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Phytochemical Composition of Extractives in the Inner Cork Layer of Cork Oaks with Low and Moderate *Coraebus undatus* Attack

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Abstract: The beetle *Coraebus undatus*, during its larval stage feeds, and excavates galleries on the cork-generating layer of *Quercus suber* L. trees, seriously affecting the cork quality with significant economic losses for the cork industry. This work compared the composition of the extracts present in the innermost cork layers (the belly) of cork planks from *Q. suber* trees with low and moderate *C. undatus* attack in one stand. The total extractives in the inner cork layer from trees with moderate and low *C. undatus* attacks were similar (on average 22% of the cork mass) with a high proportion of polar compounds (91% of the total extractives). The chemical composition of the inner cork lipophilic extractives was the same in trees infested and free of larvae, with triterpenes as the most abundant family accounting for 77% of all the compounds, predominantly friedeline. The hydrophilic extractives differed on the levels of phenolic compounds, with higher levels in the inner cork extracts of samples from trees with low attack (90.0 mg GAE g^{-1} vs. 59.0 mg GAE g^{-1} of inner cork mass) The potential toxic activity of phenolic compounds may have a role in decreasing the larval feeding.

Keywords: *Quercus suber*; cork borer; cork; chemical composition; phenolics

1. Introduction

The cork oak (*Quercus suber* L.) is an evergreen oak species adapted to dry and warm regions, distributed along the western Mediterranean basin, covering an area of approximately 2.1 million ha, in agro-forestry systems with high ecological and socio-economic importance [1,2]. Cork oak management is oriented towards the production of cork. This is one of the most important non-wood forest products that feeds a dedicated industrial chain, with wine cork stoppers as its worldwide famous product [3]. The world cork leader is Portugal, with an annual production of 100 thousand tonnes of raw cork, and second is Spain, with 62 thousand tonnes in 2010 [4].

Biotic threats to cork oak forests are a major concern since they may affect tree vitality and survival, as well as cork quality. One such biotic threat is related to infestations by the cork beetle *Coraebus undatus* Fabricius (Coleoptera, Buprestidae), named "cobrilha da cortiça" in Portugal and "culebrilla" in Spain, which negatively impacts the cork value for industrial processing [3] and, to a smaller extent, may induce stem wounds. Several reports indicated high infestation intensity in several regions of southern Spain and southern France [5,6]. *Coraebus. undatus* females lay their eggs on the surface or in cracks of the cork back (i.e., the external surface of the cork layer covering the stem) about 4 years after the previous cork extraction [7,8]. The hatched larvae perforate the layers of the cork tissue underneath the cork back to the phellogen, where they feed and grow, excavating long, sinuous galleries reaching 2 m in length and 3–4 mm in width, with a higher incidence in the stem region at a height between 0.5 m and 1.5 m [9]. Larval development lasts from

1 to 2 years, after which, the larva builds a pupal chamber within the cork layers, where it matures until the adult insect is formed and perforates the cork, emerging to the outside.

The occurrence of *C. undatus* attacks is rather elusive since the galleries are only visible when the cork is removed at the phellogen region at the end of one production cycle (on average 9 years) and appear as ribbon-like scars marked on the stem and on the inner part (i.e., the belly) of the cork plank, often showing dark fillings of larval feed excrements (Figure 1). Galleries may also be detected within the cork planks during cork processing and quality evaluation since they become embedded in the cork tissue during growth, and this constitutes a major devaluation of the cork plank [3,10,11]. Given the *C. undatus* cycle (egg laying starting in 4-year-old cork back and 2-year larval development) and the most common 9-year cork production cycle that prevails in the most important cork regions [12], it is to be expected that two to four attacks may occur during one cork production cycle. In addition to the decrease in cork quality, *C. undatus* attacks may also negatively impact tree health since, during the cork extraction, a localized phloem tearing may originate in the regions of the galleries, causing irreversible damage to the tree in that area with response to wounding and the risk for potential biological attacks.

Figure 1. Galleries of *Coraebus undatus* larvae at the time of cork extraction (**left**) on *Q. suber* trunk (**right**) showing a larva and an excrement-filled gallery on the inner side (belly) of cork planks.

Although a few studies on *C. undatus* attacks have tried to link infestation and the intensity of attacks to stand characteristics, e.g., tree density, the presence of understory, solar orientation, drought stress, or tree parameters, e.g., age, diameter, height, or health status [8,9], a clear pattern has not been obtained, in part due to a high degree between tree variation regarding attack levels: For instance, in one study in eight cork oak forest plots exploited for cork production in southern Spain (Natural Park "Los Alcornocales"; Sierra Morena, Huelva), the infestation index ranged from 0.40 to 2.32 [8].

To the best of our knowledge, no studies have related the presence of *C. undatus* with the chemical characteristics of the cork in the phellogenic region. The chemical composition of cork has been studied in detail, given its importance on the material's properties that are at the base of its applications [13], and it is known that there is a large natural variability regarding its most relevant components, namely in the content of extractives, suberin, and lignin [13,14].

Extractives are small molecules non-linked to the cell wall structural components that are soluble in the appropriate solvents. They are of particular importance since resis-

tance against pests and pathogens usually relies on high concentrations of a diverse array of plant secondary metabolites [15,16]. Secondary metabolites are essential for reducing plant palatability and affect pest growth, development, and digestion, as shown in various examples. The activity of triterpenic compounds (betulinic and ursolic acids) on insect feeding was observed against the third instar larvae of castor semi-looper (*Achoea janata* (Linnaeus) (Lepidoptera: Noctuidae)) [17]. The triterpenoid fridelin isolated from *Azima tetracantha* Lam. (Salvadoraceae) leaves showed anti-food, larvicidal, and pupicidal activities against *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) and *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) [18]. Phenolics can also be toxic to insects, for example, to the larvae of *Spodoptera litura* and its parasitoid *Bracon hebetor* (Say) (Hymenoptera: Braconidae) [19], and to the southern armyworm *Spodoptera eridania* (Cramer) (Lepidoptera: Noctuidae) [20].

Cork possesses significant amounts of secondary metabolites that are soluble compounds not chemically linked to the structural polymers of the cell walls, mainly composed of aliphatic, triterpenic, and phenolic compounds. The relative abundance and chemical composition of these compounds show a very high natural variability, even among trees of the same species and forest [21–28]. This work focuses on the content and chemical composition of the extractives present in the innermost cork layers of the cork planks, where larvae feed and excavate their galleries, taken from trees with very low *C. undatus* attack and from trees with moderate attack, as assessed at the time of cork extraction. The objective was to analyze the chemical profile of these secondary metabolites in cork in relation to the presence of the cork borer.

2. Material and Methods

2.1. Sampling

Cork planks were collected from *Q. suber* mature trees under exploitation (i.e., cork extraction) in one cork oak forest (montado), located in central western Portugal in the Coruche municipality (38°57′ N, 8°37′ W). The region has a Mediterranean-type climate with Atlantic influence, with its highest temperatures in summer (June to September) when precipitation is lowest: a mean average annual rainfall of 775 mm, 83.0% of precipitation concentrated from October to April, an average annual maximum temperature of 21 °C, and an average annual minimum temperature of 14 °C. The sampling took place in July and August during the period of cork stripping. A total of 97 cork oak trees were randomly selected from 11 five-tree plots scattered and covering all of the area of the cork oak stand and measured regarding d.b.h. (diameter at 1.30 m from the ground and cork stripping height). The mean d.b.h. was 39.4 ± 6.2 cm, and the debarking height of the cork stripping ranged from 1.2 m to 2.7 m with an average of 1.9 m $\pm$ 0.51 m.

The presence of *C. undatus* galleries was identified by visual observation of the decorked stems and the inner side (belly) of the cork planks. The classification of the intensity of attack (damage index) was based on the visual observation of the debarked stem after cork removal following the methodology used by Du Merle and Attié [29] and applied in *C. undatus* assessments [8,9]. On each tree, four vertical lines oriented to the north, east, south, and west sides of the stripped part of the stem were divided into 50 cm long sections from the soil surface to a maximum height of four levels (200 cm). The crosses between galleries and each of these lines were counted and the tree damage intensity (AI) was calculated as AI = total number of gallery intersections/4 × number of vertical levels occupied (4 is the number of orientation sections; N, S, E, and W). The plot infestation index was calculated as IP = $\sum$ AI/N, where N is the number of trees per plot. The cork samples (20 cm × 20 cm) for chemical analysis were taken from a subset of 22 *Q. suber* trees with different attack intensities that were grouped as (a) trees with very low *C. undatus* attack, corresponding to trees with a damage index between 0 and 0.06 (mean 0.03); and (b) infested trees with *C. undatus* attack corresponding to a damage index between 0.13 and 0.38 (mean 0.25). For the sake of simplicity, the two groups of samples were coded as "low attack" and "moderate attack".

The innermost cork layers of the cork plank (the belly side) were manually separated with a chisel corresponding to a removal of approximately 2 mm of thickness. This sample comprises the first few layers of phellem (cork tissue) adjacent to the phellogen and some remains of the phellogen tissue. The samples were ground individually in a Retsch (SM2000, Retsch GmbH, Haan, Germany) cutting mill, passing through a 1 mm × 1 mm sieve. The milled cork samples were dried in an oven at 60 °C and kept for analysis.

2.2. Lipophilic Compounds Extraction

The milled cork samples were Soxhlet extracted for 6 h with dichloromethane (Sigma-Aldrich, ≥99.8% purity, St. Louis, MO, USA) to recover the soluble lipophilic compounds. The extraction yield was determined by the mass difference of the solid residue after drying at 60 °C overnight and for 1 h at 105 °C and reported as a percent of the original sample. The extract was used for gas chromatography–mass spectrometry (GC–MS) qualitative and quantitative analysis.

2.3. Hydrophilic Compounds Extraction

The dichloromethane extracted samples were suspended in a 50:50 (v/v) ethanol/water mixture in an ultrasonic bath at room temperature for 16 h. The suspension was filtered through glass filter crucible G3 (pore size 15 to 30 μm) and the resulting solution was used for the quantitative analysis of total phenols, flavonoids, proanthocyanidins, and antioxidant activity. The yield of extractives solubilized by the ethanol–water mixture was determined by the mass difference of the solid residue after drying at 60 °C overnight and for 1 h at 105 °C and reported as a percent of the original sample.

2.4. Chemical Characterization of the Lipophilic Extract

The lipophilic extracts solubilized by dichloromethane were recovered as a solid residue after solvent evaporation under N_2 flow and dried overnight under vacuum at room temperature. A 2 mg extract sample was derivatized in 100 μL of pyridine (Sigma-Aldrich, ≥99.8% purity, St. Louis, MO, USA) by adding 100 μL of bis(trimethylsilyl)-trifluoroacetamide (BSTFA, Sigma-Aldrich, ≥99.0% purity, St. Louis, MO, USA) and kept for 30 min in an oven at 60 °C, by which the compounds with hydroxyl and carboxyl groups were trimethylsilylated into trimethylsilyl (TMS) ethers and esters, respectively.

The derivatized extracts were immediately injected in a GC–MS Agilent 5973 MSD with the following GC conditions: Zebron 7HG-G015-02 column (30 m, 0.25 mm; ID, 0.1 μm film thickness), flow 1 mL/min, injector 280 °C, oven temperature program, 100 °C (1 min), rate of 10 °C/min up to 150 °C, rate of 4 °C/min up to 300 °C, rate of 5 °C/min up to 370 °C, rate of 8 °C/min up to 380 °C (5 min). The MS source was kept at 220 °C, and the electron impact mass spectra (EIMS) were taken at 70 eV of energy. The compounds were identified as TMS derivatives by comparing their mass spectra with a GC–MS spectral library database (Wiley, NIST Mass Spectral Library) with over 90% similarity and by comparing their fragmentation profiles with published data [30–32]. For semi-quantitative analysis, the area of peaks in the total ion chromatograms of the GC–MS analysis was integrated, and their relative proportions were expressed as the area proportion of the total chromatogram area. Each aliquot was injected in triplicate, and the mean results are given (only a standard deviation inferior to 5% was considered).

2.5. Ethanol-Water Extract Composition

The ethanol–water extracts obtained from the dichloromethane-extracted samples were analyzed in terms of total phenolics, flavonoids, and condensed tannins. The total phenolic content (TPC) was determined according to the Folin–Ciocalteu method [33]. Briefly, 100 μL of the extract sample was added to 4 mL of diluted Folin–Ciocalteu reagent (Sigma-Aldrich, ≥99.8% purity, St. Louis, MO, USA) (1:10 v/v) and afterwards to 4 mL of aqueous sodium carbonate (Sigma-Aldrich, ≥99.9% purity, St. Louis, MO, USA) (7.5 g/L). The mixture was incubated for 30 min in the dark and was recorded with a spectropho-

tometer UV/Vis V-530 spectrophotometer (Jasco, Tokyo, Japan) at 750 nm against a blank containing only water. The TPC was calculated from a standard curve with gallic acid (Sigma-Aldrich, ≥99% purity, St. Louis, MO, USA) (range 0.014–0.762 mg/mL) and expressed as the mg of gallic acid equivalents (GAE) per gram of extract. The analyses were carried out in triplicate, and the average value was calculated.

The total flavonoid content was determined using the aluminum chloride colorimetric assay with catechin (CA) as standard [34]. Briefly, 1 mL of the extract sample was added with 0.3 mL $NaNO_2$ (Sigma-Aldrich, ≥99% purity, St. Louis, MO, USA) solution (5% w/v) and 0.3 mL $AlCl_3$ (Sigma-Aldrich, ≥ 99% purity, St. Louis, MO, USA) solution (10% w/v). The mixture was then allowed to stand for 6 min. Afterwards, 2 mL of sodium hydroxide (1 M) and 2.4 mL of water were added sequentially and vigorously shaken. The absorbance was recorded at 510 nm after 30 min of incubation against water (UV/Vis V-530 spectrophotometer). The results were calculated according to the calibration curve for catechin (Sigma-Aldrich, ≥99% purity, St. Louis, MO, USA) (0.10–1.0 mg/mL). The total flavonoid content was expressed as the mg of catechin (CE) equivalent/g of the extract. Triplicate measurements were carried out.

The total proanthocyanidin content (condensed tannins) was determined according to the vanillin-sulfuric acid method [35]. Briefly, a volume of 100 μL of the extract was mixed with 2.5 mL of 1.0% (w/v) vanillin (Sigma-Aldrich, ≥99% purity, St. Louis, MO, USA) methanolic solution and 2.5 mL of 25% (v/v) sulfuric acid in absolute methanol. The blank solution was prepared with the same procedure without vanillin. The absorbances of the extract samples and blanks were recorded at 500 nm after 15 min. Catechin standard solutions (0.1–0.6 mg/mL) were used for constructing the calibration curve, and the amount of total condensed tannins was expressed as the mg of catechin (CE) equivalent/g of the extract. Triplicate measurements were carried out.

2.6. Antioxidant Activity of Ethanol–Water Extracts

The antioxidant activity of the ethanol–water extracts was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) (DPPH, Sigma-Aldrich, ≥99.0% purity, St. Louis, MO, USA), which measures the free radical scavenging capacity [35]. Different dilutions of the extract were prepared and an aliquot of 100 μL of each solution was added to 3.9 mL of a DPPH methanolic solution (24 μg/mL). A control was prepared by adding 100 μL of methanol to 3.9 mL of a DPPH methanolic solution (24 μg/mL). The mixtures were shaken vigorously and left to stand in the dark for 30 min. The absorbance at 517 nm was measured using a UV/Vis V-530 spectrophotometer and compared to the initial absorbance of the DPPH solution using methanol as blank. The scavenging activity was estimated based on the percentage of the DPPH radical scavenged. Trolox (Sigma-Aldrich, ≥97% purity, St. Louis, MO, USA), and (+)-catechin were used as reference compounds. The IC_{50} values were determined from the plotted graphs of scavenging activity against the concentration extracts and represent the amount of extract necessary to decrease the initial DPPH concentration by 50%. Low IC_{50} values indicate high free-radical scavenging activities. The scavenging effect on the DPPH radical of the extract was also expressed as the mg of Trolox equivalent/g of the extract. All analyses were run with three replicates and averaged.

2.7. Statistical Analysis

The data were analyzed using the Sigmaplot statistical software (version 11.0, Systat Software Inc., San Jose, CA, USA). The normality of each distribution was analyzed by the Shapiro–Wilk test and the homogeneity of variances by Levene's test. Student's t test or the Mann–Whitney U test, depending, respectively, on the presence or absence of a normal distribution with equal variances. Principal component analysis (PCA) and cluster analysis (CA) were performed in order to evaluate the presence of an eventual relationship between chemical compounds present in the inner cork layer and the *C. undatus* intensity attack level and the presence of sample groups [36,37]. Samples 1 to 10 correspond to very low attack samples; sample codes from 11 to 22 correspond to moderate attack. In CA, the

Euclidean distance and single linkage methods were used. PCA and CA were performed with the Statistica[TM] software, version 7, from Statsoft (Tulsa, OK, USA).

3. Results and Discussion

3.1. Incidence of C. undatus

Previous studies showed that *C. undatus* is a very common phytophagous insect in cork oak forests in the Mediterranean region, in some sites affecting more than 90% of the sampled forests and over 70% of trees, although in many other sites, the levels of damage fluctuate from low to moderate [5,8,9]. Nonetheless, IA and IP values may underestimate the damage associated with *C. undatus* [8]. The reason for this is that the damage observed when the cork is removed (i.e., the larval paths on the phellogenic region by which the cork plank was torn away) results from the galleries of the last brood of *C. undatus*, which are observable in the decorked stem. This does not mean that older galleries generated by previous broods (from the years preceding the cork extraction) may not be present and unseen because they were incorporated into the cork tissue. However, the mere presence of one gallery highly devaluates a cork plank.

Of the 97 observed trees, 43% were affected by *C. undatus*. However, the mean stand infestation index (IP) value was very low with an IP = 0.17 ± 0.09, thereby revealing low *C. undatus* population levels. This agrees with Branco et al. [38] who reported that the percentage of trees attacked by *C. undatus* in cork oak forests in Portugal varies between 0% and 50%.

3.2. Extractable Components in the "Inner Cork"

The extraction yields of the belly of cork planks ("inner cork") from cork oak trees with low and moderate damage from *C. undatus* are shown in Table 1. The total content was similar for the two groups of cork oaks, corresponding on average to 22% of the cork mass. The difference between the two groups of samples was not statistically significant ($t = 0.479$; $p = 0.637$).

Table 1. Extractives (% of the dry mass) of the inner cork layer of cork planks extracted from trees with low attack and with moderate attack from *Coraebus undatus* (mean and standard deviation).

	Low Attack	Moderate Attack
Total extractives	21.6 [a] $\pm$ 2.7	22.0 [a] $\pm$ 1.7
Dichloromethane extractives	2.3 [a] $\pm$ 1.2	1.7 [a] $\pm$ 1.5
Ethanol-water extractives	19.3 [a] $\pm$ 3.3	20.3 [a] $\pm$ 2.5

Different letters indicate significant differences at $p < 0.05$.

In both groups of cork oaks, a striking chemical feature of the inner cork extracts was the high proportion of polar compounds soluble in ethanol–water, which accounted for 91% of the total extractives (20% of the oven dry cork mass). The lipophilic compounds soluble in dichloromethane corresponded to only 9% of the total cork extractives (2% of the cork mass). The differences between the two groups of samples with different damage indexes were not statistically significant for lipophilic ($t = 0.955$; $p = 0.351$) and hydrophilic extractives ($t = -0.0443$; $p = 0.965$).

Overall, the content in extractives of the inner cork layer is well above the average value of 16.2% reported for *Q. suber* cork, although within the range of values for the species (8.6%–32.9%) [13]. There is also a difference in the chemical profile of the inner cork extractives in relation to that of the complete cork layer since the polar extractives represent only 42%–70% of the total extractives in the complete cork layer, while 91% is in the inner cork region [13,14,39]. The inner cork region close to the phellogen contains young cellular material, some of which is still in metabolic processing, and this explains the high content in polar compounds which comprise phenolic compounds, as reported further on, and also soluble carbohydrates.

Only very few studies have addressed the radial variation of cork chemical composition across the cork plank thickness. Jové et al. [40] analyzed the cork chemical composition in three radial positions near the back, mid-cork, and belly, and observed differences with the highest extractives content in the belly layer.

3.3. Lipophilic Extractives Composition

The identified compounds in lipophilic extracts of the inner cork layer from samples with low and moderate *C. undatus* attack are given in Table 2 in proportion to the total chromatogram area grouped by chemical family.

Table 2. Chemical composition (% of all chromatogram peak areas) of the lipophilic extract of the inner cork layer of cork planks extracted from trees with very low and moderate attack from *Coraebus undatus*.

	Low Attack	Moderate Attack
Dichloromethane Extractives, % Dry Mass	2.31 [a] ± 1.2	1.65 [a] ± 1.5
Alkanols	1.48 [a] ± 0.14	1.91 [a] ± 0.35
Hexadecan-1-ol	0.01 ± 0.01	0.04 ± 0.03
Octadecan-1-ol	0.04 ± 0.05	0.03 ± 0.05
Eicosan-1-ol	0.16 ± 0.11	0.23 ± 0.14
Docosan1-ol	0.67 ± 0.39	0.97 ± 1.07
Tetracosan-1-ol	0.53 ± 0.25	0.60 ± 0.74
Hexacosan-1-ol	0.07 ± 0.04	0.04 ± 0.06
Alkanoic acids	6.05 [a] ± 0.43	8.82 [b] ± 0.49
Saturated fatty acids	2.80 [a] ± 0.36	4.57 [b] ± 0.37
Hexadecanoic acid	1.45 ± 0.67	2.62 ± 0.87
Heptadecanoic acid	0.07 ± 0.03	0.08 ± 0.02
Octadecanoic acid	0.96 ± 0.85	1.47 ± 0.73
Eicosanoic acid	0.14 ± 0.16	0.19 ± 0.09
Docosanoic acid	0.18 ± 0.07	0.21 ± 0.05
Unsaturated fatty acids	3.30 [a] ± 0.60	4.35 [a] ± 0.64
9-cis-Hexadecenoic acid	0.53 ± 0.01	0.58 ± 0.25
9,12-Octadecadienoic acid	0.52 ± 0.51	1.34 ± 1.87
9,12,15-Octadecatrienoic acid	0.07 ± 0.09	0.37 ± 0.52
9-Octadecenoic acid	1.78 ± 2.03	1.89 ± 0.51
13-Octadecenoic acid	0.35 ± 0.38	0.09 ± 0.03
Substituted fatty acids	0.93 [a] ± 0.19	1.24 [a] ± 0.37
2-Hydroxy-2-methylpropanoic acid	0.76 ± 0.09	0.84 ± 0.57
2-Hydroxy-4-methylpentanoic acid	0.17 ± 0.29	0.39 ± 0.17
Dicarboxylic acids	0.27 [a] ± 0.13	0.3 [a] ± 0.20
Saturated dicarboxylic acid	0.17 [a] ± 0.20	0.21 [a] ± 0.19
Propanedioic acid	0.04 ± 0.06	0.03 ± 0.05
Nonanedioic acid	0.13 ± 0.25	0.18 ± 0.32
Substituted dicarboxylic acid	0.10 [a] ± 0.08	0.13 [a] ± 0.22
2-Hydroxydecanedioic acid	0.10 ± 0.08	0.13 ± 0.22
Glycerol derivatives	1.90 [a] ± 1.17	1.20 [a] ±0.21
Glycerol	1.52 ± 0.93	0.91 ± 0.37
2,3-Dihydroxypropyl hexadecanoate	0.27 ± 0.19	0.14 ± 0.07
2,3-Dihydroxypropyl octadecanoate	0.11 ± 0.03	0.15 ± 0.20
Terpenes	77.95 [a] ± 3.48	76.12 [a] ± 2.29
Squalene	0.72 ± 0.95	0.62 ± 0.60
Lupeol	0.65 ± 0.15	0.74 ± 0.42
Friedelane-1-ene-3-one	17.56 ± 9.18	16.92 ± 10.06
Erythrodiol	0.28 ± 0.17	0.40 ± 0.34
Friedelin	37.56 ± 14.41	36.59 ± 5.03
Lup-20(29)-en-3-one	0.24 ± 0.19	0.19 ± 0.24
Betulin	0.97 ± 0.00	1.13 ±0.55
Betulinic acid	15.71 ± 15.3	16.43 ± 10.21
Betulinaldehyde	0.32 ± 0.30	0.21 ± 0.15

Table 2. *Cont.*

	Low Attack	Moderate Attack
D:A-Friedooleanan-28-al, 3 oxo (Canophyllal)	0.63 ± 0.22	0.87 ± 0.27
D:A-Friedooleanan-3-one, 28-hydroxy-	1.06 ± 1.28	0.68 ± 0.63
D:A-Friedo-2,3-secooleanane-2,3-dioic acid, dimethyl ester, (4R)-	2.25 ± 2.53	1.33 ± 1.18
Sterols	3.19 [a] ± 0.37	4.32 [a] ± 0.22
β-Sitosterol	1.86 ± 1.00	3.05 ± 0.62
Lanosterol	0.42 ± 0.04	0.34 ± 0.09
Cycloartenol	0.30 ± 0.14	0.30 ± 0.25
Cycloeucalenol	0.39 ± 0.00	0.50 ± 0.10
Sitosteryl-3beta-D-Glucopiranoside	0.22 ± 0.28	0.13 ± 0.17
Aromatic compounds	1.90 [a] ± 0.17	1.54 [a] ± 0.13
Benzoic acid	0.19 ± 0.11	0.21 ± 0.11
Salicylic acid	0.08 ± 0.11	0.11 ± 0.14
Vanillin	0.49 ± 0.28	0.31 ± 0.20
Vanillin acid	0.18 ± 0.08	0.18 ± 0.07
Caffeic acid	0.01 ± 0.01	0.04 ± 0.06
Caffeic acids derivatives	0.72 [a] ± 0.14	0.50 [b] ± 0.12
Caffeic acid + Triacontanoic acid	0.42 ± 0.24	0.21 ± 0.11
Caffeic acid + Dotriacontanoic acid	0.22 ± 0.18	0.17 ± 0.25
Caffeic acid + Tetratiacontanoic acid	0.08 ± 0.00	0.12 ± 0.00
Others	6.84 ± 1.53	1.94 ± 0.29
Levoglucosan	1.11 ± 2.06	0.70 ± 0.61
Quinic acid	4.68 ± 3.98	-
Myo-inositol	0.51 ± 0.00	0.66 ± 0.00
(7E,11E,15E)-3-(methoxymethoxy)-3,7,16,20-tetramethylhenicosa-1,7,11,15,19-pentaene	0.43 ± 0.06	0.45 ± 0.19
Octacosahydro-9,9′-biphenanthrene	0.11 ± 0.01	0.13 ± 0.05
Identified	96.96 ± 7.90	92.04 ± 7.27

Different letters indicate significant differences at $p < 0.05$.

The lipophilic fraction in the inner cork layer contains essentially the same compounds and in a similar proportion in samples with *C. undatus* damage. Triterpenes represented the most abundant family, accounting for 77% of all the compounds (77.95 % and 76.12%), including predominantly friedelin (37.56 and 36.59%), friedelane-1-en-3-one (17.56%–16.92%), and betulinic acid (15.71%–16.43%) that together constitute about 93% of all the identified triterpenes and triterpenoids. Other triterpenoids such as betuline and lupeol, as well as β-sitosterol were also identified in small amounts.

The long-chain lipids represented only 6.05% and 8.82% of all the compounds, including mainly fatty acids with hexadecanoic (palmitic acid) and octadecanoic (stearic acid) acids as the most abundant saturated fatty acids, and octadec-9-enoic acid (oleic acid) as the most abundant unsaturated fatty acid. Long-chain aliphatic alcohols were present in the inner cork dichloromethane extract in small amounts (1.48% and 1.91%, respectively) with docosan-1-ol and tetracosan-1-ol as the major fatty alcohols. Glycerol and two monoglycerides (monopalmitin and monostearin) were also found, representing together 1.90 and 1.20 % of the total identified compounds, respectively.

Aromatic compounds were found in minor concentrations (1.3% of total compounds), mostly vanillin and vanillinic acid. Other compounds, such as quinic acid, were identified only in the inner cork layer from trees with low *C. undatus* galleries (4.68% of all compounds).

Overall, all of the identified lipophilic compounds have been previously reported in cork extractives with small differences in composition: lipophilic extracts of corks from *Q. suber* from Bulgaria and Turkey [38] showed the dominance of triterpenoids with betulinic acid and friedelan-3-one as the main components. The inner cork compared

to the complete cork had a lower proportion of alkanoic acids (6.8% vs. 14.2% in reproduction cork) and a higher proportion of triterpenes (77% vs. 50% in reproduction cork) [23,25,26,28].

The insecticidal and phytotoxic potential against plant-pathogens of these lipophilic compounds has been reported, e.g., pentacyclic triterpenes have anti-insect properties [41] as well as friedelane triterpenes derived from the byproducts of cork processing [42]. Such compounds exist in the inner cork layer (Table 2) but in amounts without difference between trees with low and moderate *C. undatus* attacks. Therefore, the main terpenes in the inner cork layer (e.g., friedeli, friedelane-1-ene-3-one, and betulinic acid, Table 2) do not seem to have anti-food and anti-larvicidal properties against the larvae of *C. undatus*, at least in the present amounts since overall, the content of lipophilic compounds was very small (Table 1).

3.4. Composition of Hydrophilic Extractives

The composition of the hydrophilic extractives from the inner cork layer from samples with low and moderate *C. undatus* attack are presented in Table 3 regarding the content in phenolic compounds.

Table 3. Composition and antioxidant capacity of ethanol–water extracts of the inner cork layer of cork planks extracted from trees with very low and moderate attack from *Coraebus undatus* (mean and standard deviation).

	Low Attack	Moderate Attack
Total phenolics (mg GAE g^{-1} extract)	448.6 [a] $\pm$ 101.6	296.41 [b] $\pm$ 78.1
Total flavonoids (mg CE g^{-1} extract)	41.5 [a] $\pm$ 9.4	36.7 [a] $\pm$ 6.2
Proanthocyanidins (mg CE g^{-1} extract)	10.1 [a] $\pm$ 3.1	10.6 [a] $\pm$ 2.7
IC$_{50}$ (μg extract mL^{-1})	13.4 [a] $\pm$ 3.5	16.0 [a] $\pm$ 3.6

Different letters indicate significant differences at $p < 0.05$.

There was a clear difference in the levels of phenolic compounds with higher levels in extracts of inner cork from trees with low attack (448.6 mg GAE g^{-1} extract or 90.0 mg GAE g^{-1} of inner cork) as compared with samples with low attack intensity (296.4 mg GAE g^{-1} extract and 59.0 mg GAE g^{-1} of inner cork)($t = 3.773$; $p = 0.001$).

No differences were found between the two groups of samples (from trees with low and moderate *C. undatus* attack) regarding the content of flavonoids ($t = 1.333$; $p = 0.198$) or proanthocyanidins ($t = -0.345$; $p = 0.734$) nor regarding the antioxidant properties of the extract ($t = -1.035$; $p = 0.313$).

The comparison of the results obtained here for the inner-cork region with the literature data for the complete cork layer of reproduction cork shows some differences. The levels of phenolic compounds found in the inner cork layer were higher when compared to those found in extracts of reproduction cork (336.3 mg GAE g^{-1} of extract or 19.9 mg GAE g^{-1} dry cork) [43], or the mean values of 196.4 mg GAE g^{-1} of extract for ethanol–water extracts from cork [24]. The total flavonoid contents (36.7 mg and 41.5 mg CE g^{-1} extract in the inner cork layer) were lower compared with the values obtained for cork by Ferreira et al. [24]. As regards the antioxidant properties, the ethanol–water extracts of the inner cork revealed very low antioxidant activity with IC$_{50}$ values of 13.4 and 16.0 μg extract mL^{-1}, which is lower than previously reported for different cork extracts, namely 2.79 μg mL^{-1} in water extract, 3.58 μg mL^{-1} in methanol extract, and 5.84 μg mL^{-1} in methanol-water 50:50 extract [44] and for cork (3.2 μg mL^{-1}) [24]. The fact that inner cork extracts contain higher amounts of phenolics but have less antioxidant properties than complete cork extracts suggests that the antioxidant activity of the cork extracts is not directly related to the concentration of total phenolics and should result from specific phenolic compounds or from other compounds with antioxidant activity [26].

The main differences among the cork samples taken from trees with very low and moderate *C. undatus* attack are in the content of phenolic compounds (Table 3). Phe-

nolics are frequently implicated in chemical defense mechanisms against pathogens in woody plants [45] that act as anti-nutritive agents or exert toxic effects on phytophagous insects [19,46,47]. Specifically for pine species, recent studies showed that phloem chemical composition, namely, the content in phenolic compounds was negatively related to pine nematode susceptibility [48,49]. The negative impact of specific phenolic compounds against some insect pests has been reported, for instance, of ellagic acid on the larvae of *Spodoptera litura* and its parasitoid *Bracon hebetor* [19] and on the southern armyworm *Spodoptera eridania* [20]. The predominant compounds detected in *Q. suber* reproduction cork extracts are ellagic, gallic, and protocatechuic acids [26–28,43], thus supporting their role in biotic defense.

Therefore, further studies targeted to analyze the phytochemical impact on cork borers' resistance should consider a detailed analysis of the phenolic and polyphenolic composition of the metabolites present in the inner cork extracts, including their bioactive properties that could be involved in protective functions, as well as a larger sampling of trees with differing *C. undatus* attacks.

Figure 2 shows the projection of variable loads (Figure 2A) and samples (Figure 2B) on the plane defined by the two first principal components (factors 1 and 2). This plane accounts for 69.5% of the variance of original data. PCA did not differentiate the two groups of samples with different *C. undatus* attack intensities since the projection of samples on this plane did not show any defined group. The PCA only showed that contents in dichloromethane extractives, condensed tannins, and flavonoids increase along the first PC while the content of ethanol–water extractives increase in the opposite sense (along the negative part of PC1) and IC50 increases along the second PC, opposite to the total phenol content. A CA was carried out to assess the presence of groups not detected by PCA (Figure 3). The groups formed are not related to the attack level. For instance, at a distance linkage of 50, we have three clusters where low-attacked samples and attacked samples are mixed.
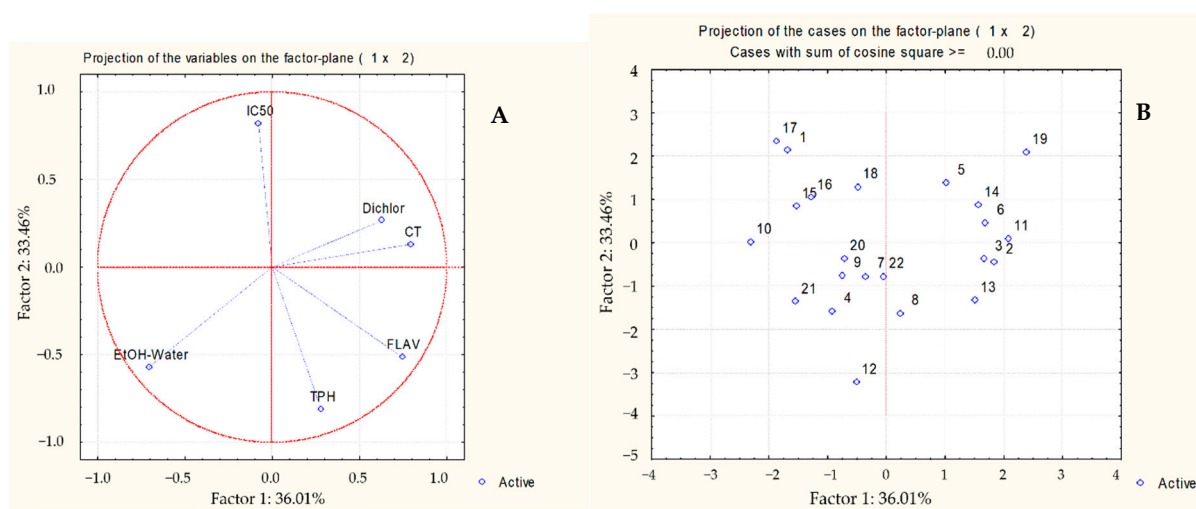

Figure 2. Principal components analysis of the chemical compounds present in the inner cork layer from tree samples with different *C. undatus* attack intensity levels: (**A**) score plots of the original variables; (**B**) samples (Samples 1 to 10 correspond to very low attack samples; sample codes from 11 to 22 correspond to moderate attack).
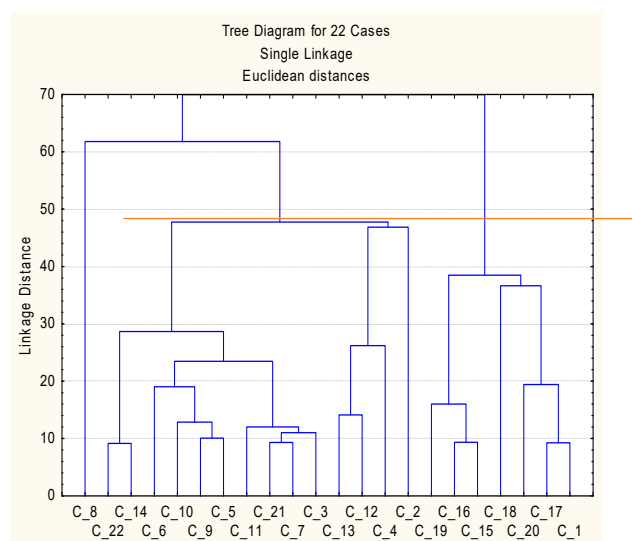
Figure 3. Dendrogram of the chemical compounds present in the inner cork layer from tree samples with different *C. undatus* attack intensity level.

4. Conclusions

This work reports for the first time the composition of extractives in the innermost cork layers (the belly) of cork planks from cork oak trees with very low and moderate *C. undatus* attack. No difference was found between the content of the total extractives, the proportion of apolar to polar compounds, and the composition of the lipophilic extractives in the inner cork layer from *Q. suber* trees with moderate or low *C. undatus* attack. However, a significant difference between the trees with moderate or very low attack was observed in the content of phenolics, which was 1.5 times higher in the trees with very low *C. undatus* attack. The potential toxic activity of phenolic compounds may have a role in decreasing larval feeding and survival. This result points out the need for targeted further research on a detailed analysis of the phytochemical composition of metabolites in the phellogenic and inner cork layers of *Q. suber* trees with differing *C. undatus* attacks. A relationship between phenolic contents and tree genome and physiological status should also be further investigated.

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