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Polyphenolic content and antioxidant activity of leaf extracts from *Quercus durifolia, Quercus eduardii, Quercus sideroxyla* and *Quercus resinosa*

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The phenolic content and antioxidant activities of extracts from *Quercus durifolia, Quercus resinosa, Quercus eduardii* and *Quercus sideroxyla* leaves were studied. Extracts were obtained by successive extraction with aqueous acetone (70%) followed by methanol (50%). Antioxidant activities were determined using a single electron transfer (SET) and hydrogen atom transfer (HAT) based assays. For SET-based the assays performed were, 2,2-diphenyl-1-picryl hydrazine (DPPH) inhibition expressed as IC_{50}, redox potential, as mV, inhibition of the low density lipoprotein (LDL) oxidation, as % of inhibition. For HAT-based assay, crocin bleaching technique was applied; a kinetics approach was used for the evaluation of total antioxidant capacity and the results presented in terms of equivalence by weight of a reference antioxidant. Acetone extracts (PFQA) from leaves of all *Quercus* species showed the best antioxidant capacity. *Q. resinosa* (PFQr) exhibited the best antioxidant capacity among the *Quercus* species analyzed either in acetone or methanol extracts (PFQM). Distinctive results are: DPPH IC_{50}, PFQrA = 78.3 µg/g, PFQrM = 250.7 µg/g; In Redox potential, PFQrA = 147.0 mV, PFQrM = 201.6 mV; In LDL oxidation inhibition: 98.2% and in inhibition of crocin bleaching, PFQrA = 1.08 g, PFQrM = 0.98 g. In conclusion, *Quercus* leaves might be used as potential source of polyphenolic antioxidants.

Key words: *Quercus sp.*, SET-based assay, HAT-based assay, phenolic compounds, antioxidant activity.

INTRODUCTION

On recent times the use of plants, vegetables, herbs and spices used in folk and traditional medicine have gained a wide acceptance as an important source for new chemicals discovery (Afolayan et al., 2008). Currently, there is an increased interest for new sources of compounds with evidenced biological activity, among which are natural antioxidants. These compounds can prevent the damage to macromolecules and cells by interfering with free radicals, usually implicated in the etiology of several diseases such as atherosclerosis,

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cancer and others (Srivastava et al., 2007).

Over eight thousand naturally occurring phenolic compounds are known (Bennick, 2002). Phenolic compounds are bioactive substances widely distributed in plants and are important constituents of the human diet because they provide a positive effect due to their antioxidant capacity (Di Majo et al., 2008) and often identified as the active principles in numerous folk herbal medicines (Rivas-Arreola et al., 2010).

The extraction of phenolic compounds from plant materials is influenced mainly by their chemical nature, extraction method, particle size, time and storage conditions as well as the presence of interfering substances (Apak et al., 2007). Phenolic extracts from plants are always a complex mixture of different classes of phenolic compounds that are selectively soluble on each solvent, where the use of an alcoholic solution has been found to be suitable for the extraction process (Koffi et al., 2010). Commonly used solvents for the extraction of these compounds from plant materials are water, aqueous mixtures of ethanol, methanol and acetone (Jakopic et al., 2009).

Polyphenols can be traditionally divided into two classes: the primary or chain-breaking antioxidants and the secondary or preventive antioxidants (Madhavi et al, 1996). Various antioxidant activity methods have been used to monitor and compare the antioxidant activity of different samples. These methods can give varying results depending on the specific free radical being used as a reactant (Miller et al., 2000). Due to the lack of a validated antioxidant assay able to reliably measure the antioxidant capacity of many samples, the use of several *in vitro* antioxidant techniques can give information about the overall antioxidant compounds present in the samples (Frankel and Meyer, 2000).

The ability of an antioxidant compound to exert free-radical-scavenging activity is determined, to a large extent, by its capacity to act as a reducing molecule, being able to either transfer electrons or hydrogen atoms to an unstable molecule (Valenzuela, 2004). Together with other dietary antioxidants, like vitamin C, antioxidants are able to protect different body organs against oxidative stress (Balasundram et al., 2006).

The state of Durango, Mexico, has extensive forest resources covered by temperate woods mainly represented by pines and oaks (Corral and Navar, 2005). Thirty-nine *Quercus* species can be found in the forest of Durango and their major non-timber uses are for food and medicine, fodder for goats and pigs, and their tannins for fiber dying and leather tanning (Luna-José et al., 2003). *Quercus* leaves can be an inexpensive, renewable and natural source of polyphenolic compounds with high antioxidant activity, contributing to the prevention or treatment of oxidative stress and chronic degenerative diseases.

Leaves from several oak species (*Quercus* spp.) have been used in traditional medicine by Mexican people for the treatment of diverse illnesses such as gastric ulcers, local inflammation (Abou-Karam and Shier, 1999) and as anticarcinogenic agents (Rocha-Guzmán et al., 2007). *Quercus resinosa* leaf infusions have proven a highly antioxidant activity due to their high phenolic content measured by *in vitro* methods (Rocha-Guzmán et al., 2009).

The aim of this work was to evaluate the total phenolic content and the antioxidant activities of polar extracts from *Q. resinosa, Quercus eduardii, Quercus durifolia* and *Quercus sideroxyla* leaves using different *in vitro* methods.

MATERIALS AND METHODS

Chemicals

Methanol, acetone, Folin-Ciocalteu reactive (2N), 2,2-diphenyl-1-picryl hydrazine (DPPH), thiobarbituric acid (TBA), trichloroacetic acid (TCA) and (+)-catechin were obtained from SIGMA (Sigma, St Louis, MO, USA). *n*-Butanol from Fermont (Fermont, Monterrey, México). 2,2´-azo-bis (2-amidinopropane) dihydrochloride (ABAP) was purchased from Wako Chemical Co., Japan, potassium hydrogen phosphate and potassium hydroxide were from Carlo Erba. Saffron (*Crocus sativus*) was obtained from Sigma, Italy. Crocin [8,8´-diapo-carotenedioic acid bis(6-*O*-D-glucopyranosyl-D-glucopyranosyl) ester] was prepared from saffron by methanolic extraction after repeated washing with ethyl ether. Ethyl ether, ethanol and methanol were from CarloErba (Milan, Italy).

Samples

Leaves from *Q. durifolia*, *Q. eduardii*, *Q. sideroxyla* and *Q. resinosa* were collected in the Mezquital and El Salto forests about 85 Km South and 95 Km west from Durango, México, respectively. Field collections and extractions with different solvents were made in November 2006. Oaks were taxonomically identified by Dr. Jeffrey Bacon from Universidad Juarez del Estado de Durango, and the voucher specimens deposited at the Herbarium of ISIMA-Universidad Juárez del Estado de Durango.

Preparation of quercus leaves samples

Oak leaves were stored in darkness and air dried at room temperature. Then dried leaves were pulverized, screened (1.13 mm), and stored in amber glass bottles until extraction.

Solid-liquid extraction

A sequential extraction from oak leaves samples was performed as described previously by Rocha-Guzmán et al. (2009). Briefly, a successive extraction was performed with a hexane degreasing step, followed by acetone 70% (1st extract) and finally methanol 50% (2nd extract). Distilled water was used in all experiments. Isolation/purification of phenolic fractions from *Quercus* leaves

(PFQs) were carried out for all *Quercus* species (*Q. durifolia* (PFQd); *Q. eduardii* (PFQe); *Q. sideroxyla* (PFQs) and *Q. resinosa* (PFQr) extracts). Phenolic fractions with aqueous acetone (PFQA) and methanol (PFQM) were obtained by extensive extraction (24h), then filtered, and rotoevaporated (Buchi Mod R205) at 40 to 45°C. Solvent free PFQA and PFQM were freeze dried and stored in amber glass vials until further use.

Yields of aqueous-extractable solids

Extract yields from different fractions were determined as follows:

$$Yield\ (\%) = \frac{Weight\ of\ dried\ \text{PFQs}\ (g)}{Weight\ of\ sample\ (g)}\ \text{X}\ 100$$

Results were reported as means of two independent extractions.

Total phenolic content

Total phenolic content was assessed with the Folin-Ciocalteu reagent by the method described by Singleton et al. (1999). Briefly, aliquots of 125 µl from samples (100 µg/mL) were mixed with 1 ml of Folin reagent (Sigma-St Louis, MO, USA). After 5 min, 300 ml of Na_2CO_3 (20%) was added. This mixture was incubated at room temperature for 2 h. The absorbance was then measured at 765 nm with a spectrometer (Agilent 8453, Palo Alto, CA, USA). Results were expressed as mg of catechin equivalents (CE)/g of dry extract.

DPPH radical scavenging assay

The antioxidant activity of the extracts from the three different samples and standard (+)-catechin was measured in terms of hydrogen donating or radical scavenging ability using the stable DPPH• method by Brand-Williams et al. (1995). The percentage of scavenging or quenching of DPPH radicals (Q) was calculated by the following equation:

$$Q = 100(A_o - A_c)/A_o$$

Where A_o is the absorbance of the control, A_c is the sample absorbance at the 'c' concentration. All experiments were performed at least in triplicate. The decrease on absorption at 515 nm was used for calculating the IC_{50} with a calibration curve of (+)-catechin diluted in methanol, and results expressed as µg catechin equivalent per gram of dry material.

Competition kinetics test

The kinetics procedure has been previously described for analyzing the antioxidant capacity of single molecules or extracts (Bors et al., 1984; Bressa et al., 1996; Tubaro et al., 1996,). In brief, the specific absorbance of the carotenoid crocin decreases at a rate V_o following the interaction with peroxyl radicals (ROO^*) and the bleaching ($-\Delta A_0/\Delta t$) slows down in the presence of an antioxidant, competing for the same radical at a rate V_a, to a new value ($-\Delta A_a/\Delta t$). The competition kinetics follows the equation:

$$\Delta A_a/\Delta t = V = V_o K_c[C]/(K_c[C] + K_a[A]) \quad (1)$$

Where ΔA_o= absorbance variation in absence of antioxidants, ΔA_a = absorbance variation in presence of antioxidants, V_o = bleaching rate in absence of antioxidants, V = bleaching rate in presence of antioxidants, $K_c = K_1\,[ROO^*]$, $K_a = K_2\,[ROO^*]$, [C] = concentration of crocin, [A] = concentration of antioxidant, K_1 = rate constant for the reaction between free radicals and crocin, and K_2 = rate constant for the reaction between free radicals and antioxidant. Thus, by transforming:

$$\Delta A_o/\Delta A_a = V_o/V = (K_c[C] + K_a[A])/K_c[C]) = 1 + K_a/K_c[A]/[C] \quad (2)$$

The slope of the straight line (K_a/K_c), resulted by fitting the experimental data from different concentrations of the antioxidant, indicates its relative capacity to interact with ROO*. When the overall antioxidant capacity of complex mixtures is analyzed, a theoretical value K_a [A] is defined by the above equation as the sum of the antioxidant capacity for each free radical scavenging. By considering the whole amount of the sample as catechin, its "theoretical" concentration (CATECHIN) is used to plot the results of crocin bleaching. Thus, the slope of the fitting of the kinetics equation data describes the antioxidant capacity of the sample in terms of the ratio between the rate constants:

$$V_o/V = 1 + (K_{CATECHIN}/K_c)([CATECHIN]/[C]) \quad (3)$$

$K_{CATECHIN}$ represents the rate constant for the interaction of CATECHIN with peroxyl radicals. Finally, since:

$$K_a[A]= K_{CATECHIN}[CATECHIN] = K_{catechin}[catechin] \quad (4)$$

And since $K_{catechin}$ is known from independent measurements, the ratio [CATECHIN]/[catechin] can be calculated. This ratio indicates the relative antioxidant capacity in terms of weight of the sample versus catechin.

Determination of the anti-oxidative activity of the extracts

Tests were carried out at 40°C in 2 ml of medium composed of phosphate buffer (0.1 M), pH 7, KCl (0.4 M), crocin (10 µM) prepared from a methanol stock solution (1 mM) ($\varepsilon = 1.33 \times 10^5\ M^{-1}$ at 443 nm) and different quantities of FPQ extract (PFQA: 50 µg/ml and PFQM: 500 µg/ml) or the reference antioxidant (catechin). The reaction was started by adding 30 µl of ABAP (from a 5 mM fresh aqueous solution). The rate of crocin bleaching was recorded at 443 nm using a spectrophotometer (Kontron Instruments, Uvikon 860) with six thermostatic cells and the linear bleaching rate, from 3 to 12 min after the addition of the diazo compound used for calculations. Before each determination, possible spectroscopic interference of extract with the crocin bleaching was ruled out. When necessary, extracts were diluted to maintain the range of absorption at an optimal level for spectrophotometric measurements, with an absorbance unity lower than 1.5 at the beginning of the reaction. The antioxidant capacity of the samples was expressed as µM catechin / µM crocin.

Redox potential

Measurements were made as proposed by Manzocco et al. (1998), using a platinum-indicating electrode and a reference electrode (Ag/AgCl, Cl-sat) connected to a voltmeter (Crison, mod. 2002, Alella, Spain). Calibration was performed against a redox standard solution (E = 468 mV at 25.8°C). Electrodes were inserted into a 50

Table 1. Extract yield and total phenolic content (TPC) of *Quercus* leaves, hexane defatted, and extracted with aqueous acetone (70%) and aqueous methanol (50%).

Sample	% Yield PFQA	% Yield PFQM	TPC PFQA (mg CE / g)	TPC PFQM (mg CE / g)
Quercus sideroxyla	17.14 ± 0.03[a]	4.10 ± 0.38[b]	353.87 ± 8.1[d]	375.50 ± 7.7[b]
Quercus durifolia	9.29 ± 0.14[c]	4.78 ± 0.18[b]	412.87 ± 1.6[c]	307.37± 11.1[c]
Quercus eduardii	8.84 ± 0.13[c]	2.62 ± 0.10[c]	752.50 ± 8.7[a]	442.00 ± 1.2[a]
Quercus resinosa	14.83 ± 0.10[b]	6.54 ± 0.16[a]	494.87 ± 7.8[b]	294.87 ± 0.6[c]

Values are means of triplicate determination ± standard error. [abc] Different letters indicate significant statistical differences ($P \leq 0.05$) with Tukey's test. PFQA: Acetonic Phenolic Fraction from *Quercus* leaves; PFQM: Methanolic Phenolic Fraction from *Quercus* leaves.

mL 3-neck flask containing 20 mL of sample at 50 µg/mL for PFQA or 500 µg/mL for PFQM. Prior to analysis, oxygen was removed from the system by continuous nitrogen flushing for 10 minutes. Millivolt values were recorded for at least 5 min, until a stable potential was reached (that is, a change of less than 1 mV in a 5 min period). The analyses were carried out in duplicate and the difference between two determinations performed on the same sample did not exceed 3%. Catechin was used as control standard.

Inhibition of human LDL oxidation

Plasma LDL was prepared according to Loy et al. (2002). The inhibition of copper-catalyzed LDL oxidation was evaluated for each of the infusions prepared at 1%, where the ability of the samples to inhibit oxidation was tested. The results were expressed as thiobarbituric acid reactive species (TBARS), measured at 532 nm with a spectrophotometer and compared to (+)-catechin.

Statistical analysis

All experiments were carried out with two replicates and each sample with two repetitions. For each set of obtained data, standard statistical methods to determine the mean and standard deviation were used. A two-way analysis of variance with a confidence interval of 95% was performed. Mean values were compared using a Tukey-Kramer test ($p<0.05$) by JMP 5.0.1 software.

RESULTS

Yields of aqueous-extractable solids

The percentage yield of *Quercus species* extracted with different solvents is shown in Table 1. The highest percentage yield was obtained for all acetone extracts; among acetone extracts, *Q. sideroxyla* was the sample that showed the highest yield, followed by *Q. resinosa* and with the lowest percentage yield were *Q. durifolia* and *Q. eduardii*. Among methanol extracts the highest percentage yield corresponded to *Q. resinosa*, followed by *Q. sideroxyla* and *Q. durifolia* and finally *Q. eduardii* with the lowest percentage yield of all extracts.

Total phenolic content

Results for total phenolic content of *Quercus* extracts with different extraction solvents are shown in Table 1. The highest phenolic content corresponds to acetone extracts; of all acetone extracts the greatest amount of phenolics was shown by *Q. eduardii* followed by *Q. resinosa*, *Q. durifolia* and finally *Q. sideroxyla* with the lowest content of phenolic compounds. Among methanol extracts, *Q. eduardii* had the highest phenolic content followed by *Q. sideroxyla, Q. resinosa* and finally *Q. durifolia* with the lowest amount of phenolics among all extracts.

DPPH radical scavenging activity

The capacity of PFQ to scavenge DPPH radical is presented on Table 2 as the half maximal inhibitory concentration (IC_{50}). PFQ acetone extracts had the best (the lowest amount needed) IC_{50} values compared to those of all PFQ methanol extracts. Among PFQ acetone extracts, PFQrA had the best results of IC_{50}, which were similar to the catechin standard; followed by PFQeA, and finally both PFQsA and PFQdA with the highest IC_{50} compared with the other *Quercus* species extracted with acetone. PFQ methanol extracts (PFQe, PFQs, PFQr, PFQd) showed similar IC_{50} values, but not as good as catechin.

Redox potential

The results of Redox potential are shown on Table 2 and are expressed as mV. The lowest value obtained for Redox potential was for acetone extracts, which is characteristic of reducing agents, and therefore higher Redox values such as methanol extracts tended to be more oxidant. Among acetone extracts, PFQrA proved to be the most reducing extract with the lowest value for Redox potential, and it is significantly lower than the

Table 2. DPPH Radical Scavenging Activity and Redox potential of phenolic fraction from *Quercus* leaves (PFQ) obtained by sequential extractions with aqueous acetone (70%) and aqueous methanol (50%).

Sample	DPPH[1] IC_{50}	Redox potential[2] Eº *vs* Ag/AgCl, Cl-sat
PFQsA	163.1 ± 2.2[a]	177.5 ± 2.5[b]
PFQdA	164.1 ± 0.5[a]	140.0 ± 9.0[c]
PFQeA	108.8 ± 4.8[b]	192.0 ± 1.0[a]
PFQrA	78.7 ± 4.1[c]	128.5 ± 4.5[c]
Catechin	71.3 ± 0.5[c]	147.0 ± 1.3[c]
PFQsM	238.7 ± 3.2[a]	215.5 ± 1.5[a]
PFQdM	263.9 ± 11.9[a]	188.2 ± 6.7[c]
PFQeM	215.8 ± 8.1[a]	198.5 ± 5.0[b]
PFQrM	250.7 ± 3.8[a]	201.6 ± 1.5[b]
Catechin	71.3 ± 0.5[b]	129.5 ± 0.9[d]

Values are means of triplicate determination ± standard error. [abcd] Different letters indicate significant statistical differences (P≤0.05) with Tukey's test. [1]Results are expressed as µg/g of dry extract. [2]Results are expressed as mV. PFQsA: Acetonic Phenolic Fraction from *Q. sideroxyla* leaves; PFQdA: Acetonic Phenolic Fraction from *Q. durifolia* leaves; PFQeA: Acetonic Phenolic Fraction from *Q. eduardii* leaves; PFQrA: Acetonic Phenolic Fraction from *Q. resinosa* leaves; PFQsA: Methanolic Phenolic Fraction from *Q. sideroxyla* leaves; PFQdA: Methanolic Phenolic Fraction from *Q. durifolia* leaves; PFQeA: Methanolic Phenolic Fraction from *Q. eduardii* leaves; PFQrA: Methanolic Phenolic Fraction from Q. leaves.

standard catechin; it was followed by PFQdA that is similar to Catechin, then PFQsA and PFQeA that had the highest Redox value, being the most oxidant among acetone extracts (Table 2). From the methanol extracts, PFQdM had the lowest Redox value, followed by both PFQeM and PFQrM with significantly higher values than catechin, and finally PFQsM with the highest redox potential as the most oxidant of all extracts (Table 2).

Inhibition of human LDL oxidation

In vitro antioxidant methods not always give accurate information about the possible *in vivo* antioxidant activity, however some assay allow a closer approach to a biological performance such as human LDL oxidation inhibition. Acetone and methanol extracts from *Quercus* species were tested on their *in vitro* inhibition of human LDL oxidation and the results are shown on Table 3. Similar inhibitory percentages were obtained between acetone and methanol PFQ extracts. The highest inhibitory percentage at a fixed concentration of 1000 µg/g of dry extract was obtained for PFQdA and PFQrA, followed by PFQsA, PFQeA and catechin with similar inhibitory percentages (Table 3). Among methanol extracts the highest inhibitory percentage was obtained for PFQrM, PFQeM and PFQdM, followed by PFQsM and the catechin standard with lower inhibition on the LDL oxidation (Table 3).

Competition kinetics test and determination of the antioxidative activity of the extracts

The kinetics plots for the competition between crocin, PFQ and catechin are shown in Figure 1. In these plots V_o/V indicates the bleaching rates in the presence of different molar ratios between the antioxidant and crocin. The data indicate that the results fit into the kinetic model (R values >0.98 and intercept close to 1) (Tubaro et al., 1996). Table 4 shows equations and R values for PFQA, PFQM and catechin. In PFQA extracts, the species *Q. resinosa* and *Q. durifolia,* and catechin had the major value for R, close to 1, followed by *Q. siderxyla* and finally with the lowest value, *Q. eduardii*. For PFQM, *Q. resinosa, Q. eduardii* and catechin obtained the highest value for R, followed by *Q. durifolia* and *Q. sideroxyla.* All results from *Quercus* species in both extracts PFQA and PFQM are inside the range, then it can be stated that they fit into the range of values accepted for the kinetic model aforementioned.

The complete kinetics analysis and the antioxidant capacity are reported on Table 4. According to the kinetic equation, the slope of the fitting line from the experimental data indicates the antioxidant capacity of the analyzed compound and a simple calculation

Table 3. Inhibition of human LDL oxidation by phenolic fraction of *Quercus* leaves (PFQ) obtained by sequential extractions with aqueous acetone (70%) and aqueous methanol (50%).

Sample		LDL oxidation inhibition[1]
Acetone	PFQ *sideroxyla*	96.0 ± 0.8[b]
	PFQ *durifolia*	98.5 ± 0.9[a]
	PFQ *eduardii*	95.6 ± 0.5[b]
	PFQ *resinosa*	98.2 ± 0.1[a]
	Catechin	95.6 ± 0.1[b]
Methanol	PFQ *sideroxyla*	96.4 ± 0.2[ab]
	PFQ *durifolia*	96.8 ± 0.6[ab]
	PFQ *eduardii*	97.2 ± 0.1[ab]
	PFQ *resinosa*	97.6 ± 0.2[a]
	Catechin	95.6 ± 0.1[b]

Values are means of triplicate determination ± standard error. [ab] Different letters indicate significant statistical differences (P≤0.05) with Tukey's test. [1]Results are expressed as the highest inhibitory percentage at 1000 µg/g of extract. PFQ: Polyphenol Fraction from *Quercus* leaves.

expressed in terms of the ratio (required weight) vs the reference antioxidant (catechin in grams). These results are expressed as grams of PFQ required to match the antioxidant capacity of one gram of standard. PFQrA and PFQrM had the best result for antioxidant capacity, since they are very close to the standard value showing no significant statistical difference between them. Following *Q. resinosa* extracts are PFQsA, PFQsM, PFQdA, PFQdM and finally with the highest required grams per gram of standard, PFQeA and PFQeM.

DISCUSSION

Polyphenols are abundant micronutrients in our diet and evidence for their role on the prevention of degenerative diseases is emerging. Different solvent systems have been used for the extraction of polyphenols from plant materials (Chavan et al., 2001). To obtain the extracts rich in phenolic compounds from *Quercus* leaves, two different solvents were used for the extraction, aqueous acetone (70%) followed by aqueous methanol (50%). The yields for the extraction step were up to 8% for PFQA and 2% for PFQM (Table 1), these differences in both extracts can be attributable to the fact that they come from successive extractions. Goli et al. (2004), report that the extraction yield depends upon the solvent and the method of extraction. The solvents were selected from experience and previous reports that recommended aqueous mixtures of ethanol, methanol, and acetone to extract phenolic compounds from plants (Sun and Ho, 2005) and according to Zhou and Yu (2004) and Karchesy et al. (1989) especially aqueous acetone for being more effective.

Analyzing the results obtained for total polyphenol concentration (Table 1) we found that PFQA showed higher concentration than PFQM, but both extracts are from the same sample by successive extraction, therefore an independent analysis cannot be performed. This means that the total concentration of polyphenolic compounds of PFQs is given by the sum of the two extracts. It was reported that *Q. resinosa* leaves show a higher phenolic content compared with other related species (Rocha-Guzmán et al., 2009). Rivas-Arreola et al. (2010) reported a lower concentration of phenolic compounds in some species of *Quercus* such as *Q. resinosa*, *Q. sideroxyla* and *Q. eduardii*, however they used leaf infusions instead of solvent extractions. Therefore a more efficient extraction of polyphenolic compounds might be possible using an aqueous solvent mixture, as reported by Turkman et al. (2006), who found that polarity of the extracting solvent significantly affected total polyphenol content and antioxidant activity of tea extracts.

DPPH radical scavenging and redox potential

The nutraceutical properties of various products depend on the presence of phenolic compounds, usually in small amounts. Among them are those with antioxidant properties that have been pointed out as adjuvants or even protectors of chronic diseases as cancer of diabetes (Santagelo et al., 2007). The antioxidant activity of the PFQ was tested to explore their possible antioxidative effect through the reduction of the DPPH radical and their reductive-oxidative potential (Table 2). The lowest IC_{50} for DPPH assay as well as the lowest Redox potential were obtained for all PFQA, meaning that the consecutive methanol extraction yield is directly related to the antioxidant activity (Table 1). Among PFQA, *Q. resinosa* showed the best results for both DPPH reduction and Redox potential suggesting an electron transferring mechanism that reduces the DPPH radical due to its highly antioxidant potential. This potential constitutes the ability of the sample to reduce or donate an electron (Table 2); additionally the antioxidant capacity of the sample does not seem to be related to its total phenolic content but to its composition (Table 1). Interestingly, the antioxidant activity of PFQeA is lower than PFQrA, requiring more amount of sample to inhibit the DPPH radical, but presenting the highest Redox potential value, which suggests a more oxidant behavior, even though it shows the highest phenolic content between the PFQA extracts.

PFQ methanol extracts show an IC_{50} two-fold higher,

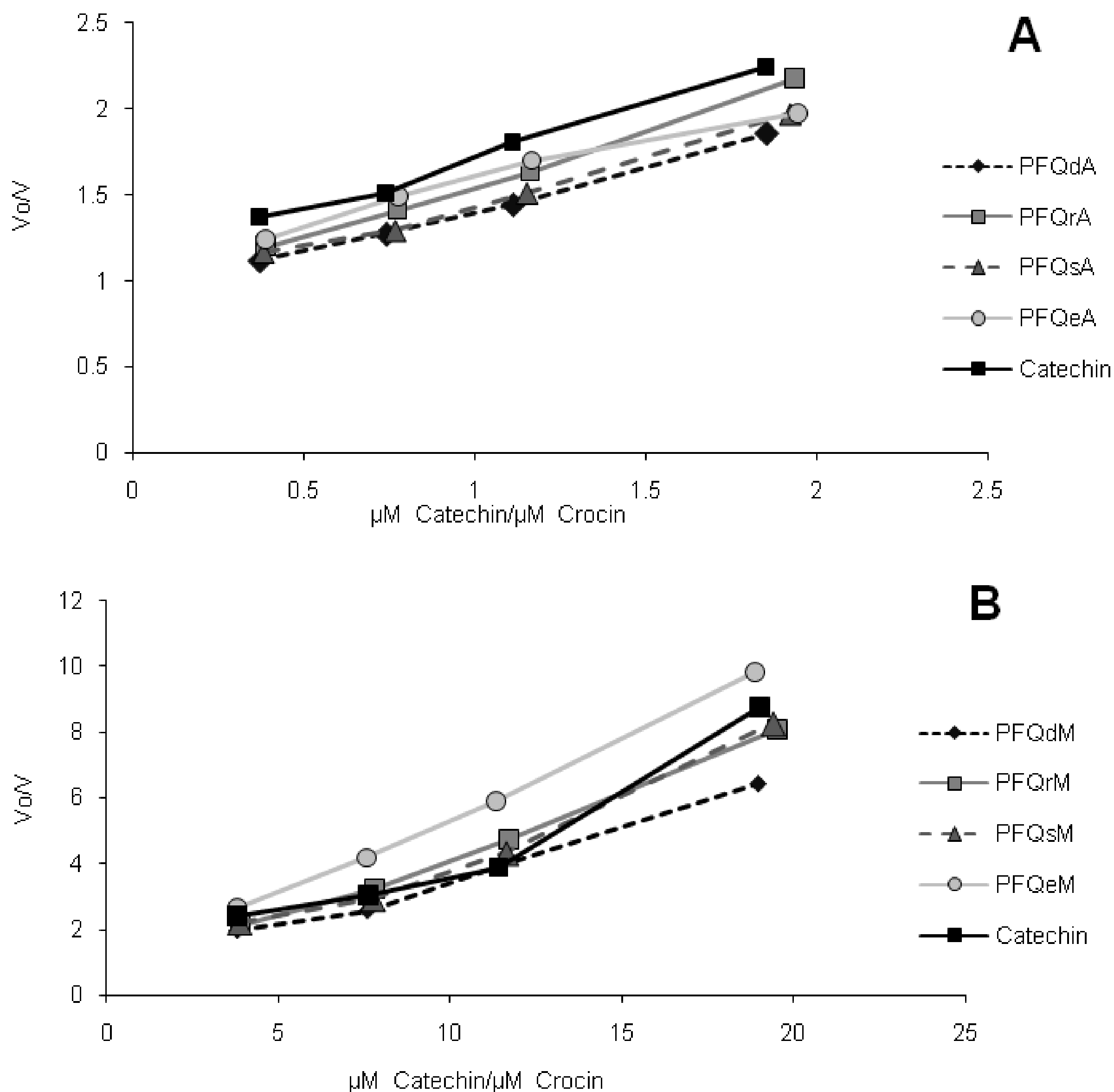

Figure 1. Competition kinetic plot of PFQA (A) and PFQM (B) extracts after 20 minutes of treatment. The slope of the regression fitting indicates, according to the kinetic equation, the antioxidant capacity of the samples. PFQA: Acetonic Phenolic Fraction from *Quercus* leaves; PFQM: Methanolic Phenolic Fraction from *Quercus* leaves.

which suggests that a greater amount of this extract is needed to reduce the DPPH radical (Table 2). Remarkably, no significant statistical difference is observed between PFQM, suggesting a common phenolic composition of such extracts, with similar antioxidant capacity. The best overall Redox potential belongs to *Q. eduardii* leaves, which are similar either in acetone or methanol extracts from the same *Quercus* samples, suggesting that methanol extract alone might yield higher phenolic contents and antioxidant activities. The most oxidative value obtained for PFQM was obtained for *Q. sideroxyla*, with the highest values between PFQA, meaning it has the lowest antioxidant potential of all *Quercus* species.

Inhibition of human LDL oxidation

LDL oxidation inhibition provides an insight on the possible reduction of cardiovascular and renal damage

Table 4. Kinetics of crocin bleaching inhibition. Statistical data of the kinetics equation plot, and antioxidant capacity of phenolic fractions from *Quercus* leaves (PFQ) reported as catechin/PFQ ratio*

Sample	Competition kinetics eq. $V_o/V = (K_a / K_c)[A]/[C] + b$	K_a/K_c	R	Catechin/PFQ*
PFQsA	y= 0.5387 x + 0.9155	0.5387 ± 0.03[a]	0.988	1.19 ± 0.07[a]
PFQdA	y= 0.5008 x + 0.9142	0.5008 ± 0.04[a]	0.995	1.33 ± 0.13[a]
PFQeA	y= 0.4652 x + 1.1025	0.4652 ± 0.01[a]	0.977	1.31 ± 0.01[a]
PFQrA	y= 0.6372 x + 0.9288	0.6372 ± 0.07[a]	0.996	1.08 ± 0.13[a]
Catechin	y= 0.6070 x + 1.1140	0.6070 ± 0.01[a]	0.990	1.00 ±0.01[a]
PFQsM	y= 0.4008 x + 0.1625	0.4008 ± 0.09[a]	0.967	1.70 ± 0.55[a]
PFQdM	y= 0.3006 x + 0.5977	0.3006 ± 0.04[a]	0.985	1.91 ± 0.37[a]
PFQeM	y= 0.4775 x + 0.6898	0.4775 ± 0.13[a]	0.997	1.92 ± 0.25[a]
PFQrM	y= 0.3878 x + 0.3929	0.3878 ± 0.10[a]	0.993	0.98 ± 0.20[b]
Catechin	y= 0.4250 x + 0.0760	0.5387 ± 0.03[a]	0.916	1.00 ± 0.08[b]

Values are means of triplicate determination ± standard error. [ab] Different letters indicate significant statistical differences ($P \leq 0.05$) with Tukey's test. *Data refers to different model system. In the equations the term "y" is referred to V_o/V and "x" to [A]/[C]. The ratio indicates the relative amount of FPQ exhibiting the antioxidant effect of catechin on a weight base expressed in grams. PFQsA: Acetonic Phenolic Fraction from *Q. sideroxyla* leaves; PFQdA: Acetonic Phenolic Fraction from *Q. durifolia* leaves; PFQeA: Acetonic Phenolic Fraction from *Q. eduardii* leaves; PFQrA: Acetonic Phenolic Fraction from *Q. resinosa* leaves; PFQsA: Methanolic Phenolic Fraction from *Q. sideroxyla* leaves; PFQdA: Methanolic Phenolic Fraction from *Q. durifolia* leaves; PFQeA: Methanolic Phenolic Fraction from *Q. eduardii* leaves; PFQrA: Methanolic Phenolic Fraction from *Q. resinosa* leaves.

(Loy et al., 2002), hence the inhibitory capacity of PFQ was evaluated at 532 nm as metal chelation and chain breaking activities and the results expressed as percentage inhibition as shown on Table 3. The highest inhibitory effect of human LDL oxidation corresponds to acetone extracts, having PFQrA and PFQdA at a concentration of 1000 µg/g producing an average 98.35% of inhibition, suggesting that at the same concentration they produce a higher inhibition.

The PFQ methanol extracts have similar inhibition percentages on human LDL oxidation to those of the acetone extracts. Interestingly, even though methanol extracts yield half the weight of phenolics than acetone extracts, they show similar results for inhibition of LDL oxidation. Rivas-Arreola et al. (2010) obtained different results for *Quercus* leaf infusions, attaining a better result for *Q. siderxyla*, followed by *Q. resinosa* and finally *Q. eduardii*. This difference on the inhibitory results of LDL oxidation can be attributed to the extraction protocol.

Competition kinetics test and determination of the antioxidative activity of the extracts

Reactive oxygen species produced as a result of the respiratory chain and oxidative phosphorylation are capable of attacking biological macromolecules like membrane lipids, proteins, and DNA. The breakdown of the latter produces single- and double-strand to break and may eventually cause cell ageing, cardiovascular diseases, mutagenic changes and cancerous tumor growth (Apak et al., 2007). Antioxidants such as phenolic compounds have been reported to be efficient on inhibiting protein and lipid oxidation (Viljanen et al., 2004), counteracting the damage caused by the alteration on the functionality of these biomolecules.

Therefore, it is important to establish methods that can measure the total antioxidant capacity directly from plant extracts containing phenolics. On the basis of chemical reactions, the major antioxidant capacity assays can be roughly divided into two categories: Single electron transfer (SET) and hydrogen atom transfer (HAT) reaction based assays. These assays differ from each other in terms of substrates, probes, reaction conditions, and quantitation methods (Frankel and Meyer, 2000). *In vivo*, mitochondria are the most important organelles that produce free oxygen and nitrogen radicals, and at the same time they are the main cellular target for free radical mediated damage (Cardenas, 2004). In normal conditions, these free radicals and non-radical reactive species derived from others exist in cells and tissues at low but measurable concentrations. Their concentrations are determined by the balance between their production and clearance rates by various antioxidant compounds and enzymes; when no regulation on the production of free radicals exists, oxidative stress develops (Dröge,

2002). The antioxidant capacity measured *in vitro* can give information on the possible effect that these compounds would perform on *in vivo* conditions.

Most HAT-based assays monitor competitive reaction kinetics and the quantitation is derived from kinetic curves, also they are generally composed of a synthetic free radical generator, an oxidable molecular probe, and an antioxidant (Huang et al., 2005). Crocin bleaching assay as a HAT-based assay, measures the inhibitory bleaching capacity of crocin from a certain sample, a naturally occurring carotenoid derivative, oxidized by the free radical generator 2,2'-Azobis-(2-methylpropionamidine) dihydrochloride (ABAP) (Huang et al., 2005).

A kinetics approach was used, for which the antioxidant capacity of PFQA and PFQM extracts were measured in terms of equivalence by weight to a reference antioxidant (catechin). For the kinetic analysis, a molar concentration of the antioxidant was required, and the whole dry matter of PFQ was considered as pure catechin; thus, the kinetic plot of the extract provides data in terms of the ratio between the rate constant (k) with peroxyl radicals of catechin and crocin. As PFQM is a product of a sequential extraction, the concentrations used for the kinetics of PFQM were 10 times higher than that of PFQA to increase its antioxidant activity closer to that of PFQA extract, as observed in previous experiments; this modification was also used for catechin in kinetics analysis of PFQM.

Total antioxidant capacity of PFQ is expressed as the ratio of the rate constants between $k_{catechin}/k_{PFQ}$. This result corresponds to the ratio of catechin required to the amount of PFQ. Consequently, because both concentrations have been calculated by using the molecular weight of catechin, the results are actually the ratio between amounts and indicate the quantity in grams of the PFQs required to achieve the same antioxidant capacity as 1 g of catechin. Results for the total antioxidant capacity ($k_{catechin}/k_{PFQ}$) reported in Table 4 did not show significant statistic differences between *Quercus* species and the catechin standard. Analyzing these results, it can be said that phenolic compounds from *Quercus* leaves, extracted with acetone (70%), given that they show important antioxidant capacity, therefore are capable of preventing the oxidation of molecule, such as crocin, avoiding alterations in their structure. The same protection can take place in molecules such as lipids and proteins avoiding their oxidation and thus preventing disorders in the functionality of these biomolecules. According to Parr and Bowell (2000), compounds that are reducing agents, and hydrogen transfer or electron donators, such a polyphenols, along with other dietary antioxidants, like vitamin C, can protect different body organs, shielding biomolecules against oxidative stress and preventing chronic diseases associated with oxidative stress, such as cancer, cardiovascular diseases, inflammation and others.

For PFQM extracts, an increase in the amount required to get the same antioxidant capacity as 1 g of catechin was observed for all samples compared to all PFQA extracts and the catechin standard. This again can be attributed to the nature of phenolic compounds present in the extract. Moreover, this data shows that PFQA and PFQM extracts from all *Quercus* species contain antioxidant molecules that are able to inhibit crocin bleaching.

Because the “antioxidant activity” measured by an individual assay reflects only the chemical reactivity under those specific conditions applied for that assay in particular, it is inappropriate and misleading to consider these data as an indicator for total antioxidant activity. However, the measurement of a sample by different assays *in vitro* allows us to comprehend the behavior of the sample, and thus predicting the possible effect on a biological system. According to our results, PFQr has the best antioxidant capacity compared to the rest of the *Quercus* species; however it is noteworthy that all the species have shown a fair degree of antioxidant capacity.

Conclusion

Extracts from *Quercus* leaves contain highly active polyphenolic compounds that confer antioxidant capacity, which was measured *in vitro* by SET- and HAT- based assays and in some cases similar to that of pure standards such as catechin, therefore this non-timber raw material can be exploited as a vast and reliable source of phenolic nutraceutical ingredients. Further work should be carried out to determine whether the activity shown *in vitro* could be extrapolated to *in vivo* conditions.

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ABBREVIATIONS

PFQs, Phenolic fractions from *Quercus* leaves; **PFQd,** *Quercus durifolia* extract; **PFQe,** *Quercus eduardii* extract; **PFQs,** *Quercus sideroxyla* extract, **PFQr,** *Quercus resinosa* extract; **PFQA,** Phenolic fractions with aqueous acetone; **PFQM,** Phenolic fractions with aqueous methanol.

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