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Chemical composition and antioxidant activity of phenolic extracts of cork from *Quercus suber* L.

Sónia A.O. Santos, Paula C.R.O. Pinto[1], Armando J.D. Silvestre*, Carlos Pascoal Neto

CICECO and Department of Chemistry, University of Aveiro, Aveiro 3810-193, Portugal

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ABSTRACT

The phenolic fraction of cork from *Quercus suber* L. was obtained following two distinct fractionation schemes, namely methanol/water extraction followed by ethyl ether fractionation and sequential extraction with methanol and water. The extracts were studied in terms of total phenolics content, using Folin-Ciocalteu method, detailed chemical analysis by HPLC–MS, and antioxidant activity. The first method underestimates both total extractives, total phenolics as well as the amounts of identified compounds. The HPLC–MS, revealed that, apart from smaller components, all the extracts displayed the same qualitative composition; 15 phenolic components were identified, with ellagic acid, followed by gallic and protocatechuic acids as the most abundant compounds. Additionally some compounds identified were reported for the first time as cork components, namely salicylic acid, eriodictyol, naringenin, quinic acid and hydroxyphenyllactic acid. The antioxidant activity of the extracts, evaluated using the 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH) radical scavenging assay, showed to be considerably higher than that of BHT, and in the range of ascorbic acid. The antioxidant potential *per* mass unit of the three extracts is in the same range, but the high extraction yields obtained by water extraction open good perspectives for the exploitation of this extract in nutraceutical applications.

1. Introduction

The up-grading of the by-products of the forestry industry constitutes an important challenge on the development of a sustainable economy and of environmentally friendly industrial processes. These by-products are seen, in recent years, as promising sources of renewable chemicals, materials and fuels and as a response to the inevitable depletion of fossil resources within the emerging "bio-refinery" concept (Kamm et al., 2006; Fernando et al., 2006).

Cork is the outer bark of *Quercus suber*, a common species in the Mediterranean region. Because of its peculiar properties (Gandini et al., 2006; Silva et al., 2005; Silvestre et al., 2008), such as high elasticity and low permeability, cork has a large variety of applications, among which the production of stoppers for wine and other alcoholic beverages is by far the most important, followed by its applications in thermal and/or acoustic insulation materials. Portugal produces about 157 000 ton of cork/year, which represents about 53% of the world production (APCOR, 2009). This industry generates substantial amounts of a residue, called "cork powder", which represents in Portugal, about 40 000 ton/year, 25% of the total cork production. This by-product, that is generally not suitable for current industrial uses, is currently mostly burned to produce energy (Gil, 1997). The full exploitation of this resource and specially the detailed study of its chemical composition is a key step towards the recovery of this sub-product.

Cork is mainly composed of lignin (~25%, w/w), polysaccharides (~20%, w/w), suberin (~40%, w/w), extractives (~15%, w/w), and inorganics (~1%, w/w) (Pereira, 1988; Pinto et al., 2009). Suberin, due to its abundance and unique composition is the most promising component for the development of new chemicals and materials from cork by-products (Gandini et al., 2006; Silvestre et al., 2008; Sousa et al., 2008; Turley, 2009). However, in an integrated biorefinery perspective, all cork components, extractives included, should be considered. Cork extractives are mainly composed of aliphatic, phenolic and triterpenic components. The detailed chemical composition of the lipophilic extractives of cork and cork by-products has been recently investigated by Sousa et al. (2006), demonstrating that this fraction could be an interesting source of bioactive triterpenic compounds.

However, the information available on the phenolic fraction of cork is scarce, despite the fact that this group of components can also be easily extracted from those residues. The total contents of polymeric polyphenols from cork (lignins and tannins), have been reported (Pereira, 1979, 1988), as well as some low molec-

* Corresponding author at: CICECO and Department of Chemistry, University of Aveiro, Campus de Santiago, Aveiro 3810-193, Portugal. Tel.: +351 234 370 711; fax: +351 234 370 084.
E-mail address: armsil@ua.pt (A.J.D. Silvestre).
[1] On Leave to LSRE and Chemical Engineering Department, Faculty of Engineering of the University of Porto, Porto 4200-465, Portugal.

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ular weight phenolic constituents of suberin (Zimmermann et al., 1985; García-Vallejo et al., 1997). Conde et al. (1997, 1998) have reported the presence of several phenolic acids, namely ellagic (also reported by Sousa et al., 2006), gallic, caffeic and protocatechuic acids, together with vanillin, protocatechuic aldehyde, coniferaldehyde and sinapaldehyde in extracts of cork from *Q. suber* L. The presence of ellagitannins, namely roburin, grandinin, and castalagin in extracts of cork (Cadahía et al., 1998) has also been reported.

The interest on natural phenolic compounds relies on the wide variety of relevant properties shown by this family, namely, among others, their antioxidant, anti-inflammatory, radical scavenger and antimicrobial properties (Balasundram et al., 2006; Noferi et al., 1997; Proestos et al., 2006). The interest in natural phenolic compounds for nutraceutical and cosmetic applications has increased considerably in recent years because of the mentioned properties but also because they do not show adverse effects as it is frequently the case of their synthetic counterparts (Ito et al., 1986).

In addition to the scarcity of studies on the composition of the phenolic fraction of cork extractives is limited; additionally, none of those studies accessed the antioxidant activity of the extracts. In this perspective, and within a wider project aiming at developing new strategies for the up-grading of cork by-products (Sousa et al., 2006, 2008; Pinto et al., 2009), in the present work we report the quantification of total phenolics content and detailed characterization of the cork phenolic fraction by HPLC–MS, obtained by methanol and water extraction. For comparative purposes the extracts and fractions obtained following a previously published procedure (Conde et al., 1997) were analyzed. The extracts were evaluated in terms of their antioxidant properties, using the 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH) radical scavenging assay (Sharma and Bhat, 2009).

2. Materials and methods

2.1. Chemicals

Gallic acid, Folin-Ciocalteu's phenol reagent, 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH) and 3,5-di-tert-4-butylhydroxytoluene (BHT) were supplied by Sigma Chemicals Co. Diethyl ether, and the HPLC-grade methanol, water and acetonitrile, were obtained from Fisher Scientific Chemicals. Formic acid, methanol, ascorbic acid and ellagic acid were purchased from Fluka Chemie. Vanillin, vanillic acid and ferulic acid were supplied by Aldrich Chemicals Co. The other phenolic compounds used, were isolated in our department from other plant sources.

All other chemicals and solvents were available in our laboratories and used without any further purification.

2.2. Raw material

Q. Suber L. natural (WNC) cork planks ("amadia" grade) were sampled from the south of Portugal (Herdade da Moinhola, Amorim Florestal). An average sample composed of fragments of several planks from different trees was milled in a Retsch cross beater mill SK1 (Haan, Germany), and the granulometric fraction of 40–60 mesh was used for analyses.

2.3. Phenolics extraction

About 20 g of the cork powder sample was submitted to a soxhlet extraction with dichloromethane for 6 h to remove the lipophilic components. Then, the solid cork residue was divided into two fractions (I and II), which followed distinct extraction pathways. Fraction I was suspended in a methanol–water mixture, 80/20 (v/v), at room temperature for 24 h under constant stirring (Conde et al., 1997). The suspension was then filtered and MeOH removed by low-pressure evaporation. The methanol free aqueous solution was then extracted three times with diethyl ether, and the solvent was then removed in the rotary evaporator yielding extract A.

Fraction II was submitted to a methanol extraction for 6 h (extract B), followed by a reflux with water for 6 h. The solvents were then removed from the liquid extracts by low-pressure evaporation and freeze drying yielding extracts B and C, respectively.

2.4. HPLC–MS analysis

Liquid chromatography of the extracts was carried out on a HPLC system HP 1050 equipped with a Rheodyne injector with a 10 μL loop, a quaternary pumping system and a UV detector. The wavelengths used to detect the phenolic compounds were 280 and 340 nm. The column used was a Discovery® C-18 (15 cm × 2.1 mm × 5 μm) supplied by Supelco. The elution was performed with water (A) and acetonitrile (B) both containing 0.1% of HCOOH; the gradient profile was as follows: 0 min, 10% B; 80 min, 100% B and then held for 30 min before returning to the initial conditions. The flow rate was 0.2 mL/min. Mass spectrometry analysis was performed using a Micromass spectrometer (Manchester, UK) equipped with an electrospray source and a triple quadrupole analyzer. The cone voltage was between 30 and 50 V, and the capillary voltage ranged from 2.6 to 2.9 kV. The source temperature was 143 °C and the desolvation temperature was 350 °C. MS/MS spectra in the negative mode were obtained using argon as collision gas with the collision energy set between 10 and 45 V.

Gallic and ellagic acids were used as reference compounds for quantitative analysis, at 280 and 340 nm, respectively and with concentration ranging between 0.05 and 1.5 mg/mL.

2.5. Total phenolic content

The total phenolic content of the extracts was determined by the Folin-Ciocalteu method (Singleton and Rossi, 1965). 2.5 mL of Folin-Ciocalteu reagent, previously diluted with water (1:10, v/v), and 2 mL of aqueous sodium carbonate (75 g/L) were added to accurately weighed aliquots of the extracts dissolved in 0.5 mL of water for the extract C and in methanol for the others, corresponding to concentration ranging between 40 and 310 μg of extract/mL. Each mixture was kept for 5 min at 50 °C and, after cooling, the absorbance was measured at 760 nm, using a UV/Vis V-530 spectrophotometer (Jasco, Tokyo, Japan). The total phenolic content was calculated as gallic acid equivalent from the calibration curve of gallic acid standard solutions (1.5–60.0 μg/mL) and expressed as g gallic acid equivalent (GAE)/g of dry extract and as g GAE/kg of dry cork. The analyses were carried out in triplicate and the average value was calculated in each case.

2.6. Antioxidant activity

The antioxidant activity of the extracts A and B was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging (Sharma and Bhat, 2009). In test tubes, 0.25 mL of DPPH 0.8 mM in MeOH was added to accurately weighed aliquots of the extracts dissolved in 3.75 mL of MeOH, corresponding to concentration ranges of extract between 0.3 and 90 μg/mL. The antioxidant activity of extract C was similarly determined. In this case 0.25 mL of DPPH 0.8 mM in MeOH was added to 1.00 mL of the aqueous solution of the extract and 2.75 mL of MeOH, corresponding to concentrations of extract between 2.5 and 10 μg/mL. After mixing, the samples were maintained in the dark, at room temperature for 30 min. The absorbance at 517 nm was measured using a UV/Vis V-530 spectrophotometer (Jasco, Tokyo, Japan) and compared with a control without extract. A blank was prepared for each sample using methanol instead of the DPPH solution. Ascorbic acid and

3,5-di-tert-4-butylhydroxytoluene (BHT) were used as reference compounds.

Antioxidant activity was expressed as a percent inhibition of DPPH radical, and calculated from the equation:

$$\text{Scavenging activity}\,(\%) = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

IC_{50} values were determined from the plotted graphs of scavenging activity against the concentration of the extracts. These values are defined as inhibitory concentration of the extract necessary to decrease the initial DPPH radical concentration by 50% and are expressed in μg/mL. Triplicate measurements were carried out. The antioxidant activity was also expressed in g of ascorbic acid equivalents/kg of dry cork (g AAE/kg dry cork).

3. Results and discussion

3.1. Extraction yields and total phenolic content

The extraction yields of the studied cork extracts and the corresponding total phenolic contents are shown in Table 1. These extraction yields were obtained after removal of the dichloromethane soluble fraction, which accounted for ~3.6% of cork weight, in agreement with previously published data (Sousa et al., 2006). The yields found are within the typical values found for "amadia" grade cork (Pereira, 1988; Sousa et al., 2006).

The total phenolic contents, determined by the Folin-Ciocalteu method for the three extracts are in range of 0.20–0.35 g of gallic acid equivalents/g of extract, however, when expressed in g GAE/kg cork, the total phenolic content is significantly higher in the water extract (C), followed by the methanol extract (B), as a consequence of the corresponding increasing extraction yields. The total phenolic content for cork extracts ranged from 2.4 to 10.6 g/kg of dry cork extracts, which are within the range of those obtained for a large variety of plant materials (Sánchez-Moreno et al., 1998; Kähkönen et al., 1999). Likewise, the phenolic content for extract A presents a value of 2.4 g GAE/kg of dry cork, which is coincident with those previously reported for diethyl ether extracts from *Q. suber* cork (Conde et al., 1997, 1998; Cadahía et al., 1998). However, when considered together, extracts B and C account for 16.5 g GAE/kg of dry cork, a value considerably higher than that of extract A, obtained through the fractionation method proposed by Conde et al. (1997). These results show that a considerable fraction of phenolic components are not extracted due to mild conditions of MeOH:H_2O extraction and also to the subsequent ethyl ether extraction, both limiting the yield of extract A.

Table 1
Extraction yields and total phenolic contents of cork extracts from *Q. suber* L.

Extract	Yield	Total phenolics content	
	(g/kg of dry cork)	g GAE/g of extract	g GAE/kg of dry cork
A	12	0.20 ± 0.04	2.4 ± 0.5
B	17	0.35 ± 0.01	5.9 ± 0.1
C	37	0.29 ± 0.02	10.6 ± 0.6

Finally, the high content of phenolic components in the water extract (C) shows that it is possible to use water instead of more harmful systems to isolate this fraction, which could be particularly interesting when nutraceutical applications are searched for these extracts.

3.2. HPLC–MS analysis

The HPLC–MS identification of phenolic compounds was carried out by comparing peak retention times and fragmentation profiles with reference compounds run under the same experimental conditions and/or with published data. The phenolic compounds identified in the studied extracts, as well as their quantification, retention time, molecular ion $[M-H]^-$ values and the corresponding MS/MS fragmentation peaks are reported in Table 2; the corresponding structures are shown in Fig. 1.

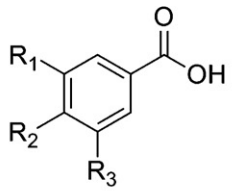
The major components identified in all fractions (A–C) were ellagic acid, followed by gallic, protocatechuic and caffeic acids and esculetin. Smaller amounts of vanillin, vanillic, coumaric, and ferulic acids were identified in extract A, and *p*-hydroxybenzoic acid in extract C. All these compounds were identified by comparison of their retention times and fragmentation patterns with those of standard compounds or by comparison with published data (see Table 2) and were previously reported in the literature (Conde et al., 1998; Mazzoleni et al., 1998) as cork components.

In addition to the known compounds, the ESI-MS analysis also allowed to identify for the first time in cork extracts several components, namely salicylic acid, eriodictyol and smaller amounts of

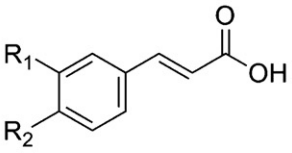
Table 2
HPLC–MS evaluation of the extracts of cork from *Q. suber* L. Expressed as mg/kg of dry cork (compounds in bold were identified for the first time in this species).

Rt (min)	Compound	Extracts			MS Data		Identified
		A	B	C	$[M-H]^-$	ESI-MS/MS fragments	
2.80	**Quinic acid**		TR		191	173, 127, 111, 93, 85	Lit.
3.86	Gallic acid	30.6	48.1	241.6	169	125	Co
6.27	Protocatechuic acid	17.5	59.0	118.3	153	109	Co
6.71	***p*-Hydroxyphenyllactic acid**	–	–	TR	181	137, 113, 109	
9.55	*p*-Hydroxybenzoic acid	–	–	1.0	137	93	Co
11.20	Esculetin	4.9	106.7	–	177	133, 105	Lit.
11.56	Caffeic acid	57.6	-	12.9	179	135	Co
13.97	Vanillin	14.3	TR	–	151	136	Co
14.96	Vanillic acid	TR	TR	–	167	152, 108	Lit.
15.13	*p*-Coumaric acid	TR	–	–	163	119, 95	Co
16.25	Ferulic acid	TR	–	–	193	178, 149, 134, 117	Co
16.42	Ellagic acid	2031.5	1576.9	526.5	301	284, 245, 229, 201, 185, 173, 157, 145	Co
20.32	**Salicylic acid**	32.7	–	–	137	93	Co
24.72	**Eriodictyol**	27.4	–	–	287	151, 135,	Lit.
28.41	**Naringenin**	2.6	–	–	271	177, 151, 119, 107	Co
	Total (mg/kg dry cork)	**2219.1**	**1790.7**	**900.3**			
	Total (mg/g extract)	**184.9**	**105.3**	**24.3**			

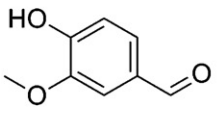
TR, identified compound but not possible to quantify by overlapping of peaks in HPLC chromatogram.
Co, identified by co-injection and ESI fragmentation of a reference sample.
Lit., identified by comparing the ESI fragmentation with the literature (please see the body of the text).



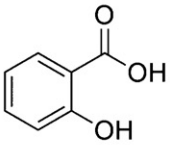
$R_1 = R_3 = H$, $R_2 = OH$ - *p*-hydroxybenzoic acid
$R_1 = R_2 = OH$, $R_3 = H$ - Protocatechuic acid
$R_1 = R_2 = R_3 = OH$ - Gallic acid
$R_1 = OCH_3$, $R_2 = OH$, $R_3 = H$ - Vanillic acid



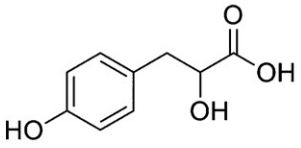
$R_1 = R_2 = H$ - Cinnamic acid
$R_1 = H$, $R_2 = OH$ - *p*-coumaric acid
$R_1 = R_2 = OH$ - Caffeic acid
$R_1 = OCH_3$, $R_2 = OH$ - Ferulic acid



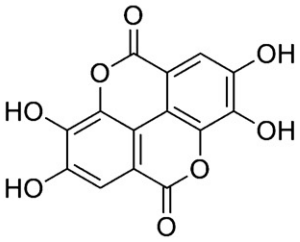
Vanillin



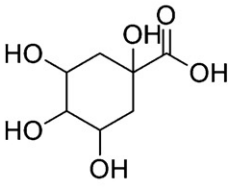
Salicylic acid



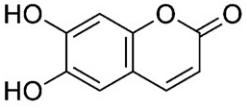
p-hydroxyphenyllactic acid



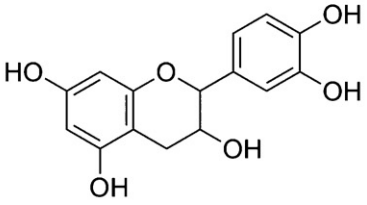
Ellagic acid



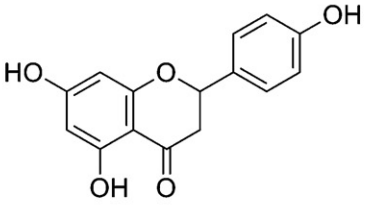
Quinic acid



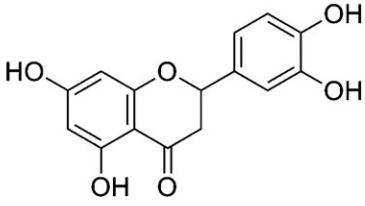
Esculetin



Catechin



Naringenine



Eriodictyol

Fig. 1. Structures of the compounds identified in the extracts of cork from *Q. suber* L.

naringenin in extract A, quinic acid in extract B and hydroxyphenyllactic acid in extract C.

Salicylic acid, shows a ESI-MS profile with a $[M-H]^-$ fragment at m/z 137, which under MS/MS conditions yields fragments at m/z 93 characteristic of this compound, as it was confirmed by using the respective standard. The $[M-H]^-$ and the fragments of naringenin at m/z 271 and 177, 151, 119 and 107, respectively, are also in tune with the fragmentation suffered by the standard compound. In the case of eriodictyol the $[M-H]^-$ fragment at m/z 287 and the corresponding daughter ions at m/z 151 and 135 are also in agreement with published data (Sudjaroen et al., 2005). Quinic acid also shows a fragmentation in accordance with the literature (Ng et al., 2004), with a $[M-H]^-$ at m/z 191 and fragments at m/z 173, 127, 111, 93 and 85. Hydroxyphenyllactic acid shows a $[M-H]^-$ fragment at m/z 181. Although there is no reference about the ESI fragmentation of this compound, its spectrum revealed the typical fragmentation of its constituents groups, with fragment ions at m/z 137 and 109, due to the losses of –COO and –CO groups, respectively.

Quinic acid is a derivative of chlorogenic acid, which can be found in many plant species (Ng et al., 2004), including teas (Dufresne and Farnworth, 2001), but it has never been reported in cork extracts. Quinic acid is used as precursor of the synthesis of compounds with pharmaceutical applications and, interestingly, in the treatment of some influenza strains (Bianco et al., 2001), however, considering its low abundance cork cannot be considered as an exploitable source of this compound. Naringenin is known to be present in citrus fruits (Bilbao et al., 2007). However, its existence together with eriodictyol, was already reported in extracts of *Populus tremula* and in some pine species (Hartonen et al., 2007; Lantto et al., 2009).

Ellagic acid is the predominant phenolic compound in all the extracts, with contents of ~2.0 g/kg of dry cork in extract A, and ~1.6 and ~0.5 g/kg of dry cork in extracts B and C, respectively; these values are in the range of those previously reported for natural cork extracts (Sousa et al., 2006; Conde et al., 1997), taking into consideration the well-known natural variability of cork composition (Conde et al., 1998).

In general, the phenolic components of the studied extracts were quantified in higher amounts considering the sum of extracts B and C (accounting for a total of ~2.7 g/kg), than in the extract A (a total of 2.2 g/kg).

However, while the increase in the total amounts of phenolic compounds detected by HPLC–MS in extracts B and C (in mg/kg dry cork) is in the order of 21%, when compared to extract A, in the case of the corresponding total phenolics contents (Table 1), the increase was considerably higher (roughly a sevenfold difference). This means that a considerable fraction of phenolic compounds was not detected even by HPLC–MS under the experimental conditions used. These should certainly correspond to high molecular weight cork tannins (Conde et al., 1998; Pereira, 1979). In addition some non-phenolic compounds, interfering in the Folin-Ciocalteu method and leading to an over-estimation of total phenolics (Prior et al., 2005) can contribute to the high values reported in both methanol (B) and water (C) extracts.

3.3. Antioxidant activity

Table 3 shows the antioxidant activity of the studied extracts, expressed in terms of the amount of extract required to reduce into 50% the DPPH concentration (IC_{50}), as well as in terms of the ascorbic acid equivalents (AAE) on a dry cork basis (mg AAE/g dry cork). The IC_{50} values for ascorbic acid and for BHT were also obtained and reported in Table 3 for comparative purposes.

These extracts have revealed an antioxidant activity considerably higher than that reported for BHT and in the range of that measured for ascorbic acid. These observations are in agreement with reported results, which demonstrate that the antioxidant activity of some phenolic compounds could be higher than ascorbic acid and BHT (Sánchez-Moreno et al., 1998; Cuvelier et al., 1992). Interestingly the antioxidant activities of the studied extracts are significantly higher than those reported for some wines, recognized for its antioxidant properties (Roussis et al., 2008).

Table 3
Antioxidant activity of the extracts of cork by DPPH radical scavenging, expressed as IC_{50} values, in μg of extract/mL, and as mg of ascorbic acid equivalents/g of dry cork.

	IC_{50} (μg/mL)	mg AAE/g dry cork
Ascorbic acid in MeOH	2.12 ± 0.06	–
Ascorbic acid in H_2O	2.46 ± 0.11	–
BHT	18.79 ± 0.22	–
Extract A	2.79 ± 0.15	9.15 ± 0.51
Extract B	3.58 ± 0.20	10.11 ± 0.54
Extract C	5.84 ± 0.29	15.59 ± 0.75

In general the obtained IC_{50} values, demonstrated a higher antioxidant activity for extract A, followed by extracts B and C. This decreasing antioxidant activity cannot be directly related with the amounts of components detected by HPLC–MS in each extract as the decrease of this fraction, (in mg/g of extract, see last row of Table 2), is much more substantial, nor with the total phenolics content (in g GAE/g of extract, Table 1), but rather it should be the result of a combination of the effect of both the detected components along with the non-detected phenolic fractions.

Finally, the antioxidant capacity as AAE on a dry cork basis, increased proportionally to the extraction yields and total phenolic content (Table 1), with extract C reaching the higher antioxidant potential. These results are promising since cork water extracts can be used with obvious advantages as natural antioxidants in nutraceutical applications, when compared to organic solvent extracts.

4. Conclusions

In this work different cork extracts from *Q. suber* L. were obtained following two distinct fractionation schemes, namely methanol/water extraction followed by ethyl ether fractionation and sequential extraction with methanol and water. The extracts were studied in terms of total phenolics (Folin-Ciocalteu method), detailed composition by HPLC–MS, and antioxidant activity. The HPLC–MS allowed to identify 15 phenolic compounds, among which ellagic acid followed by gallic and protocatechuic acids were the most abundant, and several others were reported for the first time as cork components (namely salicylic acid, naringenin, eriodictyol, quinic acid and hydroxyphenyllactic acid). To the best of our knowledge, this is also the first paper describing the phenolic compounds present in a H_2O extract of cork. The antioxidant activity of the extracts, evaluated using the DPPH radical scavenging assay, showed to be considerably higher than that of BHT, and in the range of that of ascorbic acid. The antioxidant potential *per* mass unit of the three extracts is in similar ranges, but the high extraction yields obtained by water extraction constitutes a promising result for its exploitation in nutraceutical applications, as well for the valorization of cork as a renewable resource.

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