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Antioxidant activity of fractions from *Quercus sideroxyla* bark and identification of proanthocyanidins by HPLC-DAD and HPLC-MS

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Abstract

The most active phenolics in *Quercus sideroxyla* Humb. & Bonpl. residual bark were identified and evaluated following a chromatographic fractionation. Bark powder was defatted with hexane and crude extract (CE) was obtained by extraction with aqueous acetone (70%). A liquid partition with ethyl acetate was performed to produce an organic extract (OE), which was subsequently purified by column chromatography (Toyopearl HW-40F, methanol), and resulted in six methanolic fractions (MF1 to MF6) and an oligomeric fraction (OLF) eluted with acetone 67%. Extraction yields, total phenolic and flavanol contents were determined. The antioxidant activity of bark extracts was measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic-acid)-equivalent antioxidant capacity (TEAC), and ferric ion reducing antioxidant power (FRAP) assays. Their median effective concentration (EC_{50}) data and rate constants for DPPH radical scavenging were also estimated. Identification of major phenolics was carried out by high performance liquid chromatography with diode array detection (HPLC-DAD) and high performance liquid chromatography with electrospray ionization coupled to mass spectrometry (HPLC-ESI-MS) instruments. Bioactive gallic acid, catechin, epicatechin, gallocatechin, catechin gallate, dimeric procyanidins, galloylated dimeric proanthocyanidins, trimeric procyanidins, and tetrameric proanthocyanidins were detected and identified in *Q. sideroxyla* bark extracts. MF2 was the most active fraction containing gallocatechin as its major compound; MF5 and OLF contain galloylated procyanidins, which may explain their higher antiradical activity. OLF besides galloylated procyanidins has gallocatechin, which presumably contributes to its higher antiradical activity. Consequently, *Q. sideroxyla* bark could be a good source of therapeutic health products or nutraceutical ingredients that may exert a potential prevention or treatment action against diseases in biological systems.

Keywords: antiradical activity; bark; HPLC; proanthocyanidins; *Quercus sideroxyla*.

Introduction

Proanthocyanidins are oligomers and polymers composed mainly of monomeric flavan-3-ol units; they are the distinctive polyphenolic group in condensed tannins. After lignin, proanthocyanidins are the second most abundant group of natural phenols in the plant kingdom and one of the most widespread polyphenolic biflavonoids in herbs and spices (Porter 1993). Proanthocyanidins (polyphenolics) in wood and bark of trees are in focus of research since the beginning of modern wood science. Recent publication demonstrates that their chelating and antioxidant properties are of increasing importance (Willför et al. 2004; Zulaica-Villagomez et al. 2005; Gao et al. 2006; Donoso-Fierro et al. 2009). Also in the context of the development of environmentally benign wood preservatives of extractives are the antioxidant properties and chelating characteristics of flavons and flavonoids in discussion (Binbuga et al. 2005, 2008). Polyphenolic compounds from barks are especially relevant with this regard and in terms of biorefinery (Makino et al. 2011; Telysheva et al. 2011). The availability of modern isolation and characterization methods contribute a lot for advances in this field of science. For example, Liimatainen et al. (2011) used high performance liquid chromatography with diode array detection (HPLC-DAD) and high performance liquid chromatography with electrospray ionization coupled to mass spectrometry (HPLC-ESI-MS) instruments for structural elucidation of phenolic compounds from the inner bark of *Betula pendula*. Yao et al. (2010) discovered new phenolic glucosides and flavonoids in the bark of *Eucommia ulmoides*. Reaction of polyphenols with 2,2-diphenyl-1-picrylhydrazyl (DPPH) frequently plays an important role for characterization of their biotic activities (Smeds et al. 2011).

Proanthocyanidins are divided into different classes depending on the substitution pattern of the flavan unit (Figure 1). The most common are the procyanidins, which

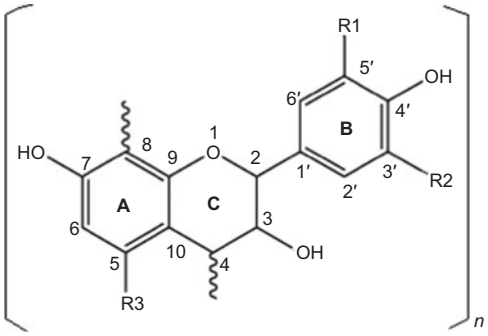

Figure 1 Chemical structure of proanthocyanidins: procyanidins (R1, R3=OH; R2=H), prodelphinidins (R1, R2, R3=OH), prorobinetidins (R1, R2=OH; R3=H), and profisetinidins (R1=OH; R2, R3=H).

are chains of catechin or epicatechin and gallic acid esters, and prodelphinidins, which consist of gallocatechin, epigallocatechin, and their derivatives as galloylated monomer units. Both proanthocyanidins are abundant in plant foods, while the leather tanning and adhesive industries utilize proanthocyanidins obtained from mimosa, which are predominantly prorobinetidins based on robinetinidol, and quebracho, which are profisetinidins based on fisetinidol (Hemingway 1989; Cheynier and Fulcrand 2003).

Proanthocyanidins are widely distributed in woody plants and are present mainly in bark, while prodelphinidins are the major constituents in leaves (De Bruyne et al. 1999). In *Quercus* species, it has been reported that flavanols and phenolic acids are in a wide range of polarity, such as gallic acid, ellagic acid, and ellagitannins, also known as hydrolyzable tannins, like vescalagin and castalagin (Peng et al. 1991; Viriot et al. 1993; Dudonné et al. 2009). It has been also reported in *Quercus* spp. that gallocatechin (Haslam 1975), procyanidins (Matthews et al. 1997), catechin, hexagalloylglucose, prodelphinidins and dimeric proanthocyanidins (Sun et al. 1987), and trimers of catechin gallate (Singh et al. 2003; Andersen and Markham 2006) are present.

All polyphenols including proanthocyanidins are able to scavenge reactive species such as $^1O_2^-$, OH·, NO·, and alkyl peroxyl radicals through electron-donating mechanisms, generating a relatively stable phenoxyl radical. The antioxidant activity of proanthocyanidins depends on factors such as structure, number of phenolic hydroxyl groups, and degree of polymerization. The antioxidant activity of these compounds is due to their radical scavenging capacity and their redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers. They also have metal-chelating properties, and it has been indicated that are effective antioxidants in blood plasma, delaying the lipid oxidation (Santos-Buelga and Scalbert 2000; Cos et al. 2004; Mayer et al. 2008). Proanthocyanidins can be absorbed by cell membranes and protect them from the action of free radicals. They have the advantage of being amphipathic molecules, having hydrophobic aromatic rings and hydrophilic weak acid hydroxyl groups, so unlike other antioxidants that do not possess this duality, they are able to cross cell membranes, which are susceptible to damage from free radicals (Lee et al. 2004).

Quercus sideroxyla (Fagaceae) is the main *Quercus* species used for wood production in Durango, Mexico's forestry industry. A great amount of residual leaves and bark is generated, and its storage or elimination represents ecological and economic problems. For this reason, an integral utilization approach of these by-products for recovering antioxidant compounds may represent a source for bioactive principles or nutraceutical ingredients (Rivas-Arreola et al. 2010; Rosales-Castro et al. 2011). Therefore, the aim of this study was to analyze the antioxidant capacity and identify the most active compounds in *Q. sideroxyla* barks by means of a chromatographic fractionation.

Materials and methods

Plant material

Bark from a *Quercus sideroxyla* Humb. & Bonpl. 70-year-old tree was collected at 1.20 m height in El Salto, Durango, about 120 km southwest from Durango, México. The sample was identified and a botanical specimen deposited at the Herbarium of CIIDIR Durango-Instituto Politécnico Nacional, voucher number 27720. The bark was air dried at room temperature (24°C), milled to mesh 40, and stored in paper bags under refrigeration until further use.

Chemicals

The radical DPPH, Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic-acid), 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS), TPTZ (2,4,6-tripyridyl-s-triazine), ascorbic acid, phosphoric acid, acetic acid, Folin's reagent and vanillin were purchased from SIGMA (Sigma, St Louis, MO, USA). For compound identification, the standards gallocatechin, catechin, epicatechin, gallocatechin gallate, catechin gallate, gallic acid, ellagic acid from Sigma-Aldrich (Sigma, St Louis, MO, USA), and the HPLC-grade solvents, water and acetonitrile, were used.

Extraction and purification

The bark powder was defatted with n-hexane (2×400 ml). A sample (40 g) was extracted twice soaking with aqueous acetone 70% (2×400 ml) at room temperature for 24 h. The extracts were combined, filtered, and then evaporated under vacuum at 30°C until acetone was removed. A portion of the remaining aqueous extract was lyophilized and identified as crude extract (CE), while another portion was subjected to liquid partition with ethyl acetate (3×200 ml). The organic phase was evaporated to dryness under vacuum at 30°C and identified as the organic extract (OE). Yields based on dry bark or dry extract basis were calculated.

Separation by column chromatography

Dried OE containing monomeric and oligomeric polyphenols was dissolved in MeOH and placed in a Toyopearl HW-40F (Tosoh Bioscience LLC, King of Prussia, PA, USA) column (50×500 mm). Elution was performed with MeOH at 1 ml min^{-1}, during 12 h, which

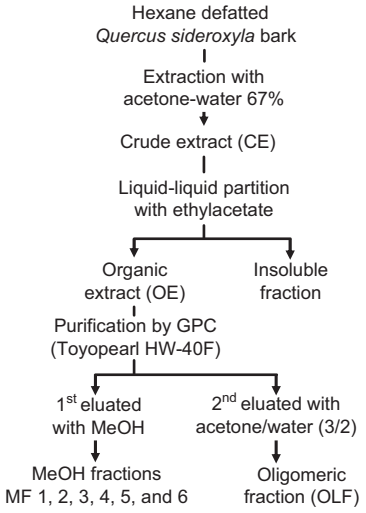

Figure 2 Chromatographic fractionation of extracts from *Quercus sideroxyla* bark.

led to six methanolic fractions (MF1 to MF6). Afterwards, the column was eluted with 1 l of acetone-water (3:2) to obtain the remaining fraction, an oligomeric fraction (OLF) as shown in Figure 2. Yields based on dry OE were calculated.

Total phenolics

Total phenolics (TP) were determined by the Folin-Ciocalteu method (Waterman & Mole 1994), with gallic acid as standard. Briefly, to a diluted sample extract (0.1 ml), 6 ml of deionized water and 0.5 ml of Folin-Ciocalteu reagent were added. The mixture was allowed to stand (6 min), and then, 1.5 ml of Na_2CO_3 (20% w/v) was added. The final volume was adjusted to 10 ml with deionized water. The mixture was allowed to stand (120 min), and the absorption was measured at 760 nm against a blank prepared similarly but containing distilled water instead of extract. The results are expressed as equivalent mg of gallic acid per g of extract (mg GAE g^{-1}).

Total flavanols

The analysis of flavanols was carried out according to Heimler et al. (2005) with (+)-catechin as standard. Briefly, to a diluted sample (50 µl), the following substances were added: 3 ml of a 4% (v/v) MeOH vanillin solution and 1.5 ml of concentrated HCl. The mixture was allowed to stand (15 min), and the absorption was measured at 500 nm against MeOH as a blank. The results are expressed as equivalent mg of catechin per g of extract (mg CEQ g^{-1}).

HPLC-diode array detector

The CE, OE, and MF2 were analyzed in a Perkin Elmer 200 HPLC system with photodiode array detector, column Perkin Elmer C_{18} (4.6×250 mm, 5 µm), flow rate 1.0 ml min^{-1} (Campos and Markham 2007). The mobile phase comprised water adjusted to pH 2.8 with phosphoric acid (A) and acetonitrile (B). A linear gradient program was applied: 0–15 min, 86–80% of A; 15–60 min, 80–30% of A. Authentic standards: Gallocatechin, catechin, epicatechin, catechin gallate, gallocatechin gallate and gallic acid. Detection: at 270 nm.

HPLC-electrospray-MS

The separation of compounds from MF5 fraction was carried out in a Phenomenex ODS-C_{18} column (4.6×250 mm, 5 µm), by means of a Thermo Finnigan Surveyor equipped with a quaternary pump. The mobile phases were water containing 0.6% acetic acid (A) and acetonitrile (B). The flow rate was1.0 ml min^{-1} for a total run time of 45 min. The gradient program: 0–25 min, 95%–70% of A; 25–30 min, 70–40% of A; 30–35 min, 40–60% of A; and 35–45 min, 60–95% of A.

HPLC-MS instrument: Finnigan LCQ Deca ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray ionization (ESI) source. Data were collected in negative mode. The sheath gas and auxiliary flow rates were set at 96 and 7 (arbitrary units), respectively. The capillary voltage was set at 29 V, and its temperature was controlled at 350°C. The ESI needle voltage was controlled at 4.5 kV. The electron multiplier voltage was set at -980 V for ion detection (Li et al. 2008).

The HPLC-MS analysis for OLF was performed in a Prominence HPLC system (Shimadzu, Columbia, MD, USA) coupled to a 4000 qTrap mass spectrometer (Applied Biosystems Inc, Concord, Ontario, Canada) equipped with a turbo ion-spray interface. Mass spectra were obtained by acquiring data between 200 and 1200 amu (m/z) in negative mode. Source temperature was set at 550°C, declustering potential (DP) at 70, entrance potential (EP) at 10, and ion-spray voltage at 4200 V. Five-microliter loop injections were measured for each fraction. HPLC grade acetonitrile (Fisher Scientific, Fairlawn, NJ, USA), HPLC-grade water (Mallinckrodt Baker Inc., Phillipsburg, NJ, USA), and 0.1% formic acid were applied.

DPPH radical scavenging

The DPPH assay was carried out according to Sanchez-Moreno et al. (1998). This assay was used to select the fractions with the highest antiradical activity after chromatographic separation. Samples of CE, OE, methanolic fractions (MF1 to MF6), and OLF were evaluated at 100 µg ml^{-1}. The DPPH radical scavenging activity of selected fractions was evaluated at three concentrations to determine their EC_{50} data. Samples and standards dissolved in MeOH (50 µl) were mixed with 1950 µl of a methanolic solution of DPPH· [2.4 mg (100 ml)$^{-1}$]. After incubation for 30 min at room temperature, the decrease in absorbance was measured at 515 nm in a Varian 50 Bio Spectrophotometer. The antioxidant (antiradical) activity was expressed as inhibition (%) or scavenging capacity, as shown in Table 1, and as the median effective concentration (EC_{50}) in Table 2.

TEAC or ABTS assay

The Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic-acid)-equivalent antioxidant capacity (TEAC) assay is based on the ability of antioxidants to scavenge the blue-green $ABTS^{\bullet+}$ radical cation (ABTS) compared with the scavenging ability of the water-soluble vitamin E analog Trolox. The $ABTS^{\bullet+}$ radical cation was generated by reaction of ABTS (7 mM) and potassium persulfate (2.42 mM) (Re et al. 1999). The absorbance at 734 nm was monitored at 1 and 6 min after the addition of $ABTS^{\bullet+}$ solution (960 µl) to 40 µl of extract or Trolox standards in ethanol. Decrease in the absorbance was read and reported as mM of Trolox equivalents (TEAC, mM).

FRAP assay

The ferric ion reducing antioxidant power (FRAP) was done according to Benzie and Strain (1996) with some modifications. The stock

Table 1 Extraction yields, contents of total phenolics and flavanols, and the antioxidant activities of extracts and purified fractions from *Quercus sideroxyla* bark.

Extract or fraction	Total yield (%)	Total phenolics[4] (mg GAE g^{-1})	Total flavanols[4] (mg CEQ g^{-1})	Antioxidant activities determined by free radical scavenging from an extract concentration of 100 μg ml^{-1}		
				DPPH[4] (%)	ABTS[4] (TEAC, mM)	FRAP[4] (AAE, μM)
CE	24.3[3]	464.0±11.6[a]	277.3±9.4[b]	29.3±1.0[a]	318.8±0.9[a]	335.2±18.1[a]
OE	2.8[2]	686.7±12.9[c]	460.7±8.4[e]	41.5±0.4[c]	420.4±2.9[c]	494.3±6.5[c]
MF1	25.0[1]	730.4±2.6[d]	660.7±4.7[g]	28.2±2.0[a]	366.3±5.8[b]	386.4±7.6[b]
MF2*	6.5[1]	665.8±13.5[c]	412.3±2.4[d]	45.4±0.7[c,d]	525.8±0.3[f]	725.2±20.3[e]
MF3	2.0[1]	607.6±14.1[b]	165.7±7.1[a]	35.6±1.3[b]	367.0±1.7[a]	408.1±4.3[b]
MF4	6.7[1]	801.3±10.3[f]	635.7±11.8[f,g]	44.3±1.0[c]	449.6±1.6[b]	627.4±20.0[d]
MF5*	5.1[1]	777.6±15.4[e,f]	609.0±12.9[f]	51.6±0.2[e]	530.4±0.7[f]	670.2±26.3[d]
MF6	25.3[1]	765.8±1.3[e]	355.7±2.4[c]	48.9±1.5[d,e]	479.5±1.1[e]	465.9±10.3[c]
OLF*	26.5[1]	719.5±15.6[d]	357.3±9.0[c]	49.2±0.6[d,e]	490.0±4.7[e]	499.0±6.7[c]

Purified fractions (MF1 to MF6) were obtained by separation on column chromatography. CE, crude extract; OE, organic extract; OLF, oligomeric fraction; GAE, gallic acid equivalents; CEQ, catechin equivalents; DPPH, diphenyl-1-picrylhydrazyl assay; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) assay; TEAC, Trolox equivalent antioxidant capacity assay; FRAP, ferric reducing ability of plasma assay; AAE, ascorbic acid equivalent. *Selected fractions with high antiradical activity; [1]based on organic extracts; [2]based on crude extracts; [3]based on dry bark; [4]Different letters in the same column mean significant difference at $p<0.05$.

solutions included 300 mM acetate buffer, pH 3.6 (3.1 g $C_2H_3NaO_2$.3 H_2O and 16 ml $C_2H_4O_2$ per liter of buffer solution); 10 mM TPTZ solution in 40 mM HCl; and 20 mM $FeCl_3$.6 H_2O solution. The fresh working solution was prepared by mixing 25 ml of acetate buffer, 2.5 ml of TPTZ solution, and 2.5 ml of $FeCl_3$.6 H_2O solution.

Aqueous solutions of known ascorbic acid concentrations, in the range of 50–500 μM, were used for calibration. A sample (100 μl) of extract, standard, or blank were added to 3.0 ml of the FRAP solution for 10 min at 25°C. Readings of the colored product were then taken at 593 nm. Results are expressed in μM ascorbic acid equivalents (AAE, μM).

Rate constant and EC_{50} for DPPH radical scavenging

The EC_{50} and rate constant for DPPH radical scavenging was measured according to Manzocco et al. (1998). The bleaching rate of the stable free radical DPPH·, at a characteristic wavelength in the presence of the sample, was monitored. In its radical form, DPPH· absorbs light at 515 nm, but upon reduction by an antioxidant or a radical species, its absorption disappears. A volume of 2900 μl of DPPH· in MeOH (6.09×10^{-5}M) was added to 100 μl of sample (at its EC_{50}). The bleaching of DPPH· was monitored at 515 nm (Varian 50 Bio Spectrophotometer) at 25°C for at least 15 min.

The following equation was chosen in order to obtain the reaction rate, *k*:

$$1/A^3-1/A_0{}^3=-3kt$$

were A_0 is the initial optical density (O.D.), and *A* is the O.D. at time *t*. The rate constant *k* was expressed as -O.D.$^{-3}$ min^{-1} mg^{-1}.

Table 2 DPPH (diphenyl-1-picrylhydrazyl) antiradical activity of the most active fractions (MF2, MF5) selected from Table 1, compared with that of crude extract (CE) and organic extract (OE) of *Quercus sideroxyla* bark.

Extract or fraction	Data obtained by DPPH scavenging	
	EC_{50} (μg ml^{-1})	Rate constant *k* (-OD^{-3} min^{-1} mg^{-1})
CE	211.7±2.5[a]	0.544±0.09[a]
OE	140.9±0.6[b]	0.617±0.02[a]
MF2	110.0±1.9[c]	1.09±0.06[b]
MF5	97.0±0.8[c]	0.573±0.01[a]
OLF	102.0±1.0[c]	1.678±0.21[c]
Gallic acid	49.7±0.7[d]	3.7±0.18[d]
Catechin	84.2±0.6[c]	0.45±0.02[a]

Different letters in the same column means significant difference, $p<0.05$. EC_{50}, median effective concentration; OD, optical density.

Results and discussion

Extraction yields, total phenolic and flavonoid contents, and the antioxidant activities of the different extracts and fractions – as determined by reaction with DPPH, ABTS, and FRAP – are listed in Table 1. The chromatographic separation resulted in an effective purification of phenolic fractions from the CE as indicated by the total phenolic and flavanol contents. The highest phenolic contents have the MF5 fraction correlating well with the antioxidant responses. The flavanol concentration is highest in MF1, MF4, and MF5, corresponding well to monomeric and dimeric procyanidins, which were detected in these fractions.

The antioxidant activity results show that *Q. sideroxyla* bark extracts are able to stabilize DPPH or ABTS radicals or can act as metal-reducing agents (as indicated by the FRAP reaction). In the DPPH scavenging assay, OE has a higher activity than CE (41.5% vs. 29.3%), and the activity is still higher in subsequent purified fractions MF2 (45.4%), MF5 (51.6%), and OLF (49.2%). This is an indication for a

successful separation of active compounds concentrated now in the purified fractions. In the ABTS assay, the more active fractions are MF2 (528.8 mM), MF5 (530.4 mM), and OLF (490 mM) as well. As expected, ABTS results reveal the same trend as those from DPPH assay but are significantly higher, which is typical of pigmented and hydrophilic antioxidant extracts (Floegel et al. 2011). Although both assays have similar mechanisms, i.e., they are relying on the transferring of hydrogen atoms (Huang et al. 2005; Friaa and Brault 2006; Sendra et al. 2006), the DPPH method is more selective than ABTS in the reaction with H-donors (Roginsky and Lissi 2005).

In the FRAP assay, the best fractions (MF2 and MF5) are the same as in the case of the ABTS assay. The responses of fractions MF2 and MF5 are outstanding and differentiated. In this assay, the reducing ability of extracts is manifest through the conversion of ion Fe^{3+} to Fe^{2+}. This reaction is nonspecific, and any reaction that has lower redox potential under the assay conditions than the ferric-ferrous half reaction, will contribute to the ferrous ion formation. The change in absorbance is, therefore, directly related to the total reducing power of the electron-donating antioxidants present in the samples of the reaction mixture (Benzie and Strain 1999).

In Table 2, the antiradical activity is presented as the median EC_{50} displayed by the more active fractions from *Q. sideroxyla* bark in the following relative order: MF5>OLF>MF2>OE>CE. Fraction MF5 presented the highest antiradical activity (97 mg ml^{-1}) with a similar response as the standard catechin. However, in the DPPH scavenging reaction, the same fractions have deviating reaction order according to their rate constants (k, -DO^{-3} min^{-1} mg^{-1}): OLF>MF2>OE>MF5>CE, where OLF has the highest k (1.678 -DO^{-3} min^{-1} mg^{-1}). The OLF displays the best overall performance either in the stoiquiometric antioxidant capacity (i.e., EC_{50}) and the antioxidant reactivity (i.e., rate constant, k) (Roginsky and Lissi 2005). More active fractions (MF5 and OLF) and MF2 were analyzed by HPLC.

The chromatographic profiles (HPLC-DAD) from CE, OE, and fraction MF2 are displayed in Figure 3. The peaks were identified based on the retention times (RTs) of the authentic standards. Gallic acid eluted at 3.3 min and gallocatechin, at 4.3 min; both were observed at low concentration in CE and OE; however, the latter one was more concentrated in fraction MF2 as the major compound. Catechin (RT 7.4 min) and epicatechin (RT 10.5 min) are detectable mainly in OE, while the former is particularly concentrated in MF2. Additionally, catechin gallate was also identified in the extracts, although at low concentration.

The HPLC profile of fraction MF5 is presented in Figure 4 and their major peaks in mass spectra in Figure 5. Molecular

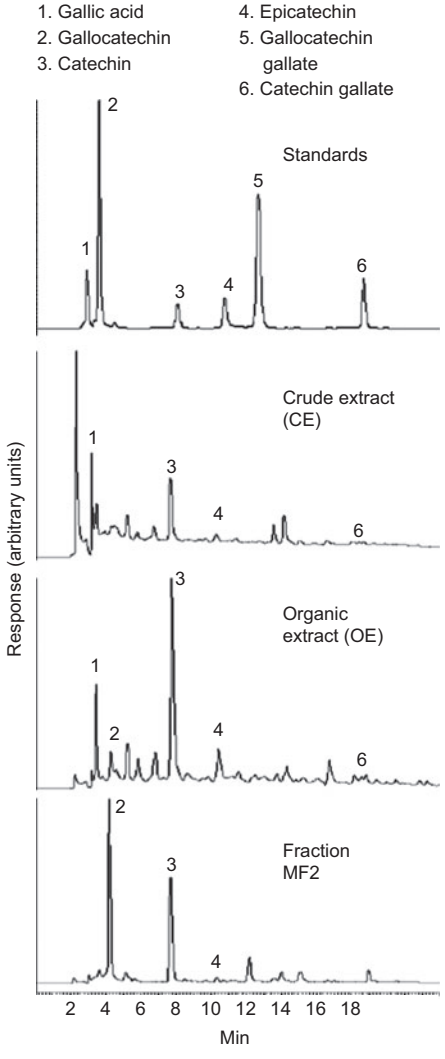

Figure 3 HPLC-DAD chromatograms recorded at 270 nm of CE, OE, and purified methanolic fraction MF2 from *Q. sideroxyla* bark.

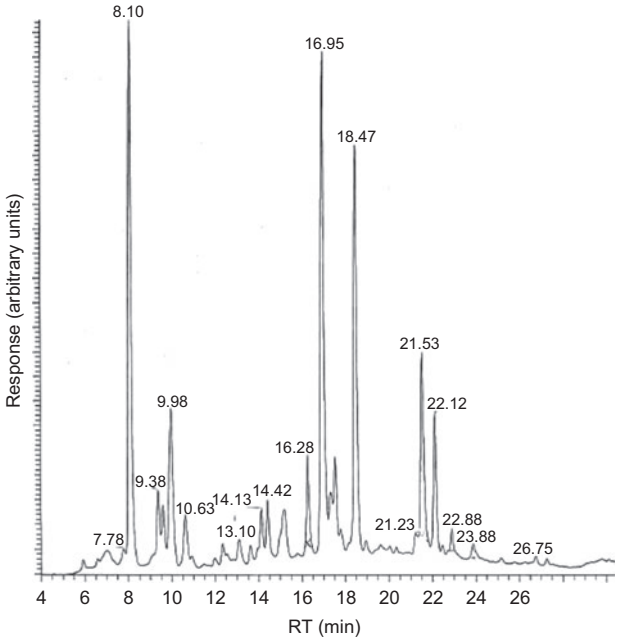

Figure 4 HPLC chromatogram of fraction MF5 from *Q. sideroxyla* bark obtained by Toyopearl HW-40F column chromatography, eluted with methanol. Mass spectra of major peaks are presented in Figure 5.

and fragment ions ([M-H]$^-$ 1187 m/z) matching to prodelphinidin and procyanidin tetramers with RT 8.1 and 10 min can be identified. Molecular ions for a dimer monogallate ([M-H]$^-$ 729 m/z) and a procyanidin dimer ([M-H]$^-$ 577 m/z) were also detected at 16.9 and 18.5 min, respectively. Ellagic acid and catechin gallate were identified as well.

The OLF was analyzed by HPLC-MS with the resulting mass spectra displayed in Figure 6. It was possible to detect molecular ions that may be assigned to proanthocyanidin tetramers ([M-H]$^-$ 1171 m/z) and trimers ([M-H]$^-$ 865 m/z), gallocatechin ([M-H]$^-$ 305 m/z), and catechin gallate ([M-H]$^-$ 441 m/z). Free radical scavenging capacity of proanthocyanidins is strongly related with their hydroxylation pattern, degree of substitution, and conjugation (Rice-Evans et al. 1996). Galloylated catechines are more bioactive than non-galloylated ones; the presence of an ortho-trihydroxyl group in the B-ring and the addition of a galloyl moiety empowers the antioxidant capacity of these compounds (Nanjo et

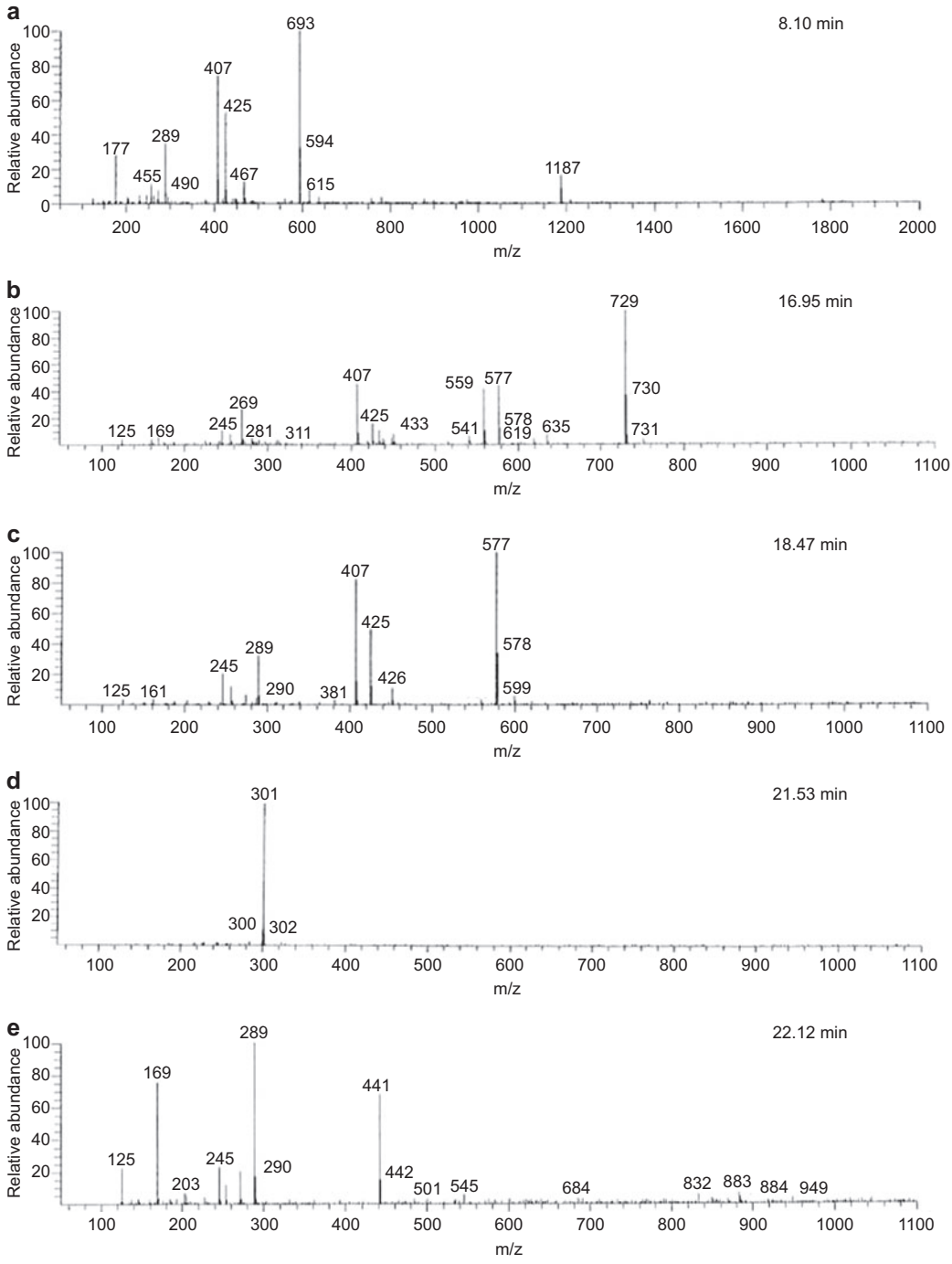

Figure 5 Mass spectra of phenolic compounds (prodelphinidin and procyanidin oligomers) in fraction MF5 from *Q. sideroxyla* bark. (a) Catechin-gallocatechin tetramer, (b) catechin monogallate dimer, and (c) procyanidin dimer. Ellagic acid (d) and catechin gallate (e).

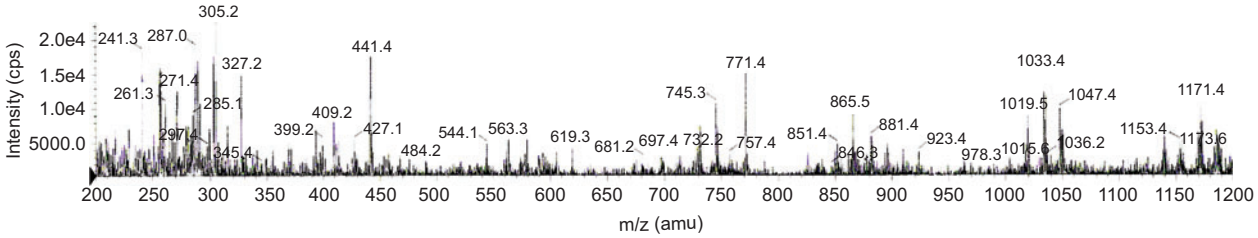

Figure 6 Mass spectra of OLF from *Q. sideroxyla* bark, obtained by Toyopearl HW-40F column chromatography eluted with acetone:water (3:2). It shows molecular ions indicating the presence of catechin or epicatechin (287 m/z), gallocatechin or epigallocatechin (305 m/z), catechin or epicatechin monogallates (441 m/z), proanthocyanidin trimers (865 m/z), and proanthocyanidin tetramers (1171 m/z).

al. 1996). Galloylated proanthocyanidins have shown higher antiradical activity against DPPH (Cos et al. 2004), and the presence of galloyl groups favored the antioxidant activity of the polyphenols in fish oil-in-water emulsions (Iglesias et al. 2010).

Fraction MF2 was the most active sample because of having gallocatechin as its major compound; also, MF5 and OLF contain galloylated procyanidins, which explains their higher antiradical activity (i.e., low EC_{50} values). Besides galloylated procyanidins, fraction OLF has gallocatechin (Figure 6, which presumably contributes to its high scavenging reaction rate.

The compounds isolated and identified from *Q. sideroxyla*, such as gallic acid, catechin, epicatechin, gallocatechin, catechin gallate, dimeric procyanidins, galloylated dimeric proanthocyanidins, trimeric procyanidins, and tetrameric proanthocyanidins, have been reported to be bioactive phytochemicals (Beecher 2004). The bioactivity is the result of their phenolic antioxidant capacity that may exert prevention against diseases in biological systems. Consequently, *Q. sideroxyla* bark is as a good source of therapeutic health products or nutraceutical ingredients.

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References

Andersen, O.M., Markham, R.K. Flavonoids chemistry, biochemistry and applications. CRC Press Taylor & Francis Group, Boca Raton, 2006.

Beecher, G.R. (2004) Proanthocyanidins: biological activities associated with human health. Pharm. Biology 42(Suppl):2–20.

Benzie, F.F., Strain, J.J. (1999) Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Methods Enzymol. 299:15–23.

Benzie, I.F.F., Strain, J.J. (1996) The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal. Biochem. 239:70–76.

Binbuga, N., Chambers, K., Henry, W.P., Schultz, T.P. (2005) Metal chelation studies relevant to wood preservation.1. Complexation of propyl gallate with Fe^{2+}. Holzforschung 59:205–209.

Binbuga, N., Ruhs, C., Hasty, J.K., Henry, W.P., Schultz, T.P. (2008) Developing environmentally benign and effective organic wood preservatives by understanding the biocidal and non-biocidal properties of extractives in naturally durable heartwood. Holzforschung 62:264–269.

Campos, M., Markham, K. (2007) Structure information from HPLC and on-line measured absorption spectra: Flavones, Flavonols and Phenolic Acids. Imprensa da Universidade de Coimbra, Coimbra. pp. 118.

Cheynier, V., Fulcrand, H. (2003) Analysis of polymeric proanthocyanidins and complex polyphenols. In: Methods in Polyphenol Analysis. Eds. Santos-Buelga, C., Williamson, G. Royal Society of Chemistry, Cambridge.

Cos, P., De Bruyne, T., Hermans, N., Apers, S., Vander Berghe, D., Vlietinck, A. (2004) Proanthocyanidins in health care: current and new trends. Curr. Med. Chem. 11:1345–1359.

De Bruyne, T., Pieters, L., Witvrouw, M., De Clercq, E., Vander, B., Vlietinck, A. (1999) Biological evaluation of proanthocyanidin dimers and related polyphenols. J. Nat. Prod. 62: 954–958.

Donoso-Fierro, C., Becerra, J., Bustos-Concha, E., Silva, M. (2009) Chelating and antioxidant activity of lignans from Chilean woods (Cupressaceae). Holzforschung 63:559–563.

Dudonné, S., Vitrac, X., Coutiere, P., Woillez, M., Merillon, J. (2009) Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC assays. J. Agric. Food Chem. 57:1768–1774.

Floegel, A., Kim, D.O., Chung S.J., Koo, S.I., Chun, O.K. (2011) Comparison of ABTS/DPPH assays to measure antioxidant capacity in popular antioxidant-rich US foods. J. Food Comp. Anal. 24:1043–1048.

Friaa, O., Brault, D. (2006) Kinetics of the reaction between the antioxidant Trolox and the free radical DPPH· in semi-aqueous solution. Org. Biomol. Chem. 4:2417–2423.

Gao, H., Shupe, T.F., Hse, C.Y., Eberhardt, T.L. (2006) Antioxidant activity of extracts from the bark of *Chamaecyparis lawsoniana* (A. Murray) Parl. Holzforschung 60:459–462.

Haslam, E. (1975) Natural proanthocyanidins. In: The Flavonoids. Eds. Harborne, J.B., Mabry, T.J., Mabry, H. Chapman & Hall, London.

Heimler, D., Vignolini, P., Dini, M., Romani, A. (2005) Rapid test to assess the antioxidant activity of *Phaseolus vulgaris* L. dry beans. J. Agric. Food Chem. 53:3053–3056.

Hemingway, R.W. (1989) Structural variation in proanthocyanidins and their derivatives. In: Chemistry and Significance of

Condensed Tannins. Eds. Hemingway, R.W., Karchesy, J.J. Plenum Press, New York.

Huang, D., Ou, B., Pryor, R. (2005) The chemistry behind antioxidant capacity assays. J. Agric. Food Chem. 53:1841–1856.

Iglesias, J., Pazos, M., Lois, S., Medina, I. (2010) Contribution of galloylation and polymerization to the antioxidant activity of polyphenols in fish lipid systems. J. Agric. Food Chem. 58:7423–7431.

Lee, J., Koo, N., Min, D.B. (2004) Reactive oxygen species, aging, and antioxidative nutraceuticals. Compr. Rev. Food Sci. Food Saf. 3:21–33.

Li, L., Tsao, R., Yang, R., Lui, C., Young, J., Zhu, H. (2008) Isolation and purification of phenylethanoids glycosides from *Cistanche deserticola* by high-speed counter-current chromatography. Food Chem. 108:702–710.

Liimatainen, J., Karonen, M., Sinkkonen, J., Helander, M., Salminen, J.-P. (2011) Characterization of phenolic compounds from inner bark of *Betula pendula*. Holzforschung.

Makino, R., Ohara, S., Hashida, K. (2011) Radical scavenging characteristics of condensed tannins from barks of various tree species compared with quebracho wood tannin. Holzforschung 65:651–657.

Manzocco, L., Anese, M., Nicoli, M.C. (1998) Antioxidant properties of tea extracts as affected by processing. LWT-Food Sci. Technol. 31:694–698.

Matthews, S., Mila, I., Scalbert, A., Donnelly, D. (1997) Extractable and non-extractable proanthocyanidins in barks. Phytochemistry 45:405–410.

Mayer, R., Stecher, G., Wuerzner, R., Silva, R., Sultana, T., Trojer, L., Feuerstein, I., Krieg, C., Abel, G., Popp, M., Bobleter, O., Bonn, G. (2008) Proanthocyanidins: target compounds as antibacterial agents. J. Agric. Food Chem. 56:6959–6966.

Nanjo, F., Goto, K., Seto, R., Suzuki, M., Sakai, M., Hara, Y. (1996) Scavenging effect of tea catechins and their derivatives on 1,1-difenil-2-picrylhydrazyl radical. Free Rad. Biol. Med. 21:895–902.

Peng, S., Scalbert, A., Monties, B. (1991) Insoluble ellagitannins in *Castanea sativa* and *Quercus petraea* woods. Phytochemistry 30:775–778.

Porter, J.L. (1993) Flavans and proanthocyanidins. In: The Flavonoids: Advances in Research Since 1986. Ed. Harborne, J.B. Chapman & Hall, London.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C. (1999) Antioxidant activity applying and improved ABTS radical cation decolorization assay. Free Rad. Biol. Med. 26:1231–1237.

Rice-Evans, C.A., Miller, N.J., Paganda, G. (1996) Structure antioxidant activity relationships of flavonoids and phenolic acids. Free Rad. Biol. Med. 20:933–956.

Rivas-Arreola, M.J., Rocha-Guzman, N.E., Gallegos-Infante, J.A., Gonzalez-Laredo, R.F., Rosales-Castro, M., Bacon, J.R., Cao, R., Proulx, A., Intriago-Ortega, P. (2010) Antioxidant activity of oak (*Quercus*) leaves infusions against free radicals and their cardioprotective potential. Pak. J. Biol. Sci. 13:537–545.

Roginsky, V., Lissi, E.A. (2005) Review of methods to determine chain-breaking antioxidant activity in food. Food Chem. 92:235–254.

Rosales-Castro, M., Gonzalez-Laredo, R.F., Rocha-Guzman, N.E., Gallegos-Infante, J.A., Peralta-Cruz, J., Morré, J., Karchesy, J.J. (2011) Chromatographic analysis of bioactive proanthocyanidins from *Quercus durifolia* and *Quercus eduardii* barks. Acta Chromatog. 23:521–529.

Sanchez-Moreno, C., Larrauri, J.A., Saura, C.F. (1998) A procedure to measure the antiradical efficiency of polyphenols. J. Sci. Food Agric. 76:270–276.

Santos-Buelga, C., Scalbert, A. (2000) Proanthocyanidins and tanninlike compounds-nature, occurrence, dietary intake and effects on nutrition and health. J. Sci. Food Agric. 80:1094–1117.

Sendra, J.M., Sentandreu, E., Navarro, J.L. (2006) Reduction kinetics of the free stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH·) for determination of the antiradical activity of citrus juices. Eur. Food Res. Technol. 223:615–624.

Singh, B., Bhat, T., Singh, B. (2003) Potential therapeutic applications of some antinutritional plant secondary metabolites. J. Agric. Food Chem. 51:5579–5597.

Smeds, A.I., Eklund, P.C., Monogioudi, E., Willför, S.M. (2011) Chemical characterization of polymerized products formed in the reactions of matairesinol and pinoresinol with the stable radical 2,2-diphenyl-1-picrylhydrazyl. Holzforschung 66:283–294.

Sun, D., Wong, H., Foo, L.Y. (1987) Proanthocyanidin dimers and polymers from *Quercus dentata*. Phytochemistry 26:1825–1829.

Telysheva, G., Dizhbite, T., Bikovens, O., Ponomarenko, J., Janceva, S., Krasilnikova, J. (2011) Structure and antioxidant activity of diarylheptanoids extracted from bark of grey alder (*Alnus incana*) and potential of biorefinery-based bark processing of European trees. Holzforschung 65:623–629.

Viriot, C., Scalbert, A., Lapierre, C., Moutounet, M. (1993) Ellagitannins and lignins in aging of spirits in oak barrels. J. Agric. Food Chem. 41:1872–1879.

Waterman, P., Mole, S. (1994) Analysis of phenolic plant metabolites. Methods in Ecology. Blackwell Sci. Pub., Boston.

Willför, S., Nisula, L., Hemming, J., Reunanen, M., Holmbom, B. (2004) Bioactive phenolic substances in industrially important tree species. Part 2: Knots and stemwood of fir species. Holzforschung 58:650–659.

Yao, L.-N., Su, Y.-F., Yin, Z.-Y., Qin, N., Li, T.-X., Si, C.-L., Liu, E.-W., Gao, X.-M. (2010) A new phenolic glucoside and flavonoids from the bark of *Eucommia ulmoides* Oliv. Holzforschung 64:571–575.

Zulaica-Villagomez, H., Peterson, D.M., Herrin, L., Young, R.A. (2005) Antioxidant activity of different components of pine species. Holzforschung 59:156–162.

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