Free Radical Scavenging Activities Measured by Electron Spin Resonance Spectroscopy and B16 Cell Antiproliferative Behaviors of Seven Plants

Claude-Alain Calliste,[†] Patrick Trouillas,[†] Daovy-Paulette Allais,[‡] Alain Simon,[§] and Jean-Luc Duroux*,[†]

UPRES EA 1085, Biomolécules et Cibles Cellulaires Tumorales, Laboratoire de Biophysique, Laboratoire de Pharmacognosie, and Laboratoire de Chimie Minérale, Faculté de Pharmacie, 2 rue du Dr. Marcland, 87025 Limoges Cédex, France

In an effort to discover new antioxidant natural compounds, seven plants that grow in France (most of them in the Limousin countryside) were screened. Among these plants, was the extensively studied *Vitis vinifera* as reference. For each plant, sequential percolation was realized with five solvents of increasing polarities (hexane, chloroform, ethyl acetate, methanol, and water). Free radical scavenging activities were examined in different systems using electron spin resonance (ESR) spectroscopy. These assays were based on the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH), the hydroxyl radicals generated by a Fenton reaction, and the superoxide radicals generated by the X/XO system. Antiproliferative behavior was studied on B16 melanoma cells. ESR results showed that three plants (*Castanea sativa*, *Filipendula ulmaria*, and *Betula pendula*) possessed, for the most polar fractions (presence of phenolic compounds), high antioxidant activities in comparison with the *Vitis vinifera* reference. *Gentiana lutea* was the only one that presented a hydroxyl scavenging activity for the ethyl acetate and chloroform fractions. The antiproliferative test results showed that the same three plants are the most effective, but for the apolar fractions (chloroform and hexane).

Keywords: *Radical scavenging; plant extracts; B16 cells; phenolic compounds; ESR*

INTRODUCTION

Reactive oxygen species (ROS) such as the superoxide anion ($O_2^{\bullet -}$), the hydroxyl radical ($OH^{\bullet}$), and hydrogen peroxide are highly reactive species that are responsible for many cell disorders through their action on proteins, DNA, and lipid peroxidation. By modifying the oxidative balance within the cells, these ROS are important mediators of cell injuries. They are assumed to play an important role in the development of many diseases. They are directly or indirectly involved in atherosclerosis, reperfusion injury, cataractogenesis, rhumatoid arthritis, inflammatory disorders, the aging process, and cancer (*1*−*6*).

We tested the antioxidant activities of plants by measuring their ability to scavenge the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) and the oxygen free radicals hydroxyl ($OH^{\bullet}$) and superoxide ($O_2^{\bullet -}$) using electron spin resonance (ESR) spectroscopy.

Then we tested on B16 melanoma cells, which were derived from C57BI/6 mouse spontaneous skin tumor cells, the cytotoxic effect of these plant extracts. This cell line is used, in our case, as a skin cell model. Skin cells are naturally exposed to several exogenous sources of free radicals such as air pollution and UV radiation. Here we look at the cytotoxicity of the plant extracts we have tested for their antioxidant properties.

In this study, we evaluated plants that grow in France (most of them in the Limousin countryside). We worked on *Betula pendula* Roth bark, *Castanea sativa* Lam. and *Juglans regia* L. leaves, *Gentiana lutea* L. root, *Filipendula ulmaria* (L.) Maxim. flowering top, *Calluna vulgaris* L. aerial parts, and *Vitis vinifera* L. marc. All of these plants have already been used in folk medicine and are suspected to contain natural antioxidants, especially phenolic compounds (see Table 1) (*7*, *8*).

To search for new antioxidant natural materials (*9*−*11*), five dry extracts are tested for each plant; they are obtained by sequential percolation with five solvents of increasing polarities (hexane, chloroform, ethyl acetate, methanol, and water). In this manner, all compounds of the plants could be evaluated for their antiproliferative and antioxidant activities.

The data obtained with the extracts are compared with the well-known results of *Vitis vinifera*.

V. vinifera contains many antioxidant molecules. The different parts of grapes (leaves, fruit, skin, seeds, etc.) have long been known as popular remedies for numerous symptoms such as inflammation, diarrhea, and cancer. For example, clinical epidemiological studies showed that the chronic intake of red wine protects against atherosclerosis and presents more generally a cardioprotective effect (*12*, *13*). One can talk about the French paradox, which is a concomitant chronic intake of red wine and a high-fat diet (generally invoked as an atherosclerosis source). In fact, many studies have shown that grape (*V. vinifera*) contains a large quantity of polyphenols. Several chromatographic studies (*14*−*20*) have established the presence, in fruits and leaves

* Corresponding author [telephone 33 (0)5 55 43 58 45; fax 33 (0)5 55 43 58 44; e-mail duroux@pharma.unilim.fr].

[†] Laboratoire de Biophysique.

[‡] Laboratoire de Pharmacognosie.

[§] Laboratoire de Chimie Minérale.

10.1021/jf010086v CCC: $20.00
Published on Web 06/08/2001

Table 1

species	chemical constituents	traditional uses
B. pendula	flavonoids, tannins, triterpenes, essential oil, arylbutanoids, glycosidic diarylheptanoids	diuretic, anti-inflammatory
Cal. vulgaris	numerous flavonoids, tannins, triterpenes (ursolic acid)	diuretic
Cas. sativa	flavonoids, tannins, triterpenes (ursolic acid)	activity on vascular tone, expectorant, sedative of cough
F. ulmaria	flavonoids, tannins, phenolic acids glycosides (derivatives of salicylic acid)	diuretic, antalgic, anti-inflammatory
G. lutea	phenolic acids, xanthones, secoiridoids, phytosterols	stimulant of the appetite
J. regia	flavonoids, tannins, naphthoquinones (juglone)	decrease in capillary permeability and fragility and increase in resistance, antidiarrheic, emolliant and antalgic (locally)
V. vinifera	numerous phenolic compounds: flavonoids, tannins (proanthocyanidins), anthocyanins, stilbenes	effect on capillary permeability and fragility (increase in vascular tone), enzyme inhibitor, free radical scavenger

of *V. vinifera*, of procyanidins (monomers, oligomers, and polymers of the flavan-3-ols). The highly standardized mixture of polyphenols obtained from grape seeds by using a patented process contains dimers and trimers, oligomers up to seven units, and small amounts of polymers. *V. vinifera* contains other phenolic compounds such as flavanoids, anthocyanins, phenolic acids, and stilbenes. All of these phenolic compounds have been studied for their antioxidant properties. So, in this paper, we will compare the results obtained with the data known about *Vitis vinifera*.

Gentiana lutea has been chosen because of the presence of chemical compounds that are different from the six other plants (Table 1).

Cal. vulgaris has previously been studied in our laboratory (*21*, *22*), and we will also see that this plant can be taken as a reference for antiproliferative tests.

MATERIALS AND METHODS

Chemicals and Reagents. Plant powder was purchased from Pharma & Plantes (Valanjou, France): *Betula pendula* Roth bark, *Castanea sativa* Lam. and *Juglans regia* L. leaves, *Gentiana lutea* L. root, *Filipendula ulmaria* (L.) Maxim. flowering top, *Calluna vulgaris* L. aerial parts, and *Vitis vinifera* L. marc. DMPO, xanthine oxidase (XO), xanthine (X), iron(II) sulfate ($FeSO_4$), and DPPH was supplied from Sigma (St. Louis, MO). Hydrogen peroxide (H_2O_2) was purchased from Prolabo (Fontenay sous Bois, France). All products for cell culture were purchased from Gibco (Cergy Pontoise, France). All solvents were purchased from Carlo Erba Reactifs (Nanterre, France).

Extraction Procedure. For each plant, 25 g of crude powder was extracted by percolation using sequentially hexane, chloroform, ethyl acetate, methanol, and water (500 mL × 5). The solvent was evaporated under reduced pressure at 45 °C to yield dry extracts. They were stored under vacuum at room temperature and protected against UV irradiation.

ESR Measurements. *DPPH Radical Scavenging Activity.* The potential antioxidant activity of plant extracts was assessed on the basis of the scavenging activity of the stable DPPH free radical. Reaction mixtures contained 100 μL test samples (0.5 mg/mL dissolved in each solvent) and 100 μL of DPPH ethanolic solution (5×10^{-4} M). Due to its paramagnetic properties DPPH exhibits an ESR signal. 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

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

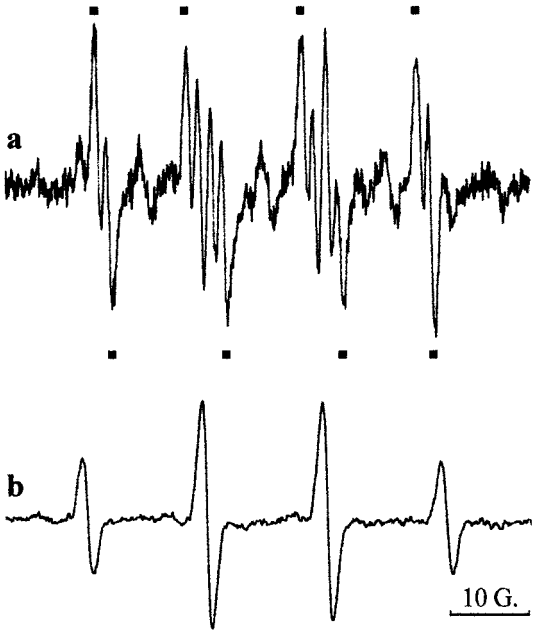

Figure 1. (a) ESR spectra obtained with X/XO system. Squares (■) represent position of the DMPO−OOH signal. (b) DMPO−OH spin adduct obtained by Fenton reaction.

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

Hydroxyl Radical and Superoxide Anion Scavenging Activity. The hydroxyl radical was generated by Fenton reaction. $OH^{\bullet}$ are identified because of their ability to form nitroxide adducts from the commonly used 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) spin trap. This spin trap has a greater ability of trapping oxygen-centered radicals than other nitrone spin traps (*23*). The adduct DMPO−OH radical exhibits a characteristic ESR response (Figure 1a). Moreover, these adducts have relatively long lives, whereas the life span of $OH^{\bullet}$ is very short. Spin trapping has become a valuable tool to characterize and quantify each oxygen radical (*24*). ESR spectra were obtained by using a Bruker ESP300E spectrometer set under the following conditions: modulation frequency, 100 kHz; microwave 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 extract (0.5 mg/mL) was mixed with H_2O_2 (10 mM, 25 μL), $FeSO_4$ (2 mM, 25 μL), and DMPO (48 mM, 50 μL) in a phosphate buffer solution (pH 7.4). Three minutes was found to be the optimum reaction time in order to obtain the best inhibition ratio with the best stability.

The superoxide anion was generated in an enzymatic system by X/XO. The resultant DMPO−OOH adduct exhibits another type of ESR response (Figure 1b), and spectra were detected

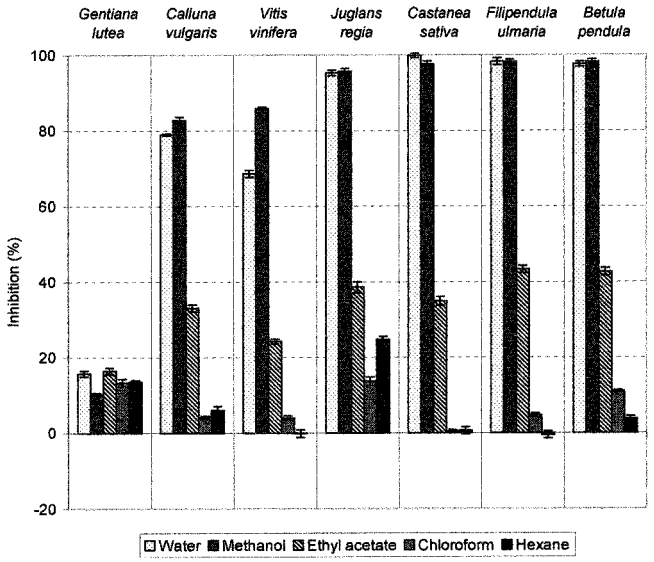

Figure 2. DPPH radical scavenging activity of plant extracts. Fractions for each plant are, respectively, water, methanol, ethyl acetate, chloroform, and hexane. Data are represented as inhibition percentage of double-integral signal of DPPH radical measured by ESR and are means ± standard deviation of four independent experiments.

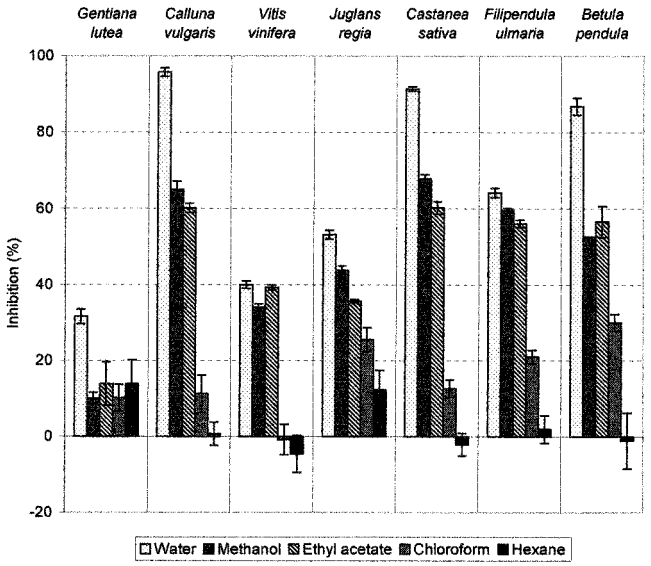

Figure 3. Inhibition of DMPO−OOH spin adduct by plant extracts. Fractions for each plant are, respectively, water, methanol, ethyl acetate, chloroform, and hexane. Data are represented as inhibition percentage of DMPO−OOH spin adduct peak height measured by ESR and are means ± standard deviation of four independent experiments.

in the same way 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 μL of solvent (reference) or extract (0.5 mg/mL) was mixed with X (2 mM, 25 μL), XO (0.5 units/mL, 25 μL), and DMPO (900 mM, 50 μL) in a phosphate buffer solution (pH 7.4).

For each radical, we measured the first derivative peak height of the spin adduct ESR signal. The inhibition ratio was determined by comparison with a solvent-treated group using eq 1. Data were the average of four measurements.

Cell Culture and Antiproliferative Measurements. B16 cells were grown in Eagle's minimum essential medium supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 units/mL), streptomycin (100 μg/mL), vitamin solution (100X), sodium pyruvate (100 mM), nonessential amino acids (100X), and L-glutamine (200 μM). They were incubated at 37 °C under a humidified atmosphere with 5% CO_2 in 12.5 cm^2 culture flasks (Nunc). They were plated at a density of 2×10^5 cells/mL for 24 h to ensure total attachment. At this time, plant extracts (0.1 mg/mL) diluted in water (for aqueous fraction) or DMSO (for methanol, ethyl acetate, chloroform, and hexane fractions) were added with cultured medium. For each solvent, control dishes were made using solvent only. After 2 days of growth, cell viability was determined by trypan blue exclusion assay.

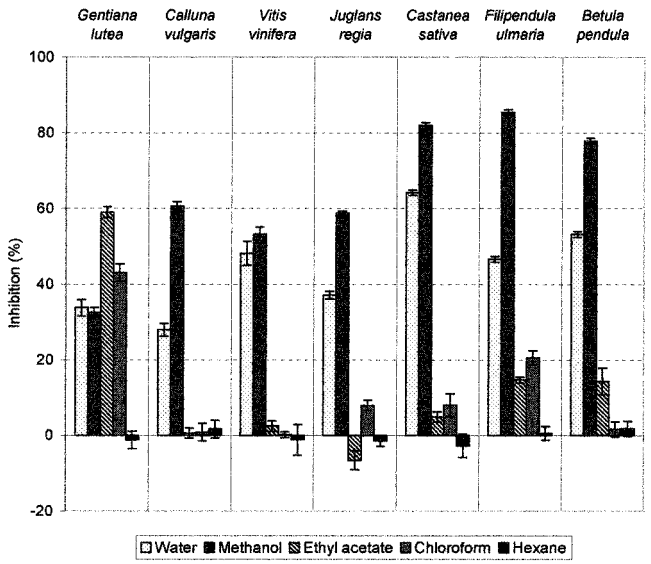

Figure 4. Inhibition of DMPO−OH spin adduct by plant extracts. Fractions for each plant are, respectively, water, methanol, ethyl acetate, chloroform, and hexane. Data are represented as inhibition percentage of DMPO−OH spin adduct peak height measured by ESR and are means ± standard deviation of four independent experiments.

RESULTS AND DISCUSSION

In Vitro Free Radical Scavenging Activities of Plant Extracts. We show in Figures 2, 3, and 4 the results of the scavenging activities of the fractions for the seven plants we studied, respectively for the three tests described under Materials and Methods.

The results we obtained must be compared with compounds having well-known antioxidant properties. A comparison with a molecule [such as α-tocopherol (vitamin E)] is difficult because each fraction could contain many molecules, and so the concentration of the extracts cannot directly be compared with the concentration of the reference molecule.

As we said above, *V. vinifera* has been extensively studied, and many chromatographic data showed the presence of polyphenolic molecules not only in the aqueous fraction and in the methanol one (*14*−*20*) but also in the ethyl acetate one (*25*, *26*). The antioxidant properties of these molecules are very well-known (*27*−*30*). These compounds are generally distributed in the three solvents as a function of polarity: phenolic acids, aglycon or monoglycosyl flavanoids, and monomers or polymers of procyanidins in the ethyl acetate fraction; phenolic acids glycosides, diglycosyl flavanoids, and oligomers of procyanidins in the methanol fraction; the most polar compounds such as triglycosyl flavanoids, galloyl tannins, and procyanidins (polymers) with high molecular weights in the water fraction.

Polyphenolic compounds were generally not found in the chloroform and hexane fractions (only very small

Table 2. Inhibition Ratio for the DPPH Radical Scavenging of the *Cas. sativa*, *B. pendula*, *F. ulmaria*, and *J. regia* Extracts, Tested with a 0.1 mg/mL Concentration[a]

species	water fraction (%)	methanol fraction (%)
Cas. sativa	68.9 ± 0.2	78.9 ± 1.2
B. pendula	33.9 ± 0.9	71.1 ± 1.7
F. ulmaria	35.0 ± 1.1	59.4 ± 2.6
J. regia	22.8 ± 0.9	40.9 ± 0.6

[a] Data are represented as inhibition percentage and are means ± standard deviation of four independent experiments.

quantities could be found in the chloroform one). In this case, *V. vinifera* is not a good reference.

The results for DPPH radical scavenging activity of the different fractions from the different plants are shown in Figure 2. We can observe that all plants except *G. lutea* possess good antioxidant activities for the concentrations of water and methanol fractions which were tested (0.5 mg/mL), in comparison with the *V. vinifera* fraction. They were able to inhibit >65% of the reference signal and 100% for *J. regia*, *C. sativa*, *F. ulmaria*, and *B. pendula*. To distinguish the activities of these four extracts, we tested them at a lower concentration (0.1 mg/mL). The results are shown in Table 2. By comparison, a 0.05 mg/mL concentration of α-tocopherol (vitamin E) inhibits 50% of the DPPH reference signal. Here we can see that the water and methanol fractions of *C. sativa* are the most effective ones, with activities twice as high as the *V. vinifera* one. The activities of the ethyl acetate fractions of these four plants are the highest ones; however, the ethyl acetate fractions of all six plants (except *G. lutea*) are able to scavenge between 30 and 45% of the control, which is less important than for the water and methanol fractions. The activities observed for the chloroform and the hexane fractions are very small, except for *G. lutea* and *J. regia* (∼20% inhibition).

The abilities of the plant extracts we tested to scavenge free radicals were further confirmed by their abilities to scavenge the superoxide anion. We show in Figure 3 the inhibition percentage of the superoxide generated by X/XO. The results show that as in the preceding test, the water and the methanol extracts are the most effective ones. In this case, the water fractions have better scavenging activities than the methanol fraction ones (*Cal. vulgaris* > *Cas. sativa* > *B. pendula* ≫ *F. ulmaria* > *J. regia* > *V. vinifera* > *G. lutea*). We can also see that the ethyl acetate extracts have interesting activities which are close to the results we obtained for the methanol fraction. Four plants are able to inhibit at least 60% of the original signal. These plants are *Cal. vulgaris*, *Cas. sativa*, *F. ulmaria*, and *B. pendula*. *G. lutea* always shows the lowest activity. For this test, *V. vinifera* and *J. regia* present a lower activity than in the preceding test.

The decrease of the DPPH signal occurs when the odd electron of the radical is paired; thus, it is representative of the capacity of the extracts to scavenge free radicals independently from any enzymatic activity. Therefore, the discrepancies with the superoxide test may be explained by the fact that the inhibition of the generation could be due either to the scavenging activity or to the inhibition of the xanthine oxidase. There is a fundamental difference on both inhibition mechanisms; whereas plant extracts contain many molecules with different chemical behaviors, both effects could occur simultaneously. This double activity has already been

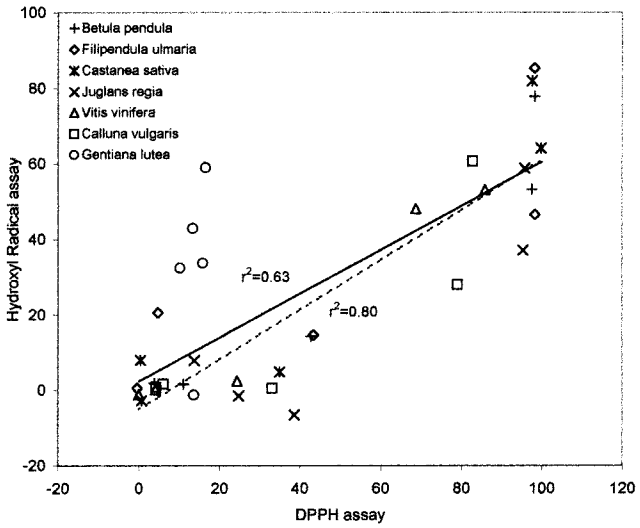

Figure 5. Correlation between DPPH and hydroxyl radical assays: solid line for all extracts and dotted line without taking account of *G. lutea*.

reported for flavonoid molecules (*31*). The differences we observed between Figures 2 and 3 show an increase in the activity of the water and ethyl acetate fractions by comparison with the methanol fraction one. This difference between both tests (DPPH, $O_2^{\bullet -}$) is generally explained by the presence of molecules with enzymatic inhibition properties, such as flavonoids that are contained in the ethyl acetate fraction.

Then we tested the scavenging activity of plant extracts against hydroxyl radicals generated by the Fenton reaction; the results are shown in Figure 4. The difference between both tests (DPPH, $OH^{\bullet}$) is only a chemical difference; there is no enzymatic process involved in the generation of radicals. For the six plants that are effective on the DPPH test, the methanol and water fractions are the most effective ones on the DMPO−OH scavenging. That is to say that, for these plants, there is no important difference in behavior in chemical mechanisms of scavenging of the two types of radicals. A moderate correlation ($r^2 = 0.63$) is shown in Figure 5. However, in opposition with the previous test (the superoxide one) and in accordance with the DPPH results, the methanol fractions have better scavenging activities than the water fractions do.

In comparison with the other plants, we note that *G. lutea* presents, especially for the ethyl acetate and chloroform extracts, an interesting hydroxyl scavenging activity. The generation of the hydroxyl radical in the Fenton reaction is due to the presence of the iron ions (Fe^{2+}). Many studies have shown that some flavonoids are good iron chelators (*27*, *32*, *33*). It was suggested that these compounds might be exerting their protective effects through chelation of metals or by altering iron redox chemistry (*34*). When this effect is observed on plants, it is often attributed to the presence of flavonoids (*35*, *36*). In our case, this effect is present for the *G. lutea* ethyl acetate and chloroform fractions. Therefore, we can reasonably consider that the activity of these two fractions of *G. lutea* is due to the presence of compounds with effective iron chelating properties. In Figure 5, we also show the correlation between DPPH and $OH^{\bullet}$ assays without taking into account *G. lutea*. The better correlation coefficient we obtained ($r^2 = 0.80$) confirms a different feature for this plant.

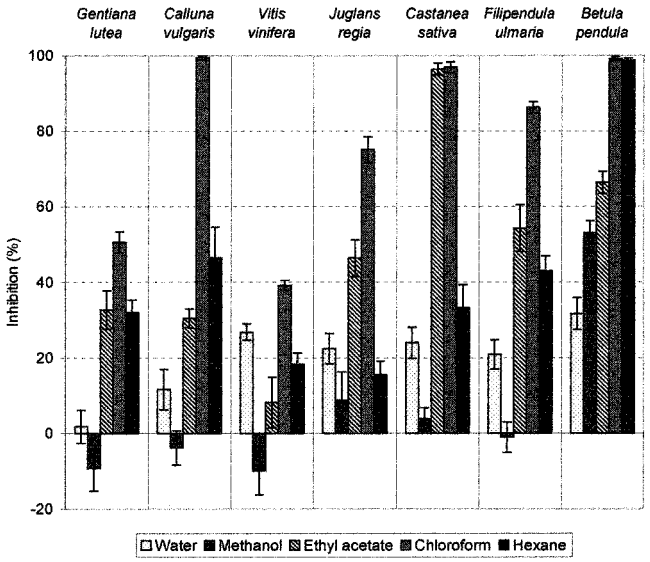

Figure 6. Cytotoxic effect of plant extracts on B16 melanoma cells. Fractions for each plant are, respectively, water, methanol, ethyl acetate, chloroform, and hexane. Data are represented as inhibition percentage of B16 cell proliferation after 2 days of growth and are means ± standard deviation of four independent experiments.

To summarize, the water and methanol fractions of the plants we tested showed an important scavenging activity for each specific radical, in comparison with the same fractions of *V. vinifera*. The antioxidant activity increases significantly with the polarity of the extracts. These results are in accordance with the literature, where correlation is made between free radical scavenging activity and the amount of polyphenols available in the fractions. As *V. vinifera* was taken as a reference in this paper, we note the higher activity, in the three tests, of *Cas. sativa*, *F. ulmaria*, and *B. pendula* in comparison with this reference. This comparison leads us to investigate furthermore the properties of the plants we studied, especially *Cas. sativa*, which presents the highest activity in the three tests. *G. lutea* is the only one that presents a hydroxyl scavenging activity for the ethyl acetate and chloroform fractions. This is probably due to the presence of iron chelators.

Antiproliferative Effects of the Plant Extracts. In biological systems, oxidative stress results from a disruption of the prooxidant/antioxidant cellular balance. This stress can lead to subsequent tissue damage. Protection against ROS within the cells is provided by antioxidant enzymes and small molecular weight antioxidants such as α-tocopherol (vitamin E) and ascorbic acid (vitamin C). They worked as preventive antioxidants by eliminating these highly reactive species. The antioxidant compounds are generally involved in cancer protection. Although they act on inhibition of ROS, their antitumoral action must be involved in the earliest stage of the cancer, during the transformation from healthy cells to tumoral cells; ROS can display their cytotoxic action during this state, for example, by modifying DNA.

We tested the seven medicinal plants (which have long been used in folk medicine and are even known for their wide range of pharmacological properties) for their cytotoxicity effects against mouse melanoma cells (B16 cells).

The cytotoxicity effect results are shown in Figure 6. The most effective fractions, for all plants, are the chloroform, ethyl acetate, and hexane ones. For *J. regia*, *Cal. vulgaris*, *Cas. sativa*, *F. ulmaria*, and *B. pendula*, the chloroform fraction inhibits >75% of B16 proliferation. The ethyl acetate fractions of *Cas. sativa* Lam., *F. ulmaria* L. Maxim., and *B. pendula* inhibit >50% of the proliferation, and the hexane part of *B. pendula* inhibits the cell proliferation by >95%. The methanol and water fractions of the plants have low antiproliferative activities against B16 proliferation.

The most effective plants were *Cal. vulgaris* aerial parts, *Cas. sativa*, *F. ulmaria* flowering top, and *B. pendula* bark as in the preceding tests, but the active fraction is in complete opposition to those of the antioxidant test. The most apolar fractions present the lowest antioxidant activity and at the same time they show the highest cytotoxic activity for the B16 melanoma cells. The chloroform fraction is the most cytotoxic for all of the plants studied. These activities may be due to, at least in part, the high hydrophobic character of the compounds extracted in these fractions, which probably can strongly incorporate into the membranes and may act as cytotoxic agent at this level. In our laboratory, previous studies have permitted us to identify in the chloroform and hexane fractions of *Cal. vulgaris* the ursolic acid, a pentacyclic triterpene acid, which is known to be a very potent cell growth inhibitor (*21*, *22*). Its action on the membrane has recently been demonstrated on Daudi cells (Burkitt's human B cells) (*37*). Therefore, it is not unreasonable to speculate that the cytotoxic activity which is observed in the most apolar fractions of the plants we tested may be due to the same kind of compounds, which act by modifying the cell membrane activity.

In conclusion, we observed some different interesting results with the seven plants we studied.

On the one hand, the water and methanol fractions of *B. pendula*, *Cas. sativa*, *J. regia*, *F. ulmaria*, and *Cal. vulgaris* show good antioxidant activities and no cytotoxic effect on B16 cells. This result is very interesting for the use of these plant extracts as protectors against oxidative stress, such as UV irradiation. Indeed, antioxidant compounds can be used to prevent the degradation induced by UV irradiation. α-Tocopherol and Trolox (a synthetic analogue of α-tocopherol) significantly reduced the cytotoxicity induced by UV irradiation (*38*). In the same way, isoflavonoids were tested for their protective effect against DNA oxidation produced by γ-radiation (*39*). The antioxidant effects of the water and methanol fractions we studied in this paper could be attributed to the presence of phenolic compounds. These fractions, particularly those of *B. pendula*, *Cas. sativa*, and *F. ulmaria* will be studied as a promising rich source of natural antioxidant compounds, and we are going to test them for their preventive action against oxidative stress caused by UV irradiation on B16 cells.

On the other hand, we observed some interesting data for the ethyl acetate fraction of *G. lutea* in which we supposed the presence of molecules with chelating properties. These explanations must to be confirmed by further chromatographic and NMR studies.

We also noted both antioxidant and cytotoxic effects for the ethyl acetate fractions of *B. pendula*, *Cas. sativa*, *J. regia*, *F. ulmaria*, *Cal. vulgaris*, and *V. vinifera*. Further studies might allow us to understand if this double activity is due to different compounds or to the association of different chemical species with each specific activity.

Finally, the high cytotoxic activity of the hexane and chloroform fractions in *B. pendula*, *Cas. sativa*, and *F. ulmaria* will lead us to fractionate further the plants in order to extract the active molecules.

LITERATURE CITED

(1) Stohs, S. J. The role of free radicals in toxicity and disease. *J. Basic Clin. Physiol. Pharmacol.* **1995**, *6* (3−4), 205−228.

(2) Aust, S. D.; Chignell, C. F.; Bray, T. M.; Kalyanaraman, B.; Mason, R. P. Free radicals in toxicology. *Toxicol. Appl. Pharm.* **1993**, *120* (2), 168−178.

(3) Aruoma, O. I.; Kaur, H.; Halliwell, B. Oxygen free radicals and human diseases. *J. R. Soc. Health* **1991**, *111* (5), 172−177.

(4) Halliwell, B. Mechanisms involved in the generation of free radicals. *Pathol. Biol. (Paris)* **1996**, *44* (1), 6−13.

(5) Knight, J. A. Diseases related to oxygen-derived free radicals. *Ann. Clin. Lab. Sci.* **1995**, *25* (2), 111−121.

(6) Gutteridge, J. M. Free radicals in disease processes: a compilation of cause and consequence. *Free Radical Res.* **1993**, *19* (3), 141−158.

(7) Bruneton, J. In *Pharmacognosy Phytochemistry Medicinal Plants*; Intercept, Lavoisier Publishing: Paris, France, 1999.

(8) Wichtl, M.; Anton, R. In *Plantes Thérapeutiques*; Technique & Documentation (Ed.), Editions Médicales Internationales: Paris, France, 1999.

(9) Liu, F.; Ng, T. B. Antioxidative and free radical scavenging activities of selected medicinal herbs. *Life Sci.* **2000**, *66* (8), 725−735.

(10) Ng, T. B.; Liu, F.; Wang, Z. T. Antioxidative activity of natural products from plants. *Life Sci.* **2000**, *66* (8), 709−723.

(11) Maulik, G.; Maulik, N.; Bhandari, V.; Kagan, V. E.; Pakrashi, S.; Das, D. K. Evaluation of antioxidant effectiveness of a few herbal plants. *Free Radical Res.* **1997**, *27*, 221−238.

(12) Bombardelli, E.; Morazzoni, P.; Carini, M.; Aldini, G.; Maffei Facino, R. Biological activity of procyanidins from: *Vitis vinifera* L. *Biofactors* **1997**, *6*, 429−431.

(13) Das, D. K.; Sato, M.; Ray, P. S.; Maulik, G.; Engelman, R. M.; Bertelli, A. A.; Bertelli, A. *Drugs Exp. Clin. Res.* **1999**, *25* (2−3), 115−120.

(14) Bombardelli, E.; Morazzoni, P. *Vitis vinifera* L. *Fitoterapia* **1995**, *66* (4), 291−316.

(15) Lunte, S. M.; Blankenship, K. D.; Read, A. R. Detection and identification of procyanidins and flavanols in wine by dual-electrode liquid chromatography-electrochemistry. *Analyst* **1988**, *113*, 99−102.

(16) Santos-Buelga, C.; Francia, E. M.; Escribano-Bailon. Comparative flavan-3-ol composition of seeds from different grape varieties. *Food Chem.* **1995**, *53*, 197−201.

(17) Fernandez de Simon, B.; Hernandez, T.; Estrella, I.; Gomez-Cordovez, C. Variation in phenol content in grapes during ripening: low-molecular-weight phenols. *Z. Lebensm. Unters. Forsch. A* **1992**, *194*, 351−354.

(18) Revilla, E.; Bourzeix, M.; Alonso, E. Analysis of catechins and proanthocyanidins in grape seeds by HPLC with photodiode array detection. *Chromatographia* **1991**, *31* (9/10), 465−468.

(19) Treutter, D.; Feucht, W.; Santos-Buelga, C. Determination of catechins and procyanidins in plant extracts—A comparison of methods. *Acta Hortic.* **1994**, *381*, 789−796.

(20) Revilla, E.; Alonso, E.; Bourzeix, M.; Kovac, V. *Extractability of Catechins and Proanthocyanidins of Grape Seeds. Technological Consequences*; Food Science and Human Nutrition; Charalambous, G., Ed.; Elsevier Science Publishers: Amsterdam, The Netherlands, 1992.

(21) Simon, A.; Najid, A.; Chulia, A. J.; Delage, C.; Rigaud, M. Inhibition of lipoxygenase activity and HL60 leukemic cell proliferation by ursolic acid from heather flowers (*Calluna vulgaris*). *Biochim. Biophys. Acta* **1992**, *1125*, 68−72.

(22) Najid, A.; Simon, A.; Cook, J.; Chable-Rabinovitch, H.; Delage, C.; Chulia, A. J.; Rigaud, M. Characterization of ursolic acid as lipoxygenase and cycloxygenase inhibitor using macrophages, platelets and differentiated HL60 leukemic cells. *FEBS* **1992**, *3*, 213−217.

(23) Makino, K.; Hagiwara, T.; Murakami, A. A mini review: fundamental aspects of spin trapping with DMPO. *Radiat. Phys. Chem.* **1991**, *37*, 657−665.

(24) Rimbach, G.; Höhler, D.; Fischer, A.; Roy, S.; Virgili, F.; Pallauf, J.; Packer, L. Methods to assess free radicals and oxidative stress in biological systems. *Arch. Anim. Nutr.* **1999**, *52*, 203−222.

(25) Pekic, B.; Kovac, V.; Alonso, E.; Revilla, E. Study of the extraction of proanthocyanidins from grape seeds. *Food Chem.* **1998**, *61* (1/2), 201−206.

(26) Vercauteren, J.; Weber, J. F.; Bisson, J. L.; Bignon, J. Compositions de dérivés phénoliques, leur préparation et leurs applications comme antioxydants. Brevet FR 2 706 478-A1, 1993.

(27) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med.* **1996**, *20* (7), 933−956.

(28) Uchida, S.; Edamatsu, R.; Hirimatsu, M. Condensed tannins scavenge active oxygen free radicals. *Med. Sci. Res.* **1987**, *15*, 831−832.

(29) Maffei Facino, R.; Carini, M.; Aldini, G.; Bombardelli, E.; Morazzoni, P.; Morelli, R. Free radicals scavenging action and anti-enzyme activities of procyanidines from *Vitis vinifera*. A mechanism for their capillary protective action. *Arzneim.-Forsch.* **1994**, *44*, 592−601.

(30) Finnen, M. J.; Lawrence, C. M.; Schuster, S. Antioxidants and phytotherapy. *Lancet* **1994**, *344*, 1129−1130.

(31) Cotelle, N.; Bernier, J. L.; Catteau, J. P.; Pommery, J.; Wallet, J. C.; Gaydou, E. M. Antioxidant properties of hydroxy-flavones. *Free Radical Biol. Med.* **1996**, *20* (1), 35−43.

(32) Morel, I.; Lescoat, G.; Cogrel, P.; Sergent, O.; Pasdeloup, N.; Brissot, P.; Cillard, P.; Cillard, J. Antioxidant and iron chelating activities of the flavonoids catechin, quercetin and diosmetin on iron loaded rat hepatocyte culture. *Biochem. Pharmacol.* **1993**, *45*, 13−19.

(33) Morel, I.; Lescoat, G.; Cillard, P.; Cillard, J. Role of flavonoids and iron chelation in antioxidant action. In *Methods Enzymol.* **1994**, *234*, 437−443.

(34) Ruiz-Larrea, M. B.; Leal, A. M.; Martin, C.; Martinez, R.; Lacort, M. Effects of estrogens on the redox chemistry of iron: a possible mechanism of the antioxidant action of estrogens. *Steroids* **1995**, *60*, 780−783.

(35) Soares, J. R.; Dinis, T. C.; Cunha, A. P.; Almeida, L. M. Antioxidant activities of some extracts of *Thymus zygis*. *Free Radical Res.* **1997**, *26* (5), 469−478.

(36) Zhonghon, G.; Kaixun, H.; Xiangliang, Y.; Huibi, X. Free radical scavenging and antioxidant activities of flavonoids extracted from the radix of *Scutellaria baicalensis* Georgi. *Biochim. Biophys. Acta* **1999**, *1472*, 643−650.

(37) Lauthier, F.; Taillet, L.; Trouillas, P.; Delage, C.; Simon, A. Ursolic acid triggers calcium-dependent apoptosis in human Daudi cells. *Anti Cancer Drug* **2000**, *11*, 737−745.

(38) Satoh, K.; Kadofuku, T.; Sakagami, H. Effect of Trolox, a synthetic analog of α-tocopherol, on cytotoxicity induced by UV irradiation and antioxidants. *Anticancer Res.* **1997**, *17*, 2459−2464.

(39) Harper, A.; Kerr, D. J.; Gescher, A.; Chipman, J. K. Antioxidant effects of isoflavonoids and lignans, and protection against DNA oxidation. *Free Radical Res.* **1999**, *31*, 149−160.

Received for review January 19, 2001. Revised manuscript received April 19, 2001. Accepted April 19, 2001. C.-A.C. received a grant from the Conseil Régional du Limousin. The expenses of this work were defrayed in part by the Ministère de l'Education Nationale, de la Recherche et de la Technologie and by the Conseil Régional du Limousin (France).

JF010086V