbohydrates. Carbohydrates included mainly glucose (50.7% of total monomers) and xylose (23.8%), with uronic (3.0%) and acetic acids (1.0%). Suberin was mainly composed of ω-hydroxyacids (48.0% of all compounds) and α,ω-diacids (19.5%). The main compounds found in the lipophilic extracts were triterpenes (43.6%–56.2% of all compounds) and alkanoic acids (32.7%–41.7%). Phenolic content was high especially in the ethanol extracts, ranging from 219.5–572.9 mg GAE/g extract and comprising 162.5–247.5 CE/g extract of flavonoids and 41.2–294.1 CE/g extract of condensed tannins. The extracts revealed very good antioxidant properties with IC_{50} values of 4.4 µg ethanol extract/mL and 4.7 µg water extract/mL. Similar anatomical, chemical, and antioxidant characteristics were found in the bark from both sites. The high phenolic content and excellent antioxidant characteristics of polar extracts showed holm oak barks to be a promising natural source of antioxidants with possible use in industry and pharmaceutical/medical areas.

Keywords: holm oak; non-wood forest products; bark anatomy; polar extracts; bioactivity; antioxidants

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1. Introduction

The use of bark evolved from ancient times to present day, expanding according to the different socioeconomic contexts, as well as the scientific and technological advances. Barks show a large diversity and have a high chemical compound richness, namely regarding extractives such as sterols, terpenes, and a large number of different phenolic compounds, allowing application in medicine and pharmacy, adhesives, formaldehyde scavengers, and antioxidants.

Quercus rotundifolia Lam., generally known as holm oak ("azinheira" in Portuguese and "encina" in Spanish) due to its leaf's resemblance to *Ilex aquifolium* L. (the common European holly used in Christmas), is taxonomically complex and either recognized as a separate species or subspecies (*Q. ilex* subesp. *ballota* (Desf.) Samp. or *Q. ilex* subesp. *rotundifolia* (Lam.) O. Schwarz ex Table Morais) belonging to the subsection *Sclerophyllodrys* O. Schwartz [1,2]. It is naturally distributed in southern Europe (Portugal, south and southeast Spain) and northwestern Africa (mainly Morocco) in the western Mediterranean basin. *Quercus rotundifolia* is the main evergreen oak, besides *Q. suber*, which is characteristic of the Mediterranean typical agrosilvopastoral system in Portugal ("montado") and Spain ("dehesa"), that populates these savanna-like ecosystems. *Quercus rotundifolia* is found

in a wide variety of soils and its drought-tolerant capacity has been well emphasized in literature due to a xylem plasticity showing greater resistance than the co-occurring evergreen *Q. ilex* L. or *Q. ilex* subsp. *ilex* L. and deciduous *Q. faginea* species (e.g., [3,4]). Several ecophysiological and biochemical studies have referred to the seedling and root performances, the stomatal responses, and the antioxidant systems as combined strategies to increase drought-tolerance of the holm oak under the Mediterranean-type climate (e.g., [5–7]). Those studies are important for the species' conservation and sustainability due to climate change and the potential decline of this forest system. However, most of the studies have dealt with *Q. ilex* or *Q. ilex* subesp. *ilex*, which are morphologically and genetically distinct from *Q. rotundifolia* and differently distributed [1,8,9].

Most reports on *Q. rotundifolia* have been historically related with the establishment, management, and maintenance of the agrosilvopastoral system. Its ecological importance is well acknowledged, while the economic importance is mainly related with acorn production for animal grazing. In fact, the *Q. rotundifolia* acorns are described as the sweetest among oaks, already used since the 18th century in the Iberian Peninsula as nuts associated with poor diets, and now gaining high value as a source of consumable flour within the local economies [10]. Another main economic interest of *Q. rotundifolia*, one that has prevailed for a long time, is related with the high density and the excellent calorific properties of the wood for charcoal production and firewood uses, for which the wood obtained from thinning or pruning operations is almost exclusively used [11].

Considering *Quercus* barks as potential resources, the case of *Q. suber* bark is highlighted due to the extensive cork layer in its periderm, which allows for sustainable cork production with major economic importance [12]. Cork has a unique set of properties, given by the material's structural and chemical features, which are at the base of a dedicated industrial sector producing known cork products worldwide [13]. *Quercus cerris* [14] and *Q. variabilis* [15], among other cork rich species [16], are also potential sources of cork, even if structural inhomogeneity must be taken into consideration before processing. However, the barks of the majority of the other oaks are not cork-rich and are referred in the literature as polyphenol-rich materials, showing excellent antioxidative capacity, namely with antimicrobial, anti-inflammatory, and antitumoral properties. Yet, little has been studied for most of the species, with some exceptions for *Q. infectoria*, *Q. coccifera,* and *Q. crassifolia* [17]. In general, most interests are related with the bark phenolic compounds, such as flavonoids and tannins [18]. In fact, the first and ancient sources for tanning included *Quercus* spp. barks in western Europe, before being replaced by non-natural agents [1,19]. While for the most valued oaks, *Q. petraea* and *Q. robur,* from which wood is the main product, a high phenolic content in bark extracts was confirmed (e.g., [20–22]) and the potential of other lesser-known oaks, such as *Q. faginea,* has also been acknowledged [23]. The lipophilic content of bark is also of high interest due to its application in food- and medicine-related areas, and compounds such as sterols and triterpenes were also found, for example, in *Q. faginea* bark [23]. Furthermore, recent studies on the potential for biorefineries of oak barks highlighted *Q. rubra, Q. laurina,* and *Q. crassifolia* due to their chemical compositions (e.g., [24–26]). This diversity of organic and inorganic components is related with their structural and physiological functions, namely the phenol-based compounds for oak wood durability and biotic defense of oak trees [27].

For the valorization and assessment of the potential of barks, it is important to have specific information on their structure and anatomy, in the line of what has been studied by this research group for *Q. faginea* [28] and *Q. cerris* [29]. Regarding holm oak, its bark anatomy was not studied yet, with exceptions for the first works of the periderms of *Q. ilex* x *Q. suber* hybrids [30], describing a rhytidome with successive and thin phellems. Studies dealing with the chemical properties are not available, with the exception of studies for the bark of *Q. ilex* L. reporting its antibacterial activity [31] and suitability for pollution biomonitoring [32]. The bark potential of *Q. rotundifolia* has not yet been analyzed and, to our knowledge, the present study is the first to characterize, in detail, the holm oak bark structure, anatomy, chemical composition, and antioxidant properties, also calling

attention to the anatomical and chemical variability of the bark of different trees at two sites. These results will contribute to more knowledge-based decision-making on future *Q. rotundifolia* management within its natural and geographic distribution range as part of the *montado*.

2. Material and Methods

2.1. Sites and Sampling

The bark samples were obtained from *Q. rotundifolia* trees selected along the species' natural distribution in Portugal—at the Perímetro Florestal da Contenda (38°03′ N, 07°06′ W; 450 m altitude; site 1), a stand under the management of the public institute ICNF (Instituto da Conservação da Natureza e das Florestas), and at Mora (38°56′ N, 8°7′ W; 130 m; site 2), a privately owned stand. For each site, legal permission was given to the sampling by ICNF. At both sites, the holm oak trees (hereafter referred to *Q. rotundifolia*, except if the opposite is mentioned) are sparse, the stands are unevenly aged and holm oaks are mixed with the dominant *Q. suber* trees. At each site, five trees were randomly selected and were sampled during February 2018 at site 1 and during October 2017 at site 2. The sampled trees are characterized in Table 1. The trees showed the characteristic holm oak architecture with an average of 7 m of tree height, a clear stem below branching of 1.6 m, and a 26.5 cm diameter at breast height (b.h., i.e., at 1.30 m above ground). Stand year plantation was not known and annual rings were not easily distinguishable in stem cross-sections (data not published). However, tree age was approximately estimated at 50–60 years and deemed to be similar at both sites. A 2 cm thick cross-sectional disc was cut from each tree at b.h. Bark thickness was measured along two cross-diameters. The samples of bark were manually removed, air-dried, and separated in two sets, one for anatomical characterization of the wood and the other for chemical analysis.

Table 1. Characteristics of the studied *Quercus rotundifolia* trees from two sites (mean and standard deviation of five trees, min and max in parenthesis).

	Site 1	Site 2
Tree age (years) *	44 ± 14 (25–65)	49 ± 18 (25–65)
Total tree height (m)	7.2 ± 0.5 (6.7–7.7)	6.1 ± 2.1 (3.9–9.2)
Stem height (m)	1.6 ± 0.2 (1.4–1.8)	1.6 ± 0.4 (1.3–1.7)
Diameter (cm) **	27.0 ± 2.2 (24.4–29.4)	25.9 ± 6.9 (17.3–32.9)
Bark thickness (mm)	11.5 ± 2.5 (8.3–15.2)	11.7 ± 1.7 (9.9–14.5)
Rhytidome thickness (mm)	4.9 ± 1.2 (2.8–6.3)	5.4 ± 1.6 (2.5–6.7)

* Measured by ring counting at b.h. ** Diameter at b.h. with bark.

2.2. Cellular Structure Characterization

The macroscopic observations were made on the bark sample cross-section after surface sanding (P 1000) using a modular stereomicroscope (Leica MZ6, Leica Microsystems Ltd., Heerburg, Germany) coupled to a digital camera (Leica DC320, Leica Microsystems Ltd., Heerburg, Germany). For the microscopic observations, the bark samples were impregnated with polyethylene glycol (DP 1500), and transverse and longitudinal microscopic sections of approximately 17 µm thickness were prepared with a sliding microtome (Leica SM 2400, Leica Microsystems Nussloch GmbH, Nussloch, Germany) using adhesive for sample retrieval. The sections were stained with a double staining of chrysodine/astra blue and Sudan 4 was used for selective staining of suberin. Individual bark specimens were also macerated for observation. Slide preparation and maceration followed standard procedures described in previous works [28]. Phloem and rhytidome were measured at two random intact points using image analysis systems (Leica Qwin Pro, v 3.5.0). Qualitative and quantitative observations were made using light microscopy (Nikon Microphot-FXA, Nikon, Japan). Bark terminology followed the IAWA List of Microscopic Bark Features [33].

2.3. Chemical Summative Analysis

The bark samples of each tree were ground separately in a cutting mill (Retsch SM 2000, Retsch GmbH, Haan, Germany) using an output sieve of 10 mm × 10 mm, followed by one of 2 mm × 2 mm, and fractionated with a vibratory system with standard sieves (Retsch AS 200, Retsch GmbH, Haan, Germany). The 40–60 mesh (0.425–0.250 mm) fractions were used for chemical analysis. The summative chemical analysis included determination of ash; extractives in dichloromethane, ethanol, and water; suberin; Klason and acid-soluble lignin; and the monomeric composition of polysaccharides. Determinations were made in duplicate samples. The methods followed the procedures adopted in our laboratory for bark chemical characterization (e.g., [23,34]) and can be briefly described as follows. The ash content was determined by incinerating 2.0 g of each sample at 525 °C overnight and weighing the residue, reported as percentage of the original samples (Tappi 211 om-02). The extractives were determined with procedures adapted from Tappi 204 cm-97, performed in a Soxhlet system with dichloromethane, ethanol, and water under reflux successively, after which the content was calculated for each solvent by mass difference of the oven-dried solid residue and reported as a percentage of the original sample. The suberin content was determined by methanolysis for depolymerization using 1.5 g of the sample of extractive-free material and is expressed as a percentage of the initial dry mass [35]. The lignin content was analyzed from the extracted and desuberinized samples by acid hydrolysis. Klason lignin was determined as the mass of the solid residue after drying at 105 °C (Tappi T 222 om-02). The acid-soluble lignin was determined by measuring the UV absorbance (TAPPI Useful Method UM 250). The remaining acid solution was kept for sugar analysis. The carbohydrates were calculated based on the amount of the neutral sugar monomers (rhamnose, arabinose, xylose, galactose, mannose, and glucose) and uronic acids (galacturonic and glucuronic acids) released by total hydrolysis, after derivatization as alditol acetates and separation by high-pressure ion-exchange chromatography with a pulsed amperometric detector (HPIC-PAD). The content of acetic acid was also determined in the hydrolysate using high-pressure ion-exclusion chromatography with a UV/visible detector (HIPCE-UV).

2.4. Composition and Antioxidant Activity of Polar Extracts

The ethanol and water extracts were obtained by successive Soxhlet extraction and analyzed in relation to total phenolics (TPC), flavonoids (FC), and condensed tannin (TC) content, as previously described [23]. Each assay was performed at least three times and at least three independent replicates were prepared for each standard and sample. The Folin–Ciocalteu method was used for TPC determination, using gallic acid as standard, and the results were reported as mg gallic acid equivalents (GAE)/g of dried bark extract. The $AlCl_3$ colorimetric assay was used for the FC determination, using catechin as standard, with the results expressed as mg of catechin equivalents (CE)/g of dried bark extract. TC was determined by the vanillin-sulfuric acid assay, using catechin as standard, and the results are expressed as mg catechin equivalents (CE)/g of dried bark extract. The antioxidant activity of the ethanol and water extracts was determined using two methods—ferric reducing/antioxidant power (FRAP), which measures the sample's ferric reducing power, and 2,2-diphenyl-1-picryhydrazyl (DPPH), which measures the free radical scavenging capacity, as previously described [23]. FRAP is expressed as Mmol Trolox equivalents/g dry mass and the DPPH is expressed in terms of the amount of extract required to reduce 50% of the DPPH concentration (IC_{50}) and Trolox equivalents on a dry extract base (TEAC).

2.5. Composition of Lipophilic Extracts

Aliquots of the dichloromethane (DCM) extracts (5 mL) were taken, evaporated under N_2 flow, and dried at room temperature under vacuum overnight. For the *GC*-MS analysis, one aliquot from the DCM extracts (2 mg) was derivatized to trimethylsilyl ethers/esters (TMS) with 100 µL of pyridine (Sigma–Aldrich, St. Louis, MO, USA) and 100 µL of BSTFA (*N*,*O*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane, Sigma–Aldrich,

St. Louis, MO, USA) at 60 °C for 30 min. After cooling at room temperature, the extracts were injected in splitless mode in a GC-MS (Agilent 7890 A-5975C MSD, Santa Clara, CA, USA) with an autoinjector and a high-temperature capillary column (Zebron 5 H T, 30 m × 0.25 mm x 0.1 µm, Anaheim, CA, USA) using He as carrier gas at a constant flow of 1 mL/min. The injector temperature was 280 °C and the oven was programmed with an initial temperature of 100 °C (1 min), 10 °C/min to 250 °C (1 min), 8 °C/min to 350 °C (5 min), and 8 °C/min to 380 °C (5 min). The MS source conditions were MSD transfer-line temperature maintained at 330 °C, the MS source at 230 °C, the quadrupole at 150 °C, and the electron ionization energy at 70 eV. The electronic impact mass spectra (EIMS) were taken over a range of m/z 40–950. For semi-quantification analysis, the GC-MS was externally calibrated with standards (hexadecanoic acid and asiatic acid) that are representative of the major families of the lipophilic extracts (saturated fatty acids and triterpenes, respectively). The respective multiplication factors needed to acquire a correct quantification of the peak areas were calculated as an average of three GC-MS runs. The compounds are expressed as a % of each peak in TIC. Each aliquot was injected and duplicated. The identification of the compounds (as TMS derivatives) was based on comparisons with standards, Wiley 6 and NIST libraries data, and interpretation of mass spectrometric fragmentation patterns.

2.6. Composition of Suberin

Aliquots of the dichloromethane extracts (5 mL) from the suberin depolymerization reaction were taken, evaporated under N_2 flow, and dried at room temperature under vacuum overnight. For the GC-MS analysis, one aliquot from the DCM extracts (1 mg) was derivatized to trimethylsilyl ethers/esters (TMS) with 100 µL of pyridine (Sigma-Aldrich, St. Louis, MO, USA) and 100 µL of BSTFA (*N*,*O*-bis(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane, Sigma-Aldrich, St. Louis, MO, USA) at 60 °C for 30 min. The subsequent procedures are described above.

2.7. Statistical Analysis

All results are expressed as mean and standard deviation. The significance of differences ($p < 0.05$) among the corresponding mean values was determined by one-way analysis of variance (ANOVA) using the SPSS statistical software (version 26).

3. Results

3.1. Structure and Anatomy

The external appearance of the bark of *Q. rotundifolia* was finely square fissured. It was similar in the trees at both sites, with 11.6 mm mean width, including equally distributed rhytidome and secondary phloem (Table 1 and Figure 1).

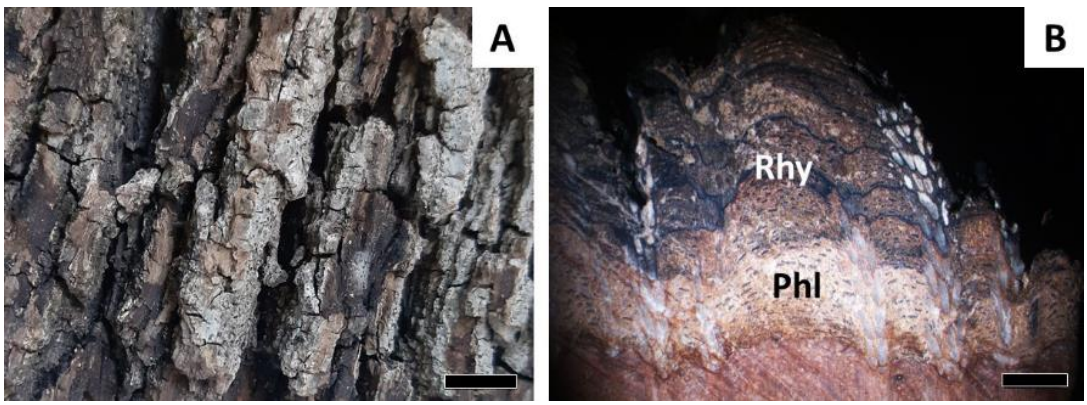

Figure 1. Bark structure of *Quercus rotundifolia*. (**A**) General aspects of the rhytidome; (**B**) phloem (Phl) and rhytidome (Rhy) on the transverse section. Scale bar: A = 1 cm; B = 2 mm.

The rhytidome of *Q. rotundifolia* was persistent, with 5.1 mm mean width (Table 1 and Figure 1), and composed of various periderms and dead secondary phloem between them (Figure 2A–C). Its qualitative anatomy did not differ among trees. The sequential periderms formed thin and undulated layers that occasionally anastomosed (Figure 2A); in each periderm, the phellem (cork) was poorly developed, comprising layers (<10 layers) of radially flattened cells arranged into a more or less distinct radial pattern with thin suberized walls or, sometimes, lignified thick walls. The phelloderm consisted of a few layers (1–3) of rectangular to round cells, also with radial alignment (Figure 2D). Both phellem and phelloderm cells may be filled with dark contents, presumably phenolic compounds. The phellogen, which gives rise to the phellem outside and phelloderm inside, is, in general, difficult to recognize but sometimes could be observed as rectangular and thin-walled cells in cross section (Figure 2D).

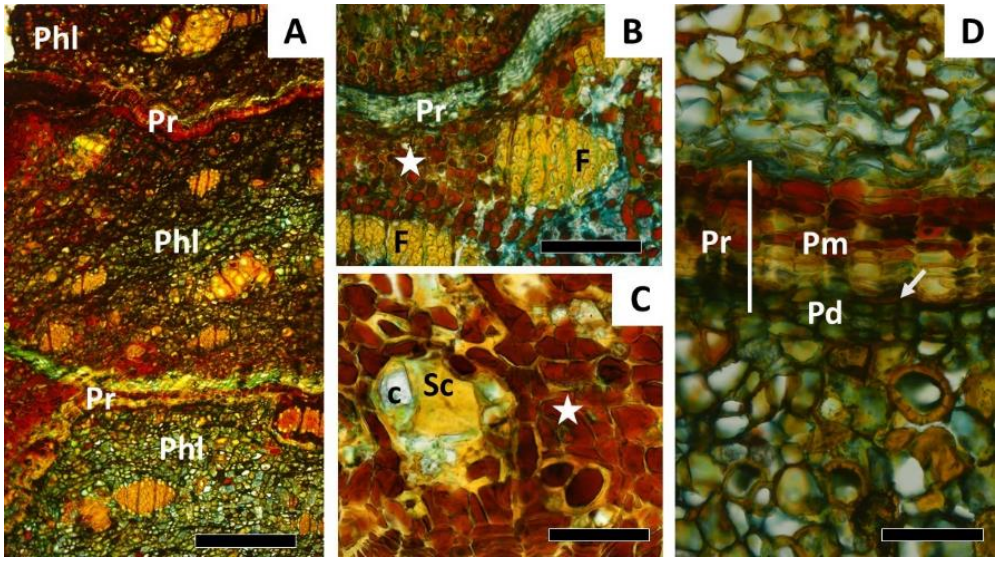

Figure 2. Transverse section of the bark of *Quercus rotundifolia*. (**A**) Rhytidome includes various periderms (Pr) and dead secondary phloem (Phl) between them; (**B**) portions of rhytidome—periderm (Pr), fibers (F), and parenchyma cells filled with extractives (asterisk); (**C**) sclereid with embedded crystal (c) and extractives in radial and axial parenchyma cells (asterisk); (**D**) a detailed view of the periderm with 7 cells of phellem (Pm) and 2–3 cells of phelloderm and the phellogen (arrow). Scale bar: A = 200 µm; B = 130 µm; C = 80 µm; D = 50 µm.

The secondary phloem (Figures 3–5) included the conducting and nonconducting phloem, with a gradual transition between these two layers. The phloem was nonlayered and the growth rings were difficult to recognize.

The conducting phloem near the vascular cambium represented a narrow portion of the entire phloem (Figure 3A) and consisted of the functional sieve tube elements and companion cells (conducting tissue), the axial parenchyma (storied tissue), rays (storage/transversal conduction), and fibers (mechanical support). The sieve tubes elements were thin, nonlignified walled cells with round to irregular shape in transverse view and were mostly tangentially or radially grouped (Figure 3D). The sieve tube elements had compound and inclined sieve plates and numerous lateral sieve areas (Figure 3E). The companion cells were recognized in transversal sections (Figure 3D). The axial parenchyma cells were nonlignified and thin-walled with a round shape of irregular size in transverse view and appeared as thin strands of a few cells located between fibers and interspersed with sieve elements (Figures 3D and 4B). The fibers were arranged in small groups as discontinuous tangential bands crossed by thin rays (Figure 2C); fibers were thick-walled and lignified, accompanied by chambered crystalliferous cells. (Figures 4A,B and 5A). The rays were comprised of two types (Figures 3A–C, 4B and 5A,B): uniseriate rays and broad rays (multiseriate, up to 20 cells wide and >100 cells high), both homocel-

lular with procumbent cells (Figure 5C). These broad rays often protrude into the xylem (Figure 3B). Sclerification of radial parenchyma cells occurred mostly in these fused broad rays (Figures 3A,B and 5B), early in the conducting phloem near the cambium (Figure 3B).

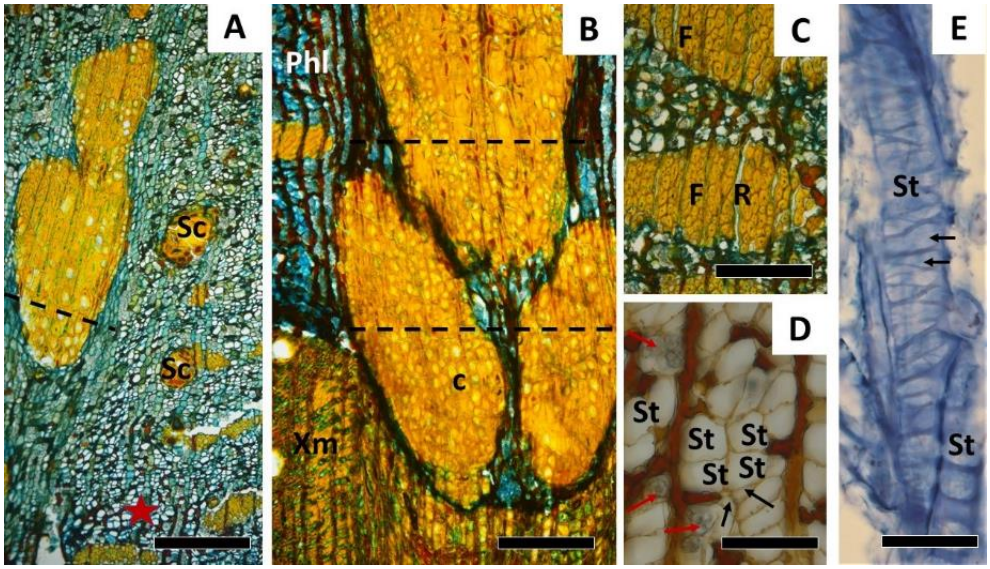

Figure 3. Transverse section of the secondary phloem of *Quercus rotundifolia*. (**A**) Nonconducting and conducting phloem (asterisk), large rays (—) with sclerified ray parenchyma cells; spherical groups of sclereids (Sc); (**B**) the broad rays (—) of the phloem (Phl) protrude into the xylem (Xm); crystals (c); (**C**) fibers (F) in tangential groups transversed by the thin rays (R); (**D**) sieve tube elements (St) with companion cells (black arrows); druses (red arrows); (**E**) sieve tube elements (St) with many lateral sieve areas (arrows). Scale bar: A = 200 µm; B = 200 µm; C = 130 µm; D = 50 µm; E = 50 µm.

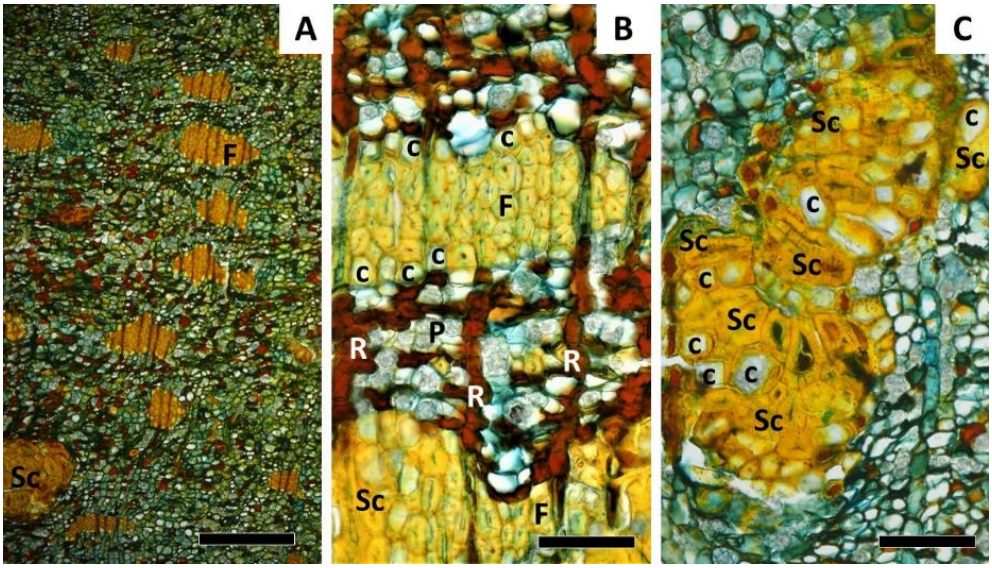

Figure 4. Transverse section of the nonconducting secondary phloem of *Quercus rotundifolia*. (**A**) Tangential groups of fibers (F) and groups of sclereids (Sc). (**B**) fibers (F) in tangential groups bordered by crystals (c); thin rays filled with extractives (R); nonlignified axial parenchyma cells (P) and sclereids (Sc). (**C**) Group of sclereids (Sc); these cells are strongly lignified with thickened walls and sometimes enclose a large prismatic crystal (c) and phenolic compounds (dark staining). Scale bar: A = 200 µm; B = 80 µm; C = 65 µm.

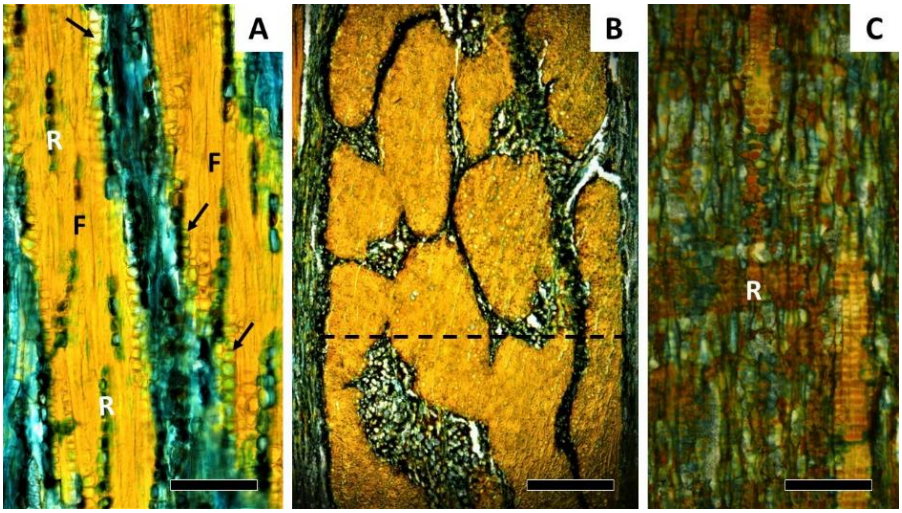

Figure 5. Longitudinal sections of secondary phloem of *Quercus rotundifolia* (tangential A,B and radial C, sections). (**A**) Uniseriate rays (R), fibers (F) bordered by a series of crystalliferous cells (arrows). (**B**) A large ray (—) with strongly lignified parenchyma cells. (**C**) Rays of homogeneous type (R) with procumbent cells filled with contents of brown color. Scale bar: A = 65 μm; B = 300 μm; C = 160 μm.

The nonconducting tissue started with the collapse of the sieve elements. At this point, the structure of the secondary phloem became disorganized with distortion and expansion of cells with subsequent sclerification, associated with the dilatation growth. Sclereids and fiber-sclereids (Figure 6A) with thick and heavily lignified cells walls appeared and often formed spherical or irregular prominent groups (Figures 3A and 6A–C), dispersed in the phloem adjacent to the fibers and near or within the broad rays. Abundant sclerification of parenchyma cells occurred in the outer portion of phloem near the inner periderm.

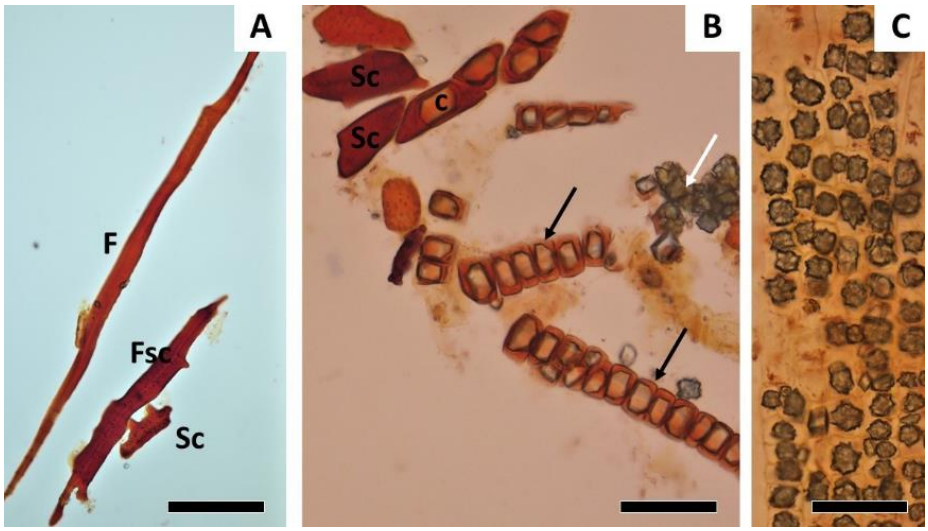

Figure 6. Individualized cells of the bark of *Quercus rotundifolia*. (**A**) Sclerenchyma tissue: secondary fibers (F), fibrosclereid (Fsc), and sclereid (Sc); (**B**) sclereid (Sc), sometimes with a prismatic crystal (c), series of parenchyma crystalliferous cells (black arrow), and druses (white arrow); (**C**) innumerous druses in nonlignified parenchyma cells. Scale bar: A = 80 μm; B = 50 μm; C = 50 μm.

Crystals, presumably of calcium oxalate, were abundant through all the bark, mostly present as abundant druses (Figures 3D and 6B,C) in axial parenchyma adjacent to sieve tube elements and as prismatic crystals in both sclereids (one large crystal/sclereid, Figures 2C, 4C and 6B) and axial parenchyma cells, e.g., crystalliferous parenchyma (Figure 6B), which bordered the fiber groups (Figures 4B and 5A). Abundant phenolic

compounds were observed by dark color staining in the secondary phloem and rhytidome e.g., axial and ray parenchyma cells, in the sclereids, phellem, and phelloderm cells (Figures 2B–D and 4A–C).

3.2. Chemical Composition

The summative chemical compositions of the *Q. rotundifolia* bark samples, from the two sites, are summarized in Table 2. The mean composition was (in % of the oven dry bark) 15.5% ash, 17.2% extractives, 3.0% suberin, 30.5% lignin, and 33.8% polysaccharides. *Q. rotundifolia* bark showed a high content of extractives of 15.1% (site 2) and 19.3% (site 1). The main contribution came from polar compounds solubilized by ethanol and water, representing 88% and 93% of the total extractives, respectively, for barks from trees of site 2 and 1; the non-polar compounds extracted by dichloromethane corresponded only to an average of 10.2% of the total extractives. Suberin content was low at 2.9% (site 2) and 3.1% (site 1), while the total lignin content was relatively high at 29.1% (site 1) and 31.8% (site 2). The ash content of *Q. rotundifolia* bark samples was particularly high. Between sites, a statistically significant difference was detected only in the content of dichloromethane and ethanol extractives.

Table 2. Chemical composition (% of o.d. mass) of *Quercus rotundifolia* bark sampled at b.h. from two sites (mean of five trees and standard deviation).

	Site 1	Site 2	*p*-Value
Ash	14.9 ± 3.1	16.0 ± 2.3	0.343
Extractives, total	19.3 ± 3.5	15.1 ± 0.8	0.001
Dichloromethane	1.3 ± 0.3	1.8 ± 0.3	0.001
Ethanol	9.1 ± 3.5	3.7 ± 2.1	0.000
Water	8.9 ± 1.0	9.6 ± 1.5	0.257
Suberin	3.1 ± 0.4	2.9 ± 0.6	0.440
Lignin, total	29.1 ± 3.5	31.8 ± 3.6	0.085
Klason lignin	25.9 ± 3.6	28.6 ± 3.7	0.092
Soluble lignin	3.3 ± 0.2	3.3 ± 0.3	0.985
Polysaccharides *	33.6 ± 2.5	34.2 ± 2.6	0.157

* by difference.

The carbohydrate composition is summarized in Table 3 in regards to the proportion of neutral monosaccharides, acetates, and uronic acids. The major monosaccharide was glucose, corresponding to 52% and 49% of the total monomers, while xylose was the dominant non-cellulosic sugar with 24%; arabinose, galactose, and rhamnose were also present (9.3%, 5.4%, and 3.3%, respectively). Uronic acids were also present, representing 5.9% of the total content of monomers as well as acetyl groups (1.0%). The carbohydrate composition was similar at both sites.

Table 3. Composition of polysaccharides (% of total monomers) determined after acid hydrolysis as neutral monosaccharides and uronic and acetic acids of *Quercus rotundifolia* bark sampled at b.h. from two sites (mean of five trees and standard deviation).

	Site 1	Site 2
Rhamnose	2.8 ± 0.6	3.7 ± 0.9
Arabinose	8.9 ± 0.8	9.6 ± 2.1
Galactose	5.2 ± 0.4	5.6 ± 1.2
Glucose	51.9 ± 1.8	49.4 ± 2.5
Xylose	24.0 ± 1.9	23.5 ± 3.0
Mannose	0.7 ± 0.2	0.9 ± 0.6
Galacturonic acid	5.3 ± 0.6	6.3 ± 1.0
Glucuronic acid	0.02 ± 0.04	0.2 ± 0.1
Acetyl groups	1.1 ± 0.1	0.8 ± 0.2

3.3. Polar Extracts Composition

The results for TPC, FC, and TC, as well as the antioxidant activity of the extracts obtained successively with ethanol and water, are shown in Table 4. The ethanol extracts contained much higher values of phenolics, flavonoids, and tannins than the subsequent water extracts. The bark ethanol extracts had a high proportion of phenolic compounds (572.8 mg GAE/g extract; 3.7 g GAE/g dry bark), in which flavonoids and condensed tannins constituted the major classes (247.6 mg CE/g extract and 294.1 mg CE/g extract, respectively). The bark water extracts contained a much lower amount of phenolic compounds: total phenolics 219.5 mg GAE/g of extract, flavonoids 162.5 mg CE/g of extract, and condensed tannins 41.2 mg CE/g of extract. Significant differences were found between sites for the ethanol and water extracts.

Table 4. Composition and antioxidant capacity of ethanol and water extracts of *Quercus rotundifolia* bark sampled at b.h. of trees in two sites (mean of five trees and standard deviation).

	Site 1	**Site 2**	***p*-Value**
Ethanol extractives			
Total phenolics (mg GAE/g extract)	561.7 ± 77	583.9 ± 58.6	0.222
Total flavonoids (mg CE/g extract)	173.8 ± 69.6	321.3 ± 28.7	0.000
Condensed tannins (mg CE/g extract)	115.8 ± 35.6	472.3 ± 189.3	0.000
Antioxidant capacity (mg TEAC/g extract)	696.9 ± 97.4	1390.6 ± 515.3	0.000
IC_{50} values (μg extract/mL) *	5.8 ± 0.9	2.9 ± 1.2	0.000
FRAP (mM TEAC/g extract)	2.25 ± 0.92	6.33 ± 3.34	0.001
Water extractives			
Total phenolics (mg GAE/g extract)	253.7 ± 26	185.3 ± 40.2	0.001
Total flavonoids (mg CE/g extract)	133.9 ± 31	191.0 ± 60	0.021
Condensed tannins (mg CE/g extract)	44.3 ± 12.1	38.1 ± 7.4	0.177
Antioxidant capacity (mg TEAC/g extract)	1109.6 ± 268.8	770.9 ± 247	0.003
IC_{50} values (μg extract/mL) *	3.4 ± 0.9	5.9 ± 1.9	0.001
FRAP (mM TEAC/g extract)	1.54 ± 0.25	1.13 ± 0.48	0.028

GAE: gallic acid equivalents; CE: catechin equivalents; TEAC: Trolox equivalent antioxidant activity; * IC_{50} Trolox in ethanol and water 3.3 and 3.6 μg Trolox/mL, respectively.

The radical scavenging activities of the bark extracts corresponded to IC_{50} values of 4.4 μg extract/mL and 4.7 μg extract/mL, respectively, for the ethanol and water extracts. The reducing ability of the ethanol extracts by the FRAP assay was, on average, 4.29 mM Trolox/g of extract, while that of the water extracts was on average 1.34 mM Trolox/g of extract. The antioxidant capacity and the reducing ability of the polar extracts were significantly different between sites.

3.4. Lipophilic Extract Composition

The compositions of the lipophilic extractives are presented in Table 5. Triterpenes and alkanoic acids were the major identified chemical classes, representing 43.7%–56.2% and 32.7%–41.7% of the total, respectively. Ursolic acid, betulinic acid, betulin, and oleanolic acid constituted the major triterpenes found; in the lipophilic extracts from *Q. rotundifolia* bark collected at site 1, ursolic, betulinic and oleanolic acids were much more common (19.8%, 18.8%, and 7.5%, respectively), whereas betulin existed in higher amounts in the lipophilic extracts from *Q. rotundifolia* bark collected at site 2 (10.3%). Among the alkanoic acids, the saturated acids predominated over the substituted acids, with, respectively, 23.7%–31.2% and 9.0%–10.4% of all compounds. Extracts from *Q. rotundifolia* barks from site 2 showed higher content in alkanoic acids (and lower triterpenes), and the major identified compounds were the hexadecenoic, docosanoic, and tetracosanoic acids, representing 10.6%, 6.5%, and 5.2%, respectively. Sterols represented between 2.6% and 7.4% of all compounds, whereas β-sitosterol was the most representative compound. Aromatics, alkanols, saturated α,ω-diacids, and ω-hydroxyacids were also present, but in small

amounts (below 1.4%). Overall, 84.4% to 99.3% of the compounds were identified in the lipophilic *Q. rotundifolia* bark lipophilic extracts.

Table 5. Composition of dichloromethane extracts from *Quercus rotundifolia* bark sampled at b.h. from trees grown at two sites, in % of the normalized peak areas in TIC (mean of five trees and standard deviation).

Compounds	Site 1	Site 2
Alkanols	0.91 ± 0.35	1.48 ± 0.50
hexadecanol	0.03 ± 0.06	0.17 ± 0.05
octadecanol	0.00 ± 0.00	0.01 ± 0.03
docosanol	0.36 ± 0.17	0.60 ± 0.22
tetracosanol	0.37 ± 0.16	0.41 ± 0.11
heptatriacotanol	0.15 ± 0.17	0.28 ± 0.35
Saturated alkanoic acids	23.72 ± 7.86	31.24 ± 10.21
decanoic acid	0.00 ± 0.00	0.02 ± 0.04
dodecanoic acid	0.05 ± 0.05	0.19 ± 0.06
tetradecanoic acid	0.61 ± 0.31	2.38 ± 1.20
pentadecanoic acid	0.01 ± 0.03	0.07 ± 0.07
hexadecanoic acid	9.68 ± 3.65	11.63 ± 4.99
heptadecanoic acid	0.01 ± 0.03	0.08 ± 0.08
octadecanoic acid	1.55 ± 0.46	2.34 ± 0.85
eicosanoic acid	0.26 ± 0.09	0.26 ± 0.13
heneicosanoic acid	0.10 ± 0.07	0.17 ± 0.11
docosanoic acid	5.14 ± 1.75	5.25 ± 2.93
tricosanoic acid	0.22 ± 0.07	0.46 ± 0.20
pentacosanoic acid	0.00 ± 0.01	0.07 ± 0.10
tetracosanoic acid	5.53 ± 2.59	7.54 ± 2.41
hexacosanoic acid	0.55 ± 0.42	0.79 ± 0.33
Substituted alkanoic acids	8.95 ± 6.20	10.43 ± 5.63
trans-9-hexadecenoic acid	0.01 ± 0.03	0.09 ± 0.09
cis-9-hexadecenoic acid	0.09 ± 0.06	0.08 ± 0.13
9,12-octadecadienoic acid	3.37 ± 2.43	3.22 ± 1.85
cis-9-octadecenoic acid	5.38 ± 5.23	6.95 ± 3.57
trans-9-octadecenoic acid	0.10 ± 0.11	0.09 ± 0.11
Saturated α,ω-diacids	0.00 ± 0.00	0.10 ± 0.14
nonanedioic acid	0.00 ± 0.00	0.09 ± 0.12
hexadecanedioic acid	0.00 ± 0.00	0.01 ± 0.02
Saturated ω-hydroxyacids	0.10 ± 0.16	0.27 ± 0.21
22-hydroxydocosanoic acid	0.10 ± 0.16	0.27 ± 0.21
Sterols	2.61 ± 0.98	7.39 ± 1.30
β-sitosterol	2.46 ± 0.90	6.84 ± 1.26
3,5-stigmastadien-7-one	0.02 ± 0.04	0.12 ± 0.17
stigmasterol	0.02 ± 0.04	0.04 ± 0.08
4-stigmasten-3-one	0.11 ± 0.11	0.32 ± 0.07
sitosteryl-3β-D-glucopiranoside	0.00 ± 0.00	0.07 ± 0.14
Terpenes/Terpenoids	56.21 ± 9.15	43.66 ± 14.23
farnesol	0.01 ± 0.02	0.00 ± 0.00
β-amyrin	0.01 ± 0.02	0.24 ± 0.53
lupenone	0.30 ± 0.43	0.30 ± 0.24
α-amyrin	0.02 ± 0.04	0.00 ± 0.00
lupeol	1.46 ± 1.15	3.10 ± 3.10
erythrodiol	2.22 ± 2.12	1.09 ± 1.40
friedelin	1.84 ± 4.01	5.01 ± 8.59
friedelan-3-ol	0.60 ± 0.84	0.11 ± 0.24
betulin	1.74 ± 3.54	10.33 ± 10.71

Table 5. *Cont.*

Compounds	Site 1	Site 2
oleanolic acid	7.48 ± 4.20	4.06 ± 1.72
ursolic acid	19.85 ± 12.15	8.22 ± 4.32
betulinic acid	18.80 ± 6.07	9.58 ± 5.80
betulinic aldehyde	0.27 ± 0.19	0.52 ± 0.35
hederagenin	0.35 ± 0.71	0.08 ± 0.12
maslinic acid	0.93 ± 1.05	0.45 ± 0.49
echinocystic acid	0.29 ± 0.32	0.59 ± 0.89
corosolic acid	0.04 ± 0.08	0.00 ± 0.00
Glycerol derivatives	0.60 ± 0.24	2.23 ± 0.63
2-monopalmitin	0.00 ± 0.00	0.01 ± 0.03
1-monopalmitin	0.28 ± 0.14	1.23 ± 0.52
1-monoolein	0.12 ± 0.14	0.07 ± 0.10
1-monostearin	0.14 ± 0.05	0.42 ± 0.31
docosyl glycerol	0.02 ± 0.04	0.16 ± 0.10
tetracosyl glycerol	0.04 ± 0.05	0.34 ± 0.18
Aromatics/Phenolics	0.56 ± 0.39	1.13 ± 0.70
3-hydroxybenzoic acid	0.04 ± 0.09	0.14 ± 0.13
vanillic acid	0.00 ± 0.00	0.02 ± 0.05
caffeic acid	0.42 ± 0.27	0.74 ± 0.64
docosyl ferulate	0.06 ± 0.10	0.13 ± 0.11
tetracosyl ferulate	0.04 ± 0.07	0.10 ± 0.10
Identified	93.65 ± 5.63	97.91 ± 1.01
Non-identified	6.35 ± 5.63	2.09 ± 1.01
Total	100	100

3.5. Suberin Composition

The results for the suberin composition of *Q. rotundifolia* bark obtained by GC–MS analysis are summarized in Table 6. The main suberin monomers were fatty acids, representing 85.2% and 83.7% of all compounds, from the samples collected at site 1 and site 2, respectively. Among them, ω-hydroxyacids and α,ω-diacids were the major compounds, representing, on average, 48% and 20%, respectively. The most abundant compounds were 9,10,18-trihydroxyoctadecanoic acid, 18-hydroxy-9-octadecenoic acid, 22-hydroxydocosanoic acid, and 9,10-dihydroxyoctanedioic acid, with 9.1%–15.7%, 13.4%–13.7%, 9.4%–10.1%, and 5.4%–7.5% of all compounds, respectively. Alkanoic acids (saturated and substituted) constituted 14.0% to 19.1% of all compounds, aromatics represented between 6.7% and 9.0%, and alkanols were around 4.4%. Trace amounts of dehydroabietic acid and β-sitosterol were also identified. In all suberin extracts, compound identification reached between 94.7% to 98.8% of all compounds.

Table 6. Suberin composition from *Quercus rotundifolia* bark sampled at b.h. from trees grown at two sites, in % of the normalized peak areas in TIC (mean of five trees and standard deviation).

Compounds	Site 1	Site 2
Alkanols	4.60 ± 0.79	4.25 ± 1.02
octadecanol	0.16 ± 0.06	0.20 ± 0.16
docosanol	1.13 ± 0.14	1.45 ± 0.35
tetracosanol	3.05 ± 0.61	2.35 ± 0.72
hexacosanol	0.04 ± 0.01	0.02 ± 0.03
3-(2-methoxyethyl)-1-octanol	0.02 ± 0.01	0.00 ± 0.00
18-methylnonadecanol	0.08 ± 0.01	0.12 ± 0.06
falcarinol	0.12 ± 0.06	0.11 ± 0.05
Saturated alkanoic acids	15.26 ± 1.30	11.40 ± 0.84

Table 6. *Cont.*

Compounds	Site 1	Site 2
octanoic acid	0.07 ± 0.01	0.04 ± 0.01
tetradecanoic acid	0.03 ± 0.01	0.01 ± 0.02
hexadecanoic acid, methyl ester	0.49 ± 0.17	0.38 ± 0.17
hexadecanoic acid	0.42 ± 0.16	0.38 ± 0.19
octadecanoic acid	0.04 ± 0.01	0.04 ± 0.02
octadecanoic acid, methyl ester	0.08 ± 0.02	0.05 ± 0.03
eicosanoic acid, methyl ester	0.13 ± 0.12	0.08 ± 0.04
docosanoic acid, methyl ester	2.39 ± 1.02	1.63 ± 0.53
tetradecanoic acid, methyl ester	0.30 ± 0.09	0.20 ± 0.06
tetradecanoic acid	1.04 ± 0.22	0.83 ± 0.14
docosanoic acid	1.52 ± 0.20	0.39 ± 0.24
tetracosanoic acid, methyl ester	7.36 ± 1.70	6.42 ± 0.67
tetracosanoic acid	0.51 ± 0.48	0.59 ± 0.34
eicosanoic acid	0.69 ± 0.46	0.34 ± 0.33
hexacosanoic acid	0.20 ± 0.38	0.01 ± 0.03
Substituted alkanoic acids	3.80 ± 1.12	2.38 ± 1.39
9,12-octadecadienoic acid, methyl ester	0.06 ± 0.01	0.06 ± 0.02
9-octadecenoic acid (Z)-, methyl ester	0.28 ± 0.04	0.20 ± 0.08
cis-9-octadecenoic acid	0.08 ± 0.03	0.08 ± 0.03
9,12-octadecadienoic acid	1.69 ± 1.35	1.52 ± 1.68
9,10-epoxyoctadecanoic acid, methyl ester	1.23 ± 0.63	0.25 ± 0.25
9-decenoic acid	0.45 ± 0.07	0.26 ± 0.17
Saturated α,ω-diacids	10.83 ± 3.24	8.02 ± 2.33
octanedioic acid	0.05 ± 0.00	0.05 ± 0.02
hexadecanedioic acid, dimethyl ester	4.97 ± 1.51	4.00 ± 1.36
eicosandioic acid, dimethyl ester	1.79 ± 2.90	0.45 ± 0.15
octanedioic acid, dimethyl ester	3.71 ± 0.65	3.27 ± 1.00
hexadecanedioic acid	0.08 ± 0.03	0.06 ± 0.04
dodecanedioic acid	0.11 ± 0.10	0.00 ± 0.00
docosanedioic acid, dimethyl ester	0.04 ± 0.04	0.13 ± 0.18
heptanedioic acid, methyl ester	0.01 ± 0.01	0.00 ± 0.00
octanedioic acid, methyl ester	0.06 ± 0.05	0.05 ± 0.05
Substituted α,ω-diacids	8.51 ± 2.66	11.31 ± 2.79
18-methoxyoctanedioic acid	0.84 ± 0.71	0.86 ± 0.58
9,10-dihydroxyoctanedioic acid, dimethyl ester	5.44 ± 1.07	7.63 ± 1.22
8,9,18-trihydroxyoctanedioic acid, methyl ester	0.99 ± 0.96	1.86 ± 1.65
16-hydroxyhexanedioic acid, methyl ester	1.23 ± 0.89	0.95 ± 0.69
Saturated ω-hydroxyacids	19.34 ± 0.95	18.81 ± 8.44
16-hydroxyhexadecanoic acid, methyl ester	3.08 ± 1.11	2.65 ± 0.61
16-hydroxydecanoic acid	0.12 ± 0.11	0.12 ± 0.10
20-hydroxyeicosanoic acid, methyl ester	1.08 ± 0.61	0.79 ± 0.29
20-hydroxyeicosanoic acid	0.03 ± 0.03	1.95 ± 4.36
22-hydroxydocosanoic acid, methyl ester	8.96 ± 1.22	8.72 ± 5.00
22-hydroxydocosanoic acid	0.46 ± 0.51	0.57 ± 0.48
24-hydroxytetracosanoic acid, methyl ester	5.28 ± 1.63	3.75 ± 2.27
24-hydroxytetracosanoic acid	0.32 ± 0.30	0.26 ± 0.24
Substituted ω-hydroxyacids	27.46 ± 4.05	30.55 ± 7.86
18-hydroxy-9-octadecenoic acid, methyl ester	13.70 ± 1.72	13.76 ± 1.42
9,10-epoxy-18-hydroxyoctadecanoic acid	0.05 ± 0.07	0.07 ± 0.07
9,10,18-trihydroxyoctadecanoic acid, methyl ester	9.14 ± 0.70	14.93 ± 6.63
9,10-epoxy-18-hydroxyoctadecanoic acid, methyl ester	4.57 ± 3.11	1.79 ± 1.07
Sterols	0.01 ± 0.02	0.03 ± 0.04
β-sitosterol	0.01 ± 0.02	0.03 ± 0.04

Table 6. *Cont.*

Compounds	Site 1	Site 2
Terpenes/Terpenoids	0.02 ± 0.02	0.03 ± 0.03
dehydroabietic acid	0.02 ± 0.02	0.03 ± 0.03
Aromatics/Phenolics	8.96 ± 1.50	6.98 ± 1.33
4-methoxybutanoic acid	0.04 ± 0.02	0.01 ± 0.02
4-hydroxy-benzoic acid, methyl ester	0.03 ± 0.02	0.03 ± 0.03
3,5-bis(1,1-dimethylethyl)-4-hydroxy-benzoic acid, ethyl ester	0.32 ± 0.16	0.13 ± 0.06
2,5-dihydroxypyrazine	0.05 ± 0.01	0.02 ± 0.03
3,4-dihydroxybenzoic acid, methyl ester	0.25 ± 0.43	0.04 ± 0.05
vanillic Acid	0.07 ± 0.01	0.07 ± 0.03
isovanillic acid	0.27 ± 0.09	0.29 ± 0.35
coniferyl aldehyde	0.07 ± 0.03	0.04 ± 0.05
3,5-dimethoxy-4-hydroxybenzoic acid	0.02 ± 0.01	0.03 ± 0.02
2,5-dimethoxymandelic acid	0.06 ± 0.02	0.07 ± 0.05
ferulic acid, methyl ester	4.25 ± 1.40	4.64 ± 0.94
ferulic acid	3.53 ± 0.64	1.60 ± 0.67
Identified	98.79 ± 0.51	93.77 ± 3.59
Non-identified	1.21 ± 0.51	6.23 ± 3.59
Total	100	100

4. Discussion

4.1. Structure and Anatomy

The finely square fissured external appearance of the bark of *Q. rotundifolia* (Figure 1) is in accordance with the available description [36]. The bark is quite different from that of other oaks, and its grey to dark color is also a distinctive characteristic. Bark thickness is highly variable within the species, namely in relation to tree age; a similar bark thickness (2–6 mm) was reported at 1.5–1.8 m of stem height for 50–100 years old trees [32]. The holm oak bark thickness and the rhytidome were narrower compared to *Q. petraea* (10.5–29.1 mm and 3.7–23.3 mm, respectively) [37] and similar to *Q. faginea* (5 mm to 14 mm) [28]. The anatomical features of the secondary phloem of the bark of *Q. rotundifolia* (Figures 3A–E, 4A–C, 5A–C and 6A–C) were similar to those of other *Quercus* species: *Q. robur* [38,39], *Q. sessiliflora* [40], *Q. petraea* [37,41], *Q. faginea* [28], *Q. cerris* [29], *Q. infectoria*, *Q. alnifolia*, and *Q. rubra* [36], and other ten *Quercus* species [42]. The main similarities were in the type of rays (uni/multiseriate), the strong sclerification of cells in the broad multiseriate rays, the occurrence and formation of large groups or clusters of sclereids, and the location of crystals (prismatic crystals and druses). The pattern of rhytidome development with thin sequential periderms in *Q. rotundifolia* (Figure 2A) was similar to that in various other *Quercus* species, leading to a low proportion of phellem in these barks (Figure 2A,B,D) and corroborating early findings from holm oak growing in Portugal [30]. Among oaks, one exception is the cork-rich bark of *Q. cerris*, which may have a substantial proportion of phellem in the rhytidome [29], and *Q. suber* [13] and *Q. variabilis* [15,43], with only one periderm with a continuous and thick cork layer.

4.2. Chemical Composition

The high extractives content and their substantial polar fraction were in line with the anatomical observations of phenolic deposits in the secondary phloem and rhytidome cells (Figures 1B–D and 3A–C). This result aligns with results found in barks of other *Quercus* spp., such as *Q. faginea* (total extractives 13.2%, with an 85.6% proportion of ethanol and water extractives) [23], *Q. rubra* (12.1%, with 9.4% of ethanol–water extract) and *Q. robur* (23.0%, with 21.9% of ethanol–water extract) [26], *Q. laurina* (14.2% hot water and 13.6% ethanol extractives), *Q. crassifolia* (20.7% hot water and 11.0% ethanol extractives), and *Q. scytophylla* (6.8% hot water and 4.4% ethanol extractives) [44], or *Q. laurina* (19.3%

total extractives) and *Q. crassifolia* (12.7% total extractives) [25]. The polar extractives content was particularly high at site 2 and the significant differences between sites might be related with the effect of growth season sampling time and/or drought on the formation of these secondary metabolites. In a recent study, Leite et al. [16] analyzed the effect of drought on cork chemical composition and showed that drought enhanced the amount of the polar extractives soluble in ethanol.

The *Q. rotundifolia* bark was low in suberin (about 3%), a value that fit well with the small amount of phellem tissue in the periderms of in the rhytidome (Figure 2A,B,D). Suberin is the chemical fingerprint of phellem (cork) cells and is not present in other cell types. Therefore, the suberin content is low in barks with little cork development, as shown in other *Quercus* spp., e.g., *Q. faginea* (3.9%) [23], *Q. rubra* (3.7%), and *Q. robur* (3.9%) [26]. Barks with a substantial proportion of cork have a high content of suberin, e.g., *Q. laurina* (26.6%, in outer bark) and *Q. crassifolia* (20.1%, in outer bark) [25]. For the cork tissue, the suberin content is higher, e.g., *Q. suber* (44.8%) [13], *Q. variabilis* (39.2%) [15], *Q. cerris* (28.5%) [45].

The lignin was relatively high (29%, Table 2), which was in agreement with the thick-walled lignified fibers, sclereids, and fiber-sclereids observed and described above. The bark lignin content was similar to that of *Q. faginea* bark (28.2%) [23], *Q. rubra* bark (32.8%) and *Q. robur* bark (29.1%) [26], *Q. laurina* outer bark (36.9%), and *Q. crassifolia* outer bark (39.6%) [25]. The carbohydrate composition (Table 3) showed a predominance of cellulose and of xylan-based hemicelluloses of the arabino-xylose type, with significant contents of galacturonic acid, rhamnose, and acetyl groups (the sum of xylose, arabinose, galacturonic acid, rhamnose, and acetyl represented 43% of the monomers). The polysaccharide monomeric composition was similar to that reported for barks of *Q. faginea* [23] and *Q. cerris* [45], as well as for other species from different taxon, such as *Betula pendula* [46].

The ash content was very high (Table 2), which is in accordance with the abundant crystals observed mostly in sclereids and in axial parenchyma adjacent to fiber and sieve tube elements.

4.3. Polar Extract Composition

The bark of *Q. rotundifolia* was very rich in phenolic compounds that may be solubilized by polar solvents. When considering the extraction of polar compounds successively with ethanol and water, the results showed that most of the phenolics were extracted by ethanol (Table 4), mostly constituted by flavonoids (247.4 mg CE/g extract) and condensed tannins (294.1 mg Ceq/g extract). The subsequent water extraction solubilized the remaining phenolic compounds with much lower proportions of flavonoids (162.5 mg Ceq/g extract) and condensed tannins (41.2 mg Ceq/g extract). Similar reports on high TPC have been given in the literature for *Quercus* spp. bark polar extracts, even if the extraction procedures differed. For ethanol:water extracts (50:50), Ferreira et al. [23] reported for *Q. faginea* bark 630.3 mg GAE/g of extract of total phenols, 207.7 mg CE/g of extract of flavonoids and 220.7 mg CE/g of extract of condensed tannins, and Sillero et al. [26] for *Q. rubra* and *Q. robur* 276.5–610.6 mg GAE/g of extract of total phenolics and 650.4–1021.8 mg CE/g of extract of flavonoids. Valencia-Avilés et al. [44] compared the ethanol and hot water extraction for *Q. laurina*, *Q. crassifolia,* and *Q. scytophylla* barks and reported high TPC (from 329 to 756 mg GAE/g extract) but lower flavonoids (12.9 to 25.4 mg QE, (quercetin)/g extract) and condensed tannins (12.6 to 53.5 mg CChE (cyanidin chloride equivalents)/g extract). For methanol:water extracts of *Q. suber* cork, Santos et al. [47] reported 200–350 GAE/g of extract of phenolics.

The *Q. rotundifolia* bark extracts showed very good antioxidant properties (Table 4). The IC_{50} values demonstrated this high antioxidant activity (IC_{50} = 4.4 µg ethanol extract/mL and 4.7 µg water extract/mL) when compared to the antioxidant standard Trolox (IC_{50} = 3.3 and 3.6 µg Trolox/mL, in ethanol and water, respectively). Sillero et al. [26] and Ferreira et al. [23] reported very similar antioxidant properties of ethanol:water bark extracts of *Q. rubra* and *Q. robur* (399.62 mg TEAC/g extract and 1521.25 mg TEAC/g ex-

tract, respectively) and *Q. faginea* (1576.12 mg TEAC/g extract; IC_{50} = 2.63 µg extract/mL). Santos et al. [47] reported for *Q. suber* cork an IC_{50} value of 2.8 µg water extract/mL, 3.6 µg methanol extract/mL and 5.8 µg methanol–water extract/mL. The results of the FRAP assay follow a similar trend to the one observed in the results obtained for DPPH. The ethanol extracts showed higher FRAP activity than the water extracts with 4.3 mM Trolox/g of extract and 1.3 mM Trolox/g of extract, respectively. In a sequential extraction, water extracts had lower antioxidant properties than ethanol extracts, but they still had compounds with antioxidant capability that were not solubilized in ethanol. The reducing ability by FRAP of both extracts was similar to the values of 4.44 mM TEAC/g of extract for *Q. faginea* bark ethanol:water (50:50) extracts [23].

4.4. Lipophilic Extracts Composition

The compositions of lipophilic extractives of *Q. rotundofolia* bark are shown in Table 5. The main constituents were triterpenes (49.9% of all compounds), namely ursolic, betulinic, and oleanolic acids, as well as betulin, with quantitative differences between the two sites. At site 1, the triterpenic acids were much more relevant than betulin, with ursolic, betulinic, and oleanolic acids constituting 35.2%, 33.4%, and 13.3% of all triterpenes, respectively, whereas betulin was only found in small amounts (3.1% of all triterpenes). However, the opposite was observed in the *Q. rotundifolia* bark samples collected at site 2—oleanolic acid was found in smaller amounts (only 9.3% of all triterpenes) and ursolic acid comprised 18.8% of all triterpenes identified, whereas betulinic acid and betulin represented 21.9% and 23.7% of all triterpenes, respectively. Other *Quercus* species showed different lipophilic compositions, e.g., *Q. suber* cork also included triterpenes belonging to friedelane, lupine, and steroid families, as well as long alkanoic chains [48], but in different amounts; *Q. cerris* cork also showed high amounts of botulin [49]; and *Q. variabilis* cork also showed a high triterpenic content, representing around 52.0% of all compounds [43]. Friedelin was found in a fair quantity in the dichloromethane extracts of *Q. rotundofolia* bark (between 1.8% and 5.0%), which was slightly higher than the reported for *Q. suber* cork by Castola et al. ([48,50]) and Sousa et al. [51], while it was the main constituent of *Q. cerris* cork (representing 6.2%) [14]. *Q. rotundofolia* bark lipophilic extracts also included long-chain lipid compounds e.g., saturated alkanoic acids (23.7%–31.2% of all compounds), constituting 72.4%–74.0% of all long-chain acids. Substituted alkanoic acids, α,ω-diacids and ω-hydroxyacids also existed but in smaller amounts. Similar results have been reported in the literature for *Q. faginea* barks, where saturated alkanoic acids also constituted an abundant group of compounds (around 25.0%), with hexadecanoic acid and docosanoic acid as the most representative elements of this family of compounds [23].

4.5. Suberin Composition

The results for the suberin composition obtained by GC–MS analysis are summarized in Table 6. The main suberin monomers of *Q. rotundifolia* bark were fatty acids, representing 84.0% of all compounds, in the samples collected at both sites. Among them, ω-hydroxyacids were the major compounds, representing 46.8% and 50.2% in the samples from site 1 and 2, respectively. The most abundant compounds were 9,10,18-trihydroxyoctadecanoic acid at site 2 and 18-hydroxy-9-octadecenoic acid at site 1, representing 31.3% and 29.3% of all ω-hydroxyacids, respectively. α,ω-Diacids (saturated and substituted) were also abundant, with hexadecanedioic acid and 9,10-dihydroxyoctanedioic acid as the most representative at rates of 25.7% and 19.4% of all α,ω-diacids at site 1 and 28.1% and 38.7% α,ω-diacids at site 2, correspondingly. The literature on suberin composition of other oaks shows that it is species specific, i.e., it varies between *Quercus* species. For instance, in *Q. faginea* bark, suberin is mainly composed by fatty acids, namely ω-hydroxyacids (between 40%–50% of all compounds) and substituted α,ω-alkanoic diacids [23]. In *Q. suber*, for instance, the main constituents of suberin are substituted α,ω-diacids with mid-chain epoxy or diol substitutions [52]. In *Q. cerris*, however, ω-hydroxyacids represent 90% of the long chain monomers [45], as well as in *Q. variabilis*

where ω-hydroxyacids are also the major compounds identified (around 58.7%), followed by α,ω-substituted diacids (19.5%) [43]. Alkanoic acids, aromatics, and alkanols are also present, in amounts varying from around 4.0%–19.0%. Trace amounts of dehydroabietic acid and β-sitosterol were also identified, which was somewhat common in *Q. faginea* barks, where no sterols or triterpenes were identified, as reported by Ferreira et al. [23].

5. Conclusions

The general structure and anatomy of *Q. rotundifolia* bark were described for the first time and proved similar to most *Quercus* spp. The main pattern of *Q. rotundifolia* bark showed a rhytidome with sequential undulated and anastomosed periderms with a small proportion of cork, while the phloem included broad rays with strong cell sclerification, groups of sclereids with embedded large prismatic crystals, and abundant druses in parenchyma cells. The chemical composition of *Q. rotundifolia* bark was also studied for the first time, including variability of composition of lipophilic and polar extracts, and antioxidant activity, as well as suberin composition. The polar extractive content was high, especially for ethanol extracts in regards to total phenolics, flavonoids, and condensed tannins. The *Q. rotundifolia* bark extracts showed very good antioxidant properties and therefore should be considered as a relevant natural source. Certainly, further studies are needed for the identification of single compounds and to gather information on their structural elucidation and their biological activity. Suberin levels were low, in accordance with the bark anatomical characteristics, mainly composed by fatty acids and substituted α,ω-alkanoic diacids. as in various other *Quercus* spp., but also confirming the between-species variation. These findings constitute the grounds for the valorization of *Q. rotundifolia* bark, which may be integrated into a biorefinery approach with a first step of polar extracts fractionation, thereby contributing to local communities' economies and to the overall sustainability of this species and the *montado* ecosystem in the western Mediterranean area.

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