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Castanea sativa Mill. Leaves as New Sources of Natural Antioxidant: An Electronic Spin Resonance Study

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The antioxidant potential of *Castanea sativa* Mill. leaf (sweet chestnut) was explored as a new source of active extracts. The capacity of the different fractions issued from aqueous, methanol, and ethyl acetate extracts to inhibit the stable free radical 2,2-diphenyl-1-pycryl-hydrazyl, superoxide anion, and hydroxyl radical was measured by electronic spin resonance. Their scavenging potential was analyzed versus their amount of phenolic compounds. Among the active fractions, the most effective one was A6, an ethyl acetate fraction, which contained a high level of total phenolic compounds (29.1 g/100 g). Thus, a different extraction procedure was performed to concentrate the active compounds of A6 in the new *C. sativa* leaf extract (CSLE). Compared to reference antioxidants (quercetin and vitamin E) and standard extracts (Pycnogenol, from French *Pinus maritima* bark, and grape marc extract), it was observed that A6 and CSLE have high antioxidant potentials, equivalent to at least those of reference compounds.

KEYWORDS: ***Castanea sativa*; ESR; antioxidant; phenolic compounds**

INTRODUCTION

Although oxygen is essential for aerobic forms of life, oxygen metabolites are highly toxic. In healthy individuals, free radical production is continuously balanced by natural antioxidative defense systems. Disruption of the balance between reactive oxygen species (ROS) production and elimination, due to, among other things, aging, leads to the process called oxidative stress. As a consequence, ROS are known to be implicated in many cell disorders and in the development of many diseases including cardiovascular diseases, atherosclerosis, cataracts, chronic inflammation, or neurodegenerative diseases such as Alzheimer's or Parkinson's disease (*1*−*5*).

To counteract deleterious effects of ROS, phenolic compounds, naturally distributed in plants, are effective (*6*, *7*). Epidemiological studies pointed out that diets rich in fruits and vegetables (rich in phenolic compounds) could prevent certain diseases in which free radicals are implicated (*8*, *9*). Because purified phenolic compounds are difficult to obtain and because extracts sometimes have antioxidant activities higher than those of pure molecules, there is a growing interest for the use of plant extracts. Among them, Pycnogenol from French *Pinus maritima* bark, oligomeric proanthocyanidins from *Vitis vinifera* marc or seed, and *Ginkgo biloba* leaf extract are used in pharmacology and cosmetology or as a food supplement. All of them have demonstrated high scavenging potential against ROS such as hydroxyl ($OH^{\bullet}$) and superoxide ions ($O_2^{\bullet -}$) (*10*−*13*). They also prevent low-density lipoprotein (LDL) oxidation, involved in atherosclerosis (*10*, *14*, *15*). Additionally, they inhibit platelet aggregation (*15*−*17*) and *G. biloba* and *V. vinifira* extracts are known to reduce myocardial ischemic reperfusion injury (*10*, *18*). It was observed that Pycnogenol is effective in the treatment of patients with chronic venous insufficiency (*19*, *20*). The *G. biloba* standardized extract improves cognitive functions in patients with age memory impairment and dementia, including Alzheimer's disease (*21*, *22*). Moreover, it is accepted that procyanidins from *V. vinifera* are significantly implicated in the cardioprotective effect of red wine known as the "French paradox" (*8*, *23*).

To find new natural sources of active extracts, we decided to explore the antioxidant potential of *Castanea sativa* Mill. (sweet chestnut). This plant is very common in the Limousin countryside and covers nearly 50000 ha. Many parts of this plant are exploited. The wood is used for timber, paper, or fuel; fruits are consumed roasted or boiled and as sweetened chestnut spread or jam. In this study, we focused on *C. sativa* leaves (CSL) used in folk medicine as a tea in France to treat hacking cough and diarrhea. Although it has already been demonstrated that CSL contain phenolic compounds (*24*), little is known about their antioxidant potential. It was recently demonstrated that CSL extract possesses a pronounced in vitro antibacterial effect (*25*). In a previous study, we demonstrated that the aqueous, methanol, and ethyl acetate extracts of CSL had good antioxidant

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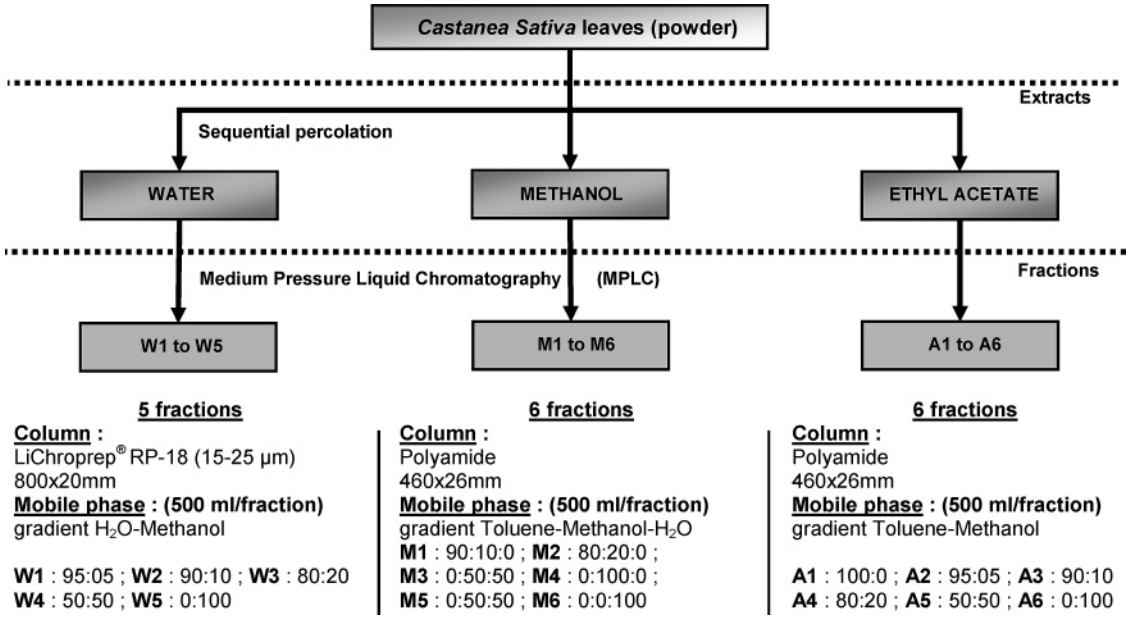

Figure 1. Extraction and fractionation procedure performed on raw *C. sativa* leaf powder.

potential as compared to *Vitis vinifera* (*26*). In this paper, we measured the capacity of the different fractions issued from these extracts to inhibit the stable free radical 2,2-diphenyl-1-pycrylhydrazyl (DPPH), superoxide anion ($O_2^{\bullet -}$), and hydroxyl radical ($OH^{\bullet}$) by electronic spin resonance (ESR). Their scavenging potential was analyzed versus their amount of phenolic compounds. Additionally, an extract was made from raw leaves to concentrate the most active compounds of CSL and tested for its free radical scavenging capacity. The composition was analyzed and compared to that of the other extracts/fractions. Results are discussed and compared to those obtained for reference antioxidant molecules (quercetin, α-tocopherol) and extracts (Pycnogenol, *V. vinifera* marc extract).

MATERIALS AND METHODS

Chemicals and Reagents. Plant powder was purchased from Pharma & Plantes (Valanjou, France). Pycnogenol was a gift from ORPHAG Research (Cointrin, Geneva, Switzerland). Grape marc extract was purchased from SEFCAL (St. Julien de Peyrolas, France). 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO), xanthine oxidase (XO), xanthine (X), iron(II) sulfate ($FeSO_4$), phosphate-buffered saline solution (PBS), and DPPH were supplied from Sigma (St. Louis, MO). Hydrogen peroxide (H_2O_2), LiChroprep RP-18, and Folin−Ciocalteu reagent were purchased from MerckEurolab (Fontenay sous Bois, France). Polyamide SC-6 was purchased from Macherey-Nagel (Hoerdt, France). All solvents were purchased from Carlo Erba reactifs (Nanterre, France).

Preparation of *C. sativa* Mill. Leaf Fractions and Extracts. Crude powder (250 g) was extracted by percolation sequentially using hexane, chloroform, ethyl acetate, methanol (5000 mL × 4), and water (2000 mL). The solvent was evaporated under reduced pressure at 45 °C. This procedure yielded hexane (8 g), chloroform (6 g), ethyl acetate (2.3 g), methanol (60 g), and water (15 g) dry extracts. The next separation step was done on water (3 g), methanol (3 g), and ethyl acetate (2 g) crude extracts by medium-pressure liquid chromatography (MPLC) using a LiChroprep RP-18 (Ø 15−25 µm) column (800 × 20 mm) for water extract and polyamide SC-6 (Ø < 70 µm) column (460 × 26 mm) for methanol and ethyl acetate. Fractions were obtained successively by using 500 mL of different mobile phases listed in **Figure 1**. The solvent was evaporated under reduced pressure at 45 °C. The 17 CSL fractions obtained were stored under vacuum and protected from UV irradiation until they were used.

The *C. sativa* leaf extract that concentrates the most active compounds (CSLE) was prepared as infusion. Plant powder (25 g) was extracted by 250 mL of distilled water/acetone (1:2) maintained at 60 °C under gentle agitation for 30 min. Plant powder was removed by filtration and acetone evaporated under reduced pressure at 45 °C. Phenolic compounds (monomers and small polymers) were separated from tannins by washing the filtrate three times with 150 mL of ethyl acetate. The aqueous fraction was discarded, and the ethyl acetate fraction was evaporated to dryness. The residue was dissolved in 150 mL of water/acetone (50:50), and chlorophyll was removed by extraction with 50 mL of chloroform and hexane. The water/acetone fraction was collected and evaporated to dryness under reduced pressure at 45 °C, stored under vacuum, and protected from UV light until it was used. This extraction yielded 0.85 g of CSLE from 25 g of leaf powder.

Phenols Quantification. Total phenolic (TP), tannins (T), and other phenolic compounds (OTH) were quantified for most active fractions according to the Folin−Ciocalteu method using pyrogallol as a standard (*27*). Absorbance was measured at 760 nm with a Uvikon 930 UV−vis spectrophotometer (Kontron Instruments), and the results were expressed as pyrogallol equivalents in grams per 100 g of dry material.

ESR Measurements. *DPPH Radical Scavenging Activity.* The potential antioxidant activities of fractions were assessed on the basis of the scavenging activity of the stable DPPH free radical. Reaction mixtures contained 50 µL of test samples dissolved in distilled water (water fractions) or DMSO (other fractions) and 50 µL of DPPH ethanolic solution (5×10^{-4} M). Due to its paramagnetic properties, DPPH exhibits a characteristic ESR signal (**Figure 2**). ESR spectra were obtained with a Bruker ESP300E spectrometer using microsampling pipets at room temperature under the following conditions: modulation frequency, 100 kHz; microwave frequency, 9.78 GHz; microwave power, 2 mW; modulation amplitude, 1.97 G; time constant, 10.24 ms. All spectra were recorded 3 min after agitation.

The inhibition ratio was calculated using eq 1

$$\text{inhibition ratio} = \frac{(\text{ref} - \text{extract})}{(\text{ref} - \text{bg})} \quad (1)$$

where ref is the double integral of the reference signal (DPPH + solvent), extract is the double integral of the test signal (DPPH + solvent + plant extract), and bg is the background signal. The data were the average of three measurements.

Hydroxyl Radical and Superoxide Anion Scavenging Activity. The hydroxyl radical was generated by Fenton reaction. $OH^{\bullet}$ radicals are identified because of their ability to form nitroxide adducts from the commonly used DMPO spin trap. The adduct DMPO−OH radical exhibits a characteristic ESR response (**Figure 2**). ESR spectra were obtained by using a Bruker ESP300E spectrometer set under the following conditions: modulation frequency, 100 kHz; microwave

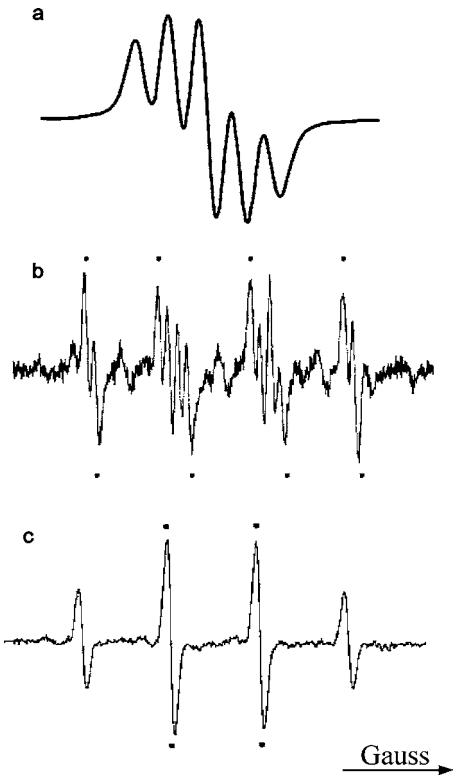

Figure 2. ESR spectra of DPPH radical (**a**), DMPO–OOH (**b**), and DMPO–OH (**c**) spin adducts obtained by the xanthine/xanthine oxidase system ($O_2^{\bullet -}$) and Fenton reaction ($OH^{\bullet}$), respectively. Squares (■) represent positions of peaks used for the calculation.

frequency, 9.78 GHz; microwave power, 4 mW; modulation amplitude, 0.987 G; time constant, 10.24 ms. The ESR spectrum was recorded at room temperature, 3 min after 4 μL of solvent (reference) or fraction [distilled water for aqueous fractions and water/acetone (50:50) for less polar extracts] was mixed with H_2O_2 (10 mM, 25 μL), $FeSO_4$ (2 mM, 25 μL), phosphate buffer salts (PBS; 10 mM) (120 mM NaCl, 2.7 mM KCl; pH 7.4, 46 μL), and DMPO (48 mM in PBS, 50 μL).

Superoxide anion was generated in an enzymatic system composed of X and XO. The resultant DMPO−OOH adduct exhibits another type of ESR response (**Figure 2**), and spectra were detected under the following conditions: modulation frequency, 100 kHz; microwave frequency, 9.78 GHz; microwave power, 10 mW; modulation amplitude, 0.495 G; time constant, 0.16 ms. The ESR spectrum was recorded at room temperature, 1 min after 4.5 μL of solvent (reference) or fractions (dissolved in water or DMSO) was mixed with X (10 mM, 22.5 μL), XO (1 unit/mL, 22.5 μL), DMSO (18 μL), phosphate buffer solution (PBS; pH 7.4; 15 μL), and DMPO (900 mM, 30 μL). X was dissolved in distilled water and DMPO in PBS. Spectra were recorded at 1 min because of the short half-life of the DMPO−OOH adduct.

For both radicals, we measured the peak high of the spin adduct (**Figure 2**). The inhibition ratio was determined by comparison with a solvent-treated group using the formula mentioned previously. Data were the average of three measurements.

HPLC and HPLC-MS Analyses. Each tested component (20 μL, 0.5 mg/mL) was injected into a Waters HPLC analytical system equipped with a 600 model pump, a variable-wavelength photodiode array detector (PDA 996), and a 600 model controller. The column used was a 250 × 4.6 mm i.d., 10 μm, μBondapak C18 cartridge (Waters). The mobile phase consisted of 100% methanol (A) and 1% aqueous acetic acid (B). Analyses were performed using a linear gradient from 30% A to 80% A during 60 min at 1 mL/min. Mass spectroscopy was performed on a Waters Alliance system equipped with a Waters electrospray interface. The source was operated in the negative and positive ion modes (ES^- and ES^+) with a 40 V cone voltage.

Table 1. Radical Scavenging Activity against DPPH, Superoxide, and Hydroxyl Radicals of Reference Antioxidants and Different Extracts and Fractions Obtained from Raw *C. sativa* Leaf Powder

	antioxidant activity (IC_{50}, μg/mL)		
	DPPH (SD ± 1 μg/mL)	superoxide (SD ± 0.2 μg/mL)	hydroxyl (SD ± 10 μg/mL)
aqueous extract	**71**	**2.2**	**330**
W1	>250	2.7	~500
W2	**47**	**1.7**	**400**
W3	**38**	**1.8**	**210**
W4	**37**	**1.9**	**170**
W5	>200	nd	500
methanol extract	**35**	**30.7**	**240**
M1	>250	≫30	>500
M2	>250	≫30	>500
M3	>250	≫30	>500
M4	**69**	**34.5**	>500
M5	**45**	**33.0**	**460**
M6	**31**	**28.9**	**160**
ethyl acetate extract	>250	20	nd
A1	>250	>20	nd
A2	>250	≫20	nd
A3	>250	≫20	nd
A4	>250	≫20	>500
A5	>250	≫20	>500
A6	**17**	**1.6**	**310**
Pycnogenol	**25**	3.4	**132 ± 5**
GME	**17.4** ± 0.5	3.0	**105** ± 5
CSLE	**16.8** ± 0.5	3.0	**91** ± 5
quercetin	**12.0** ± 0.5	2.3	**34** ± 5
vitamin E	**25.0** ± 0.5	nd	nd

RESULTS AND DISCUSSION

Radical Scavenging Activity. *DPPH Scavenging Activity*. It was observed that antioxidant activity was concentrated in 7 of the 17 fractions (**Table 1**). The IC_{50} values for these fractions (W2, W3, W4 M4, M5, M6, and A6) ranged from 69 μg/mL (M4) to 17 μg/mL (A6). Among these 7 fractions, 5 were more effective than the corresponding extracts. These fractions were W2, W3, W4, M6, and A6. Their IC_{50} values were 47, 38, 37, 31, and 17 μg/mL, respectively. The most effective one was A6, the only active ethyl acetate fraction.

Superoxide Anion Inhibition. This test measured the ability of fractions/extracts to scavenge the superoxide anion generated by the X/XO system. Activity could be attributed to direct scavenging or to enzymatic inhibition. Compared to the DPPH test, the most active fractions were the 7 fractions mentioned previously, with the addition of W1. The most effective ones were W2, W3, W4, and A6 with IC_{50} equal to 1.7, 1.8, 1.9, and 1.6 μg/mL, respectively. The aqueous extract was equally effective, with an IC_{50} equal to 2.2 μg/mL.

Hydroxyl Scavenging Activity. The capacity of fractions/extracts to inhibit hydroxyl radical generated by the Fenton reaction (Fe^{2+}/H_2O_2) was determined. This activity could be due to a direct scavenging effect and/or to inhibition of hydroxyl generation. The second mechanism occurs by ferrous ion chelation. As we observed in the other two tests, the same fractions were the most effective (except M4, which was, in this case, less active than the other fractions) (**Table 1**). Four fractions had a better activity compared to the corresponding extract: W3, W4, M6, and A6, with IC_{50} values of 210, 170, 160, and 310 μg/mL, respectively.

Comparison between Antioxidant Activity and Phenolic Amounts. We speculated that free radical scavenging activity of water, methanol, and ethyl acetate fractions was essentially due to the presence of phenolic compounds, and we investigated

Table 2. Phenolic Compounds Determined as Pyrogallol Equivalents in Grams per 100 g of Dry Material by Folin–Ciocalteu Method

	phenolic compounds (g/100 g)		
	total	tannins	others
aqueous extract	11.5	5.5	6.6
W1	2.2		2.2
W2	**14.4**	**3.0**	**11.4**
W3	**19.5**	**6.6**	**12.9**
W4	**22.4**	**9.6**	**12.8**
W5	4.8	1.8	3.0
methanol extract	14.7	5.3	9.4
M1	1.8	0.3	1.5
M2	3.5	0.1	3.4
M3	2.3		2.3
M4	9.4	0.8	8.4
M5	**12.6**	**1.6**	**11.0**
M6	**24.7**	**2.4**	**22.3**
A6	**29.1**	**2.8**	**26.3**
Pycnogenol	**30.7**	**17.9**	**12.8**
GME	**39.6**	**14.4**	**25.2**
CSLE	**27.5**	**7.9**	**19.6**

the phenolic composition of the most active fractions/extracts. **Table 2** shows the total phenolic composition and distinguishes tannins and other phenolic compounds.

Effect of MPLC Separation. For water extracts, phenolic compounds were concentrated in the W2, W3, and W4 fractions. The total phenolic composition was 14.4, 19.5, and 22.4 g/100 g of dry extract, respectively, whereas the other two fractions contained 4.8 g/100 g at most. Tannin amounts were 3.3, 6.6, and 9.6 g/100 g in W2, W3, and W4, respectively. Other phenolic compounds were nearly 12 g/100 g for these fractions.

The separation procedure for the methanol extract produced six fractions. Three of them contained a total phenolic amount up to 9 g/100 g. These fractions were M4 (9.4 g/100 g), M5 (12.6 g/100 g), and M6 (24.7 g/100 g), the most polar ones. Tannins represented <2.5 g/100 g of the phenolic amount. Compared to the crude methanol extract, M6 was the only fraction that had a higher phenolic compound ratio. Tannins represent a very minor part of the methanol fractions.

The ethyl acetate extract yielded six fractions. The amount of polyphenols was determined only for A6 because it was the only one that showed a consistent free radical scavenging activity. A6 contained the highest level of total phenolic compounds (29.1 g/100 g) among all fractions tested. Tannins represented only 2.8 g/100 g, whereas the amount of the other phenolic compounds was 26.3 g/100 g of the dry fraction.

Comparison between Free Radical Scavenging Activity and Phenolic Amounts. Among all of the extracts/fractions analyzed, a significant phenolic content (>12 g/100 g of dry extract) and good radical scavenging activity were found for six fractions and one extract. In general, the higher the free radical scavenging activity was, the higher the phenolic content was (**Tables 1** and **2**). Correlation between phenolic content and scavenging activity cannot be directly demonstrated, but we plotted $1/IC_{50}$ for DPPH versus phenolic amount. $1/IC_{50}$ is representative of the antioxidant activity because the more $1/IC_{50}$ increases, the more efficient is the extract. **Figure 3** shows a good correlation in the DPPH test between $1/IC_{50}$ and phenolic compounds ($R^2 = 0.88$), especially for phenolic compounds other than tannins [including flavonoid oligomers (OPC), flavonoids, and phenolic acids)] ($R^2 = 0.90$). No correlation was observed with tannins ($R^2 = 0.12$).

The superoxide scavenging activity correlated less with phenolic content ($R^2 = 0.34$). Interestingly, the R^2 coefficient

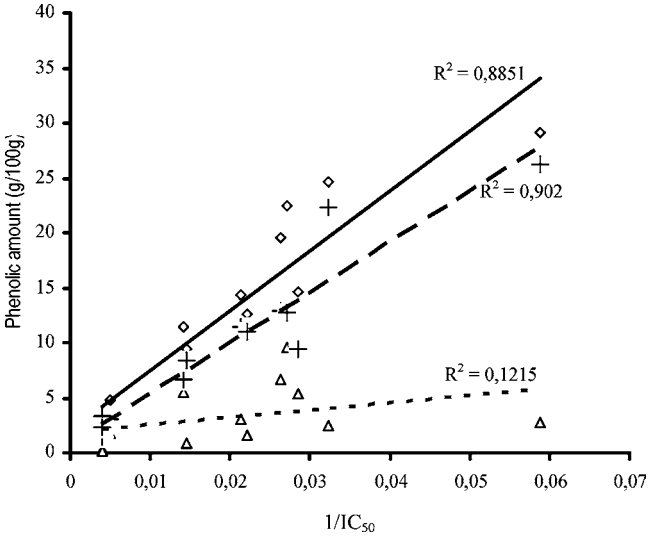

Figure 3. Correlation between DPPH scavenging activity (represented by $1/IC_{50}$) and phenolic composition of each extract and fraction. Solid line and circles represent total phenolic (TP), dashed line and crosses represent other phenolic compounds (OTH), and dotted line and triangles represent tannins (T).

recalculated without data concerning the methanol extract correlated well ($R^2 = 0.80$ for TP, 0.92 for OTH) except for tannins ($R^2 = 0.34$).

No correlation existed between $1/IC_{50}$ corresponding to the hydroxyl activity and the phenolic composition of extracts/fractions ($R^2 < 0.6$). A difference in correlation between the three antioxidant tests has already been observed by Pajero et al. (*28*) and by our laboratory (*26*) with plant extracts. A possible explanation is that the DPPH scavenging activity exclusively depends on redox potential, whereas superoxide and hydroxyl scavenging activity could occur concomitantly by direct radical quenching and inhibition of the radical generation. Iron chelating properties of tannins (*29*) could explain the increase we observed for hydroxyl scavenging activities of W2, W3, and W4 versus the increase in tannin composition [from 3.0 (W2) to 9.6 g/100 g (W4)].

In light of our results (**Table 2**) and compared to the classical extraction procedures used in industry (*30*, *31*), there is evidence that high phenolic polymers are concentrated in water fractions/extracts, whereas the degree of polymerization decreases from aqueous to methanol and ethyl acetate fractions/extracts. Phenolic acids such as acid phenols, flavonoid monomers, monoglycosylated flavonoids, and oligomers are probably concentrated in A6 and M6, whereas tannins are concentrated in water fractions. This could explain the high activity of A6 against superoxide radicals because flavonoids are known to be radical scavengers as well as xanthine oxidase inhibitors (*32*). Moreover, the scavenging activity of A6 against hydroxyl radicals can be attributed to the capacity of the flavonoids to scavenge free radicals and to chelate iron during the Fenton reaction (*33*). As mentioned previously, separation steps concentrate high phenolic polymers in water fractions; OPC and small polymers are concentrated in methanol fractions/extracts and monomers in ethyl acetate fractions/extracts. Phenolic distribution could explain the relatively poor superoxide scavenging activity of methanol fractions/extract compared to DPPH and hydroxyl scavenging activities. This could be attributed in part to lowest xanthine oxidase inhibition, high redox potential, and the iron chelating effect of OPC.

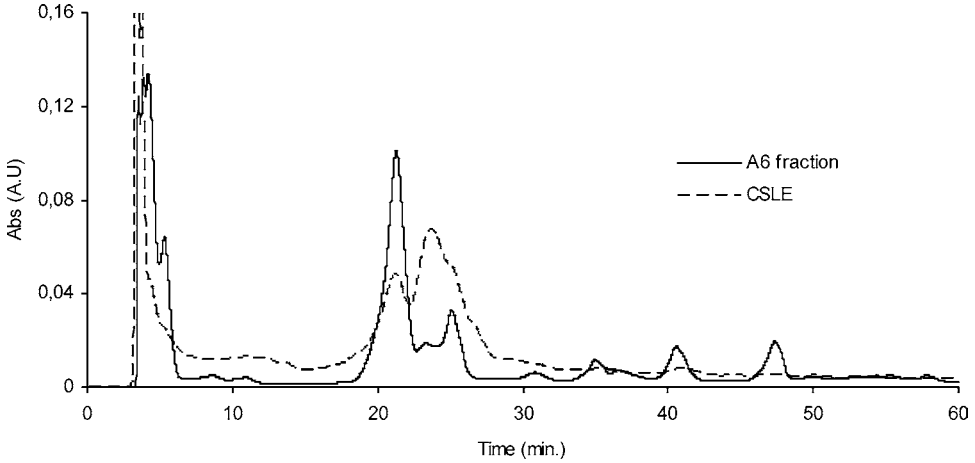

Figure 4. HPLC chromatogram of A6 fraction (solid line) and CSLE (dashed line). Conditions: wavelength, 254 nm; column, μBondapak C-18 10 μm (250 × 4.6 mm); mobile phase, methanol/water; gradient: 30:70 to 80:20 during 60 min.

Comparison between Fractions/Extracts Antioxidant Activity and Reference Antioxidants. We compared our results to reference antioxidants of natural origin. Quercetin, one of the most studied flavonols, known for its antioxidant power (*34*), and the chain-breaking antioxidant vitamin E were used as standard molecules. Vitamin E was used in only the DPPH test because it is a lipophilic antioxidant, insoluble in solvents used in superoxide and hydroxyl tests. Pycnogenol (from French *P. maritima* bark) and grape marc extract were used as standard extracts.

Quercetin IC_{50} values were 12, 2.3, and 34 μg/mL for DPPH, superoxide, and hydroxyl assays, respectively, and the DPPH vitamin E IC_{50} was 25 μg/mL (**Table 1**). The IC_{50} values of Pycnogenol and grape marc extracts were 25 and 17.4 μg/mL in the DPPH test and 3.4 and 3 μg/mL in the superoxide assay, respectively, and activity against hydroxyl radical was 132 and 105 μg/mL (**Table 1**). Standard activities were close to each other, even if quercetin was the most potent antioxidant in each test, particularly for the hydroxyl radical scavenging activity.

Compared to fractions/extracts, DPPH scavenging activities for W3, W4, M5, M6, A6, and methanol extracts were relatively close to those of standards (**Table 1**). They were at most 2-fold less active than vitamin E and 3-fold less active than quercetin. Considering that fractions or extracts are complex mixtures of compounds and assuming that certain compounds have low antioxidant activity, the radical scavenging potential of *C. sativa* leaf extracts is interesting. A6 was the most powerful, with IC_{50} values of 17 μg/mL (DPPH) and 1.6 μg/mL ($O_2^{\bullet -}$), very close to those of quercetin and more effective than vitamin E. Concerning hydroxyl scavenging activity, W3, W4, and M6 fractions were the most effective ones. W4 and M6 activities were ~5-fold less active than those of quercetin.

***C. sativa* Leaf Extracts.** Data obtained for fractions demonstrated that the most polar part of the ethyl acetate extract (A6) was the most antioxidant. Thus, a different extraction procedure was performed to concentrate the active compounds of A6 in the new *C. sativa* leaf extract (CSLE) (see Material and Methods).

Comparison of CSLE and Standard Antioxidants. CSLE demonstrated a strong antioxidant potential (**Table 1**). It was effective against DPPH with an activity range between those of quercetin and vitamin E (IC_{50} = 16.8 vs 12 and 25 μg/mL for quercetin and vitamin E, respectively). Its superoxide activity was close to that of quercetin (3.0 vs 2.3 μg/mL), whereas it was 3 times less active for hydroxyl inhibition (91 vs 34 μg/mL).

Compared to the reference extracts, the free radical scavenging activity of CSLE was similar to that of grape marc extract (GME) and Pycnogenol in each test performed (IC_{50} values were 16.8, 17.4, and 25 μg/mL in the DPPH test; 3.0, 3.0, and 3.4 μg/mL in the superoxide assay; and 91, 105, and 131 μg/mL in the hydroxyl radical scavenging activity for CSLE, GME, and Pycnogenol, respectively) (**Table 1**).

The phenolic composition of CSLE was slightly lower than that of GME and Pycnogenol (27.5 vs 39.6 and 30.7 g/100 g of pyrogallol equivalent) (**Table 2**). Phenolic compounds other than tannins represented 71.3, 63.6, and 41.7% of the total phenolic composition for CSLE, GME, and Pycnogenol, respectively. These differences could explain in part the relatively low activity of Pycnogenol compared to CSLE and GME extracts because it is assumed that phenolic monomers and oligomers are more reactive against free radicals compared to tannins. Nevertheless, Pycnogenol is well-known for its antioxidant activity reliability, in terms of both batch-to-batch variation and over time (season and year). This feature of Pycnogenol remains to be demonstrated for CSLE. However, these results demonstrate that CSLE has an attractive potential as a natural antioxidant.

Comparison of CSLE and A6 Fraction. The CSLE extract had the same activity on DPPH as A6 [~17 μg/mL (IC_{50})]. Comparatively, CSLE was 2 times less effective than A6 on superoxide and 3 times more effective on hydroxyl scavenging activity [3.0 vs 1.6 μg/mL (superoxide) and 91 vs 310 μg/mL (hydroxyl)]. The total phenolic composition of CSLE (27.1 g/100 g) was close to that of A6 (29.1 g/100 g). However, tannins and other phenolic compound ratios were different. Tannins represented 28.7 g/100 g of CSLE phenolic compounds and 9.6 g/100 g for A6. As mentioned previously, differences between mechanisms implicated in antioxidant activity observed in superoxide and hydroxyl tests could be explained in part by the ratios of tannins/other phenolic compounds.

CSLE was prepared to obtain the active molecules present in A6 directly from crude leaves. The HPLC profiles are compared in **Figure 4**. Analysis of the A6 chromatogram indicates the presence of three different groups of compounds. The first group had retention times (t_R) between 3 and 10 min, between 28 and 38 min for the second group, and betwee 34 and 50 min for the last one. Two of the three groups of compounds (t_R = 3−10 min and 28−38 min, respectively) were present in CSLE (**Figure 4**). Analysis of UV spectra and retention times indicated that the first group was composed, for the major components, of phenolic acids derived from benzoic acid characterized by one or two absorption bands in the 235−

305 nm range. UV spectra for the second group (t_R = 28−38 min) displayed two major absorption bands in the 240−400 nm region characteristic of flavones and flavonols (Band I in the 320−385 nm region representing B ring absorption, and Band II in the 250−285 nm representing A ring absorption) (*34*, *35*). For all groups of compounds, the band I position in the 350−360 nm range is in favor of flavonols instead of flavones. This absorption range is equally characteristic of flavonols without a hydroxyl group on B ring or C-3 substituted. The major component of this group (t_R = 21.4 min) was analyzed by ES-MS. The negative and positive ES-MS spectra gave molecular ions at *m*/*z* 463 $[M - H]^-$, *m*/*z* 465 $[M + H]^+$, and *m*/*z* 465 $[M + Na]^+$, respectively, suggesting the molecular formula $C_{21}H_{20}O_{12}$ corresponding to isoquercitrin (quercetin 3-glucoside), which was previously described in *C. sativa* leaves (*24*). This result was confirmed by the molecular ion at *m*/*z* 465 $[M + H]^+$ corresponding to quercetin and by analysis of UV spectra and retention time of standard isoquercitrin injected in the same chromatographic system as A6 and CSLE. The third group of compounds (t_R up to 34 min) has not been identified at present.

Comparison of antioxidant potential and HPLC chromatogram of A6 and CSLE demonstrated that the procedure used to obtain CSLE is adapted for direct extraction of the major active compounds of A6.

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LITERATURE CITED

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