Comparative Study of Polyphenol Scavenging Activities Assessed by Different Methods

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The effect of procyanidin solutions on superoxide anion radicals was studied with an enzymatic method and their EC_{50} values were determined. A comparative study of the results suggested that the free radical scavenger potential of these phenolic compounds closely depends on their chemical and stereochemical structures. Oligomeric procyanidins were isolated in different fractions from grapes and wines by low- and high-pressure liquid chromatography. These compounds were found to be efficient free radical scavengers even for the weak concentrations in wines. Their activity in grapes or wines was much stronger than that of other commercially available natural antioxidants (such as ascorbic acid and gallic acid). The effect of tannins isolated from grapes on different radicals was analyzed according to three distinct methods: an enzymatic method for superoxide anion radicals ($O_2^{\bullet -}$), a chemical method for the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH$^\bullet$), and an immunochemical method to study the scavenging activity of seed procyanidins on DNA lesions induced by the radical HO$^\bullet$.

Keywords: *Grape seeds; wine; procyanidin; radical scavenger; DNA lesions*

INTRODUCTION

Recently, radical scavengers have attracted special interest because they can protect the human body from free radicals that may cause many diseases including cancer and lead to the aging process (Sorata et al., 1984; Halliwell and Gutteridge, 1989; Nakayama et al., 1993). Moreover, it has been revealed that various phenolic antioxidants, such as flavonoids and tannins, scavenge active oxygen species and thus prevent cell damage. They are viewed as promissing therapeutic drugs for free radical pathologies (ischemia, anemia, etc.) (Cheng et al., 1986; Nakayama et al., 1992; Hertong et al., 1993). On the other hand, phenolic compounds are widely distributed in the plant kingdom and found in a number of fruits (grapes, persimmon, etc.) and beverages (teas, etc.) (Pratt, 1965; Zhao et al., 1989; Kanner et al., 1994). A balanced diet can thus constitute a natural source of antioxidants able to protect the human body from several diseases attributed to the reactions of radicals (Loliger, 1991). Various studies, however, have shown that the ability of phenolic compounds to scavenge free radicals is dose-dependent and can vary according to their structure, their substituents and their degree of polymerization (Uchida et al., 1987; Kitagawa et al., 1992).

In this paper, we have restricted ourselves to the study of radical scavenging activities of phenolic compounds from grapes and wine to quantify the antioxidant potential of these food products. The aim of this study is to determine the radical quenching activity of several procyanidins using different methods to ascertain whether the radical scavenger ability of these compounds depends on their chemical constitution, the nature of the radical ($O_2^{\bullet -}$, DPPH$^\bullet$, $^\bullet$OH, and $^\bullet$OOH), and their repartition (in grape seeds or wine).

Table 1. Concentrations of Pure Monomeric and Dimeric Procyanidins Causing a 50% Decrease of $O_2^{\bullet -}$ EC_{50} Values in an HPX/XOD System

compound	EC_{50}[a] (mg L^{-1})	EC_{50}[a] (μM L^{-1})	% purity[b]
(+)-catechin	73 ± 2 (6)	251 ± 7 (6)	99.9
(−)-epicatechin	61 ± 2 (6)	210 ± 7 (6)	99.9
dimer B_1	49 ± 2 (5)	85 ± 3 (5)	89.4
dimer A_2	400 ± 5 (4)	692 ± 8 (4)	91.2
dimer B_2	117 ± 4 (5)	202 ± 7 (5)	92.7
dimer B_3	50 ± 2 (5)	87 ± 3 (5)	93.8
dimer B_4	100 ± 3 (5)	173 ± 5 (5)	88.9

[a] Numbers represent means ± SD of results from four to six (numbers in parentheses) different preparations. [b] Percentage of purity obtained by ^{1}H NMR (Bruker DPX 400).

First, we tested the radical scavenging activities of pure dimer procyanidins to determine their concentration for 50% inhibition (EC_{50}) of superoxide anions ($O_2^{\bullet -}$) generated by the hypoxanthine (HPX)/xanthine oxidase (XOD) system (Hodgson and Fridovich, 1976).

Second, we extracted and isolated proanthocyanidins from grape seeds and wines according to their degree of polymerization (Saint-Cricq de Gaulejac et al., 1998a), and we examined the effects of these phenolic compounds on $O_2^{\bullet -}$. We then calculated their $O_2^{\bullet -}$ scavenging activities at their real concentration in grapes or wine.

Third, to evaluate their scavenging reactivities on different radicals, we analyzed the effect of tannins isolated from grapes on the stable 1,1-diphenyl-2-

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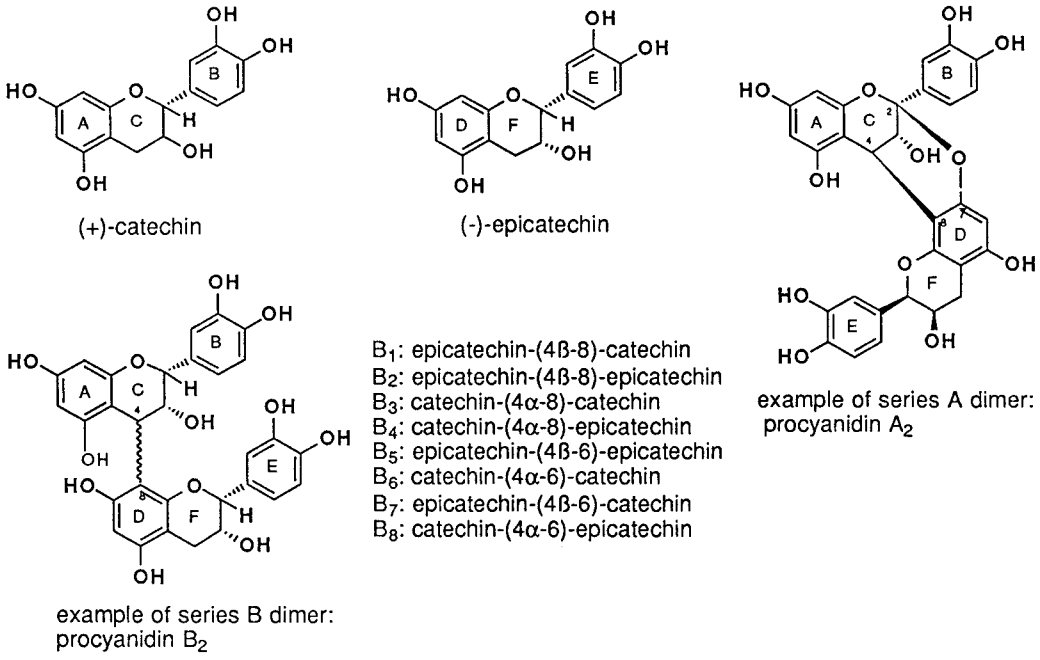

Figure 1. Chemical structures of the tested compounds.

picrylhydrazyl (DPPH•) radical (Brand-Williams et al., 1995) and on •OH radicals generated by Fenton's reaction.

Lastly, we analyzed these phenolic compounds by a damaged DNA detection assay (3D assay) using plasmid DNA adsorbed on sensitized microplates as the substrate for an in vitro repair reaction (Salles et al., 1995). This enabled us to detect the inhibition of tannins on DNA damage.

MATERIALS AND METHODS

Reagents and Equipment. *Phenolic Compounds.* Monomer procyanidins [(+)-catechin and (−)-epicatechin] were purchased from Extrasynthèse SA (Genay, France). Dimer procyanidins were obtained by hemisynthesis (procyanidins B_1-B_4) according to the conditions described by Feirriera et al. (1989). The pure procyanidin A_2 was extracted from horse chestnut shells (*Aesculus hyppocastanum*) in the conditions described by Vivas et al. (1996). These monomeric and dimeric procyanidins are shown in Figure 1, with the other dimers (procyanidins B_5-B_8), which are present in the extracts analyzed too. The structure and purity of hemisynthesized products were verified by ^{1}H NMR (Bruker DPX 400). The purity of hemisynthetic procyanidins are reported in Table 1.

Reagents. For the enzymatic test, HPX and XOD were purchased from Sigma Chemical Co. (St. Quentin Fallavier, France). For the chemical test, the methanol used was of spectrophotometric grade (990 g L^{-1}) from Merck (Chelles, France). 2,2-Diphenyl-1-picrylhydrazyl (DPPH•, 950 g kg^{-1}) was also from Sigma. For the bioassay, the 96 well microtitration plates were supplied by Dynatech and the chemical reagents by Sigma. Nucleotides and the DIG-11-dUTP came from Boehringer Mannheim. The Lumi-Phos 530 came from Lumigen.

LPLC Equipment. The samples were injected into a low-pressure column (1.6 × 35 cm) of gel TSK Toyopearl HW-40-(S). They were eluted with methanol with a flow rate at 0.8 mL min^{-1}. Detection was monitored using a UV detector (Gilson, model 111) at 280 nm. The low-pressure chromatograms (Figures 3 and 4) were recorded using a Linseis (model L203) instrument.

LSIMS Equipment. LSIMS spectra were recorded using a VG-Autospec EQ instrument, fitted with a cesium ion gun in negative mode: cesium ion beam energy, 35 keV (2 μA); temperature, 40 °C. Calibration was performed with cesium iodide salt (150−3500 Da). Thioglycerol was used as matrix. Data acquisition was performed using a Vax Station 3100 (Digital Equipment, OPUS system).

HPLC Equipment. The samples were injected into two Beckman ultrasphere ODS C_{18} (250 × 4.6 mm; 5 μm) columns in series at 20 °C (±1 °C), eluted with a flow rate at 1.0 mL min^{-1} with the composition of two solvents: solvent A was formic acid/water (2.5:97.5 v/v); solvent B was solvent A/acetonitrile (20:80 v/v). Detection was monitored at 280 nm.

Equipment for Enzymatic and Chemical Tests. All spectrophotometric data were acquired using a Uvikon 810 Kontron spectrophotometer. Disposable cuvettes (1 cm × 1 cm × 4.5 cm) from Muller Ratiolab (Dreieich, Germany) were used for visible absorbance measurements.

Bioassay Equipment. The luminescent signal was detected with a limax 2 luminometre market by the SFRI.

Preparation and Quantification of Seed and Wine Extracts. The seeds were taken from *Cabernet sauvignon* L. cv. grapes (1997). To prepare the low-pressure liquid chromatography (LPLC) sample, the seeds were recovered, freeze-dried, and reduced to powder. Four gram of powder was mixed with 10 mL of ethanol 95% vol and 10 mL of an aqueous solution containing 1 g/L of $NaHSO_3$ (antioxidizing agent) using an automatic mixer (Ultra-Turrax, 2 min). We added 20 mL of chloroform and continued mixing for 1 min. The resulting mixture was then centrifuged for 10 min (3000*g*). Two phases were separated out by an interface made up of the solid matter. The green lower phase (containing chloroform, lipids, pigments, etc) was eliminated. The yellow superior phase (hydroalcoholic solution) containing the phenolic constituents was recovered. This extraction was repeated six times on the powder remaining in the centrifuger tube. All of the hydroalcoholic extracts were collected and evaporated to remove ethanol (rotary evaporator, temperature = 30 °C). The resulting aqueous solution was then filtered and extracted with ethyl acetate (6 × 20 mL). The organic phases were collected, and the solvent was evaporated (rotary evaporator, temperature = 30 °C). The wine sample (20 mL of Cabernet sauvignon, 1997) was straight-extracted with ethyl acetate (6 × 20 mL). The organic phases were collected and treated as previously. The extracts obtained were dissolved in 5 mL of methanol before being injected into a low-pressure column.

The extracts obtained in the conditions previously described were fractionated by LPLC. For each fraction collected by LPLC, the solvent was evaporated. The samples were then

dissolved in the minimum quantity of anhydrous methanol required to solubilize the polyphenols and were analyzed by liquid secondary ion mass spetrometry (LSIMS). After identification, the fractions containing oligomer procyanidins were then collected. The solvent was completely evaporated (rotary evaporator, temperature = 30 °C). The extracts were dissolved in 0.5 mL of methanol to be quantified by high-performance liquid chromatography (HPLC) and to test their scavenging activities. For the quantification by HPLC, 20 μL of extract was injected into chromatographic columns. The anaytical method used was very similar to the procedure described by Ricardo da Silva et al. (1991). The gradient conditions were

temp (min)	0	5	90	95	100	105
% of A	93	93	80	0	0	93
% of B	7	7	20	100	100	7

Dimer procyanidins levels were quantified using standard curves developed from reference standards given by Freitas (University of Porto).

Effects of Phenolic Compounds on Superoxide Anion Radicals Generated by the HPX/XOD System (Hodgson and Fridovich, 1976). A tetrazolium blue solution (NBT, 10^{-3} M) was prepared in a sodium phosphate buffer T (0.05 M; pH 7.4). Hypoxanthine solution (HPX, 0.5×10^{-2} M) and xanthine oxidase solution (XOD, 1.67 units mL^{-1}) were solubilized by T. The phenolic compounds tested are monomeric and dimeric procyanidins (B_1-B_4 and A_2) and dimeric procyanidic fractions extracted from grape seeds and wine. These samples were solubilized in H_2O.

cuvette	T (μL)	NBT (μL)	HPX (μL)	XOD (μL)	samples (μL)
0	2400	100	500		
1	2300	100	500	100	
2	2200	100	500	100	100

The decrease in absorbance was determined at 560 nm every minute for a 10 min period.

The slope of cuvette 1 (P_1) represents the maximum effect of superoxide anions $O_2^{\bullet-}$ (≈100%)

Those of cuvette 2 (P_x) represent the $O_2^{\bullet-}$ scavenging effect of phenolic compounds.

The amount of residual $O_2^{\bullet-}$ can be calculated as

$$O_2^{\bullet-} \text{ remaining} = (P_x - P_0)/(P_1 - P_0) \times 100$$

Antiradical activity was defined as the amount of antioxidant required to lower the initial $O_2^{\bullet-}$ concentration by 50% (efficient concentration = EC_{50} (mol L^{-1} phenolic compounds).

Reaction of Phenolic Compounds on the Stable Radical DPPH• (Brand-Williams et al., 1995). Radical activities were determined using DPPH• as a free radical. For the dimeric procyanidic fraction extracted from grape seeds, different concentrations were tested. This phenolic compound solution (0.1 mL) was added to 3.9 mL of a 6×10^{-5} mol/L methanol DPPH• solution. The decrease in absorbance was determined at 515 nm at 0 min, 1 min, and every 15 min until the reaction reached a plateau. The exact initial DPPH• concentration ($C_{DPPH\bullet}$) in the reaction was calculated from a calibration curve with the equation

$$Abs_{515nm} = 12509 \times (C_{DPPH\bullet}) - 2.58 \times 10^{-3}$$

as determined by linear regression.

For each antioxidant concentration tested, the reaction kinetics were plotted. From these graphs, the percentage of DPPH• remaining at the steady state was determined, and the values were transferred onto another graph showing the percentage of residual steady state DPPH• as a function of the molar ratio of antioxidant to DPPH•. Antiradical activity was defined as the amount of antioxidant required to lower the initial DPPH• concentration by 50% [EC_{50} (mol L^{-1} phenolic compounds)].

Immunochemical System: 3D Test (Salles et al., 1995). *Target DNA Adsorption in the Microtitration Plate Wells.* Ultrapurified plasmid DNA (pBS) (mostly in supercoiled form) was applied to the microplate wells sensitized with polylysine gently stirred for 30 min at 30 °C, at 1 μg mL^{-1} concentration in a volume of 50 μL. In these conditions, DNA adsorption is quantitative (~40 ng per well). A positive repair control consisting of a plasmid damaged with UVC (called pBS^{UV}) was added. After incubation, the wells were washed twice with a PBS solution containing 0.1% Tween 20.

Generation of •OH Radicals Using Hydrogen Peroxide (H_2O_2). A stock solution of 30% H_2O_2 (w/w; 8.8 M) was diluted to a concentration of 5 mM in a 1 μM $FeCl_2$ solution. Fifty microliters of the solution was incubated for 30 min at 30 °C, gently stirred constantly, on the plasmid DNA in the presence or absence of the samples to be tested.

Dilution of Test Samples. Sample (grape seed extract containing dimeric procyanidins) was tested with the following dilutions: 1×10^{-2}, 1×10^{-3}, 1×10^{-4}, 1×10^{-5}, 1×10^{-6}, and 1×10^{-7}. All points were duplicated.

Contact with DNA. Sample dilutions were mixed with H_2O_2 and put into contact with the DNA adsorbed for 30 min at 30 °C to measure the protective capacity of the sample. They were then put into contact directly with the (undamaged) DNA adsorbed for 30 min at 30 °C, so as to measure possible damage to the DNA caused by the sample. Finally, they were put into contact directly with the (predamaged) DNA adsorbed for 30 min at 30 °C; in this way, a possible indirect effect of the sample on the test can be measured. For instance, inhibition of the control repair signal of the predamaged DNA can be caused by a direct interaction of the sample with the DNA, which prevents repair enzymes from reaching the damaged areas.

RESULTS AND DISCUSSION

Effect of Pure Procyanidin Solutions on Superoxide Anion Radicals Generated by the HPX/XOD System. To start, we investigated the $O_2^{\bullet-}$ scavenging effect of pure monomer procyanidins [(+)-catechin and (−)-epicatechin] and dimers (B_1, B_2, A_2, B_3, and B_4) shown in Figure 1. Superoxide anions $O_2^{\bullet-}$ can be generated by HPX/XOD. At pH 7.4, $O_2^{\bullet-}$ reduces the tetrazolium blue to formasan blue (max λ_{560}), but after addition of some radical scavengers (phenolic compounds), the formation of formasan blue is restricted and the absorption at 560 decreases. Therefore, absorption at 560 nm was proportional to the amount of residual $O_2^{\bullet-}$. The phenolic compounds mentioned above were tested with this method in a series of concentrations from 1.5 to 400 mg L^{-1}. As shown in Figure 2 for some procyanidins, these phenolic compounds scavenged $O_2^{\bullet-}$ dose-dependently. Some of them (B_2 and A_2, Figure 2B,C) became prooxidizing in a range of concentrations. The concentrations of phenolic compounds causing a 50% decrease of $O_2^{\bullet-}$ (EC_{50} values) were determined, as shown in Figure 2A for procyanidin B_3 and are listed in Table 1. Among the various procyanidins tested, dimer B_1 was the most potent scavenger of the $O_2^{\bullet-}$ radical. The concentration of this tannin required for 50% inhibition was 50 times more effective than that of vitamin E and up to 20 times more effective than that of SO_2, the antioxidizing agent usually used in wines (Vivas et al., 1997). The inhibitory action on $O_2^{\bullet-}$ radicals by the monomers, (+)-catechin and (−)-epicatechin, was much the same, although (−)-epicatechin seems to be a little more efficient. The different structural configuration of these two molecules may have led us to this conclusion. Indeed, it is interesting to note that (−)-epicatechins have a more significant area of charge delocalization (Freitas, 1996). A comparison of

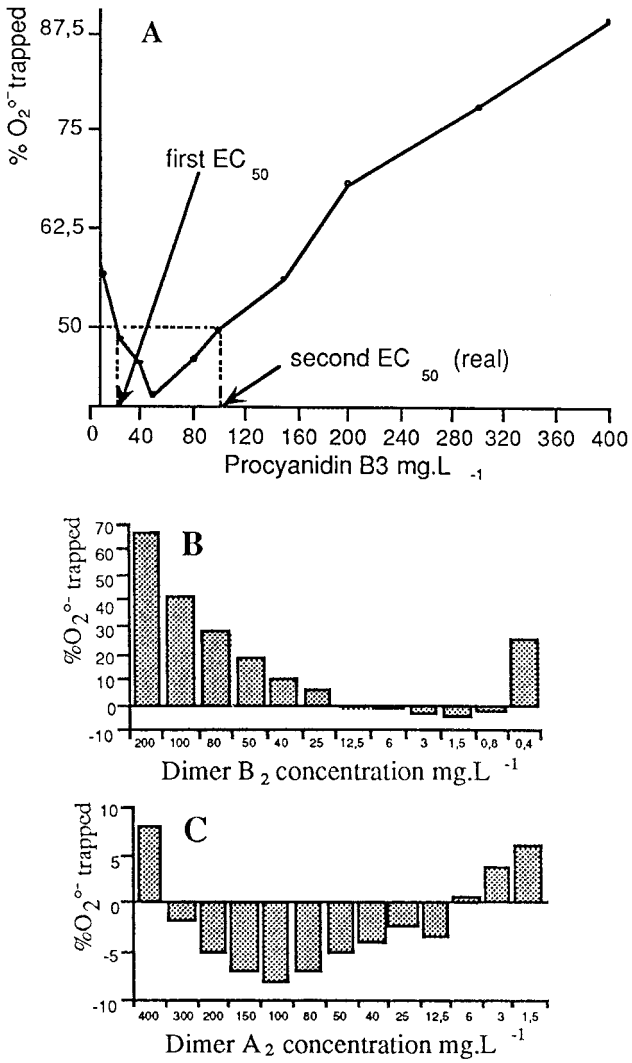

Figure 2. Example of dose-dependent $O_2^{\bullet-}$ scavenging effect of several procyanidins: (A) procyanidin B_3 (concentration causing a 50% decrease of $O_2^{\bullet-}$ (IC_{50} value) was defined as the concentration required to bring about a 50% decrease of the intensity of the signal of trapped $O_2^{\bullet-}$); (B and C) prooxidizing effect of procyanidins B_2 and A_2.

the monomers with dimers suggested that inhibition of the $O_2^{\bullet-}$ radical by these tannins tends to increase in the order of polymerization. Moreover, this hypothesis is verified by the works of Saint-Cricq de Gaulejac et al. (1998b) on a fractionation of seed procyanidins from $n = 1$ to $n = 10$. However, a comparison between B_2 (2 units of epicatechin with a linkage in the C_4-C_8 position) and A_2 (the same structure as B_2 with an additional C_2-O-C_7 ether linkage) showed that procyanidin B_2 had greater $O_2^{\bullet-}$ scavenging activities than A_2. The activity of A_2, which has a C_2-O-C_7 ether linkage instead of a phenolic OH group as in B_2, was much weaker; these results suggest that this phenolic OH group is an important one in scavenging $O_2^{\bullet-}$.

Extraction and Quantification of Dimer Procyanidins in Grape Seeds and Wine. Figures 3 and 4 represent, respectively, the chromatograms of seed and wine extracts obtained on a gel TSK Toyopearl HW-40-(S) column in the conditions previously described. The particular feature of polyphenols is to adsorb themselves on this gel by hydrophobic linkages. As a result, the elution of phenolic compounds is performed according

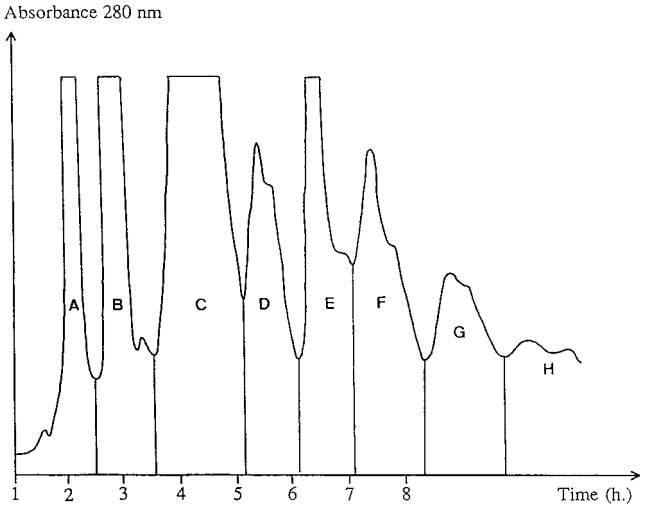

Figure 3. Low-pressure chromatogram of a Cabernet sauvignon (1997) grape seed extract [gel TSK toyopearl -40(S); column (1.6 × 35 cm); solvent, MeOH; flow, 0.8 mL min^{-1}) (for composition of fractions A−H, cf. Table 2)].

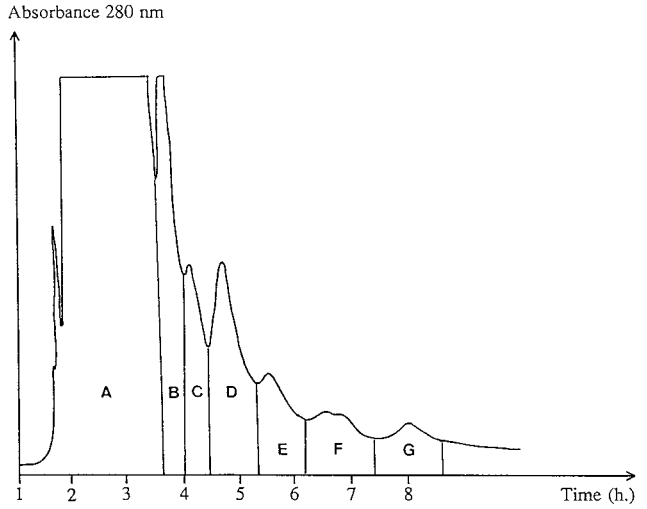

Figure 4. Low pressure chromatogram of a Cabernet sauvignon (1997) wine extract. (gel TSK toyopearl -40(S), column (1.6 × 35 cm), solvent: MeOH, flow: 0.8 mL.min^{-1}).

Table 2. Composition of Various Phenolic Fractions from Low-Pressure Chromatograms of Seed Extract (Cabernet Sauvignon, 1997) (Freitas, 1995)

seed fraction	nature of molecules
A	*p*-coumaric acid, gallic acid, and caffeic acid
B	(+)-catechin and (−)-epicatechin
C	procyanidins B_1, B_2, B_3, B_4, B_5, and B_7; monogalloyl (−)-epicatechin
D	procyanidins B_6, B_8 and trimer C_1
E	trimer procyanidins; monogalloyl dimers B_1, B_2, B_3 B_4, and B_5
F	trimer procyanidins; monogalloyl dimer B_6
G	trimer procyanidins; monogalloyl trimer procyanidins; tetramer procyanidins
H	digalloyl procyanidins B_1, B_2, B_3, and B_7; tetramer procyanidins

to their increasing molecular mass (Karchesy et al., 1989; Biau, 1996).

The seed sample (Figure 3) contained eight different fractions (A−H). Each fraction was recovered and directly analyzed by LSIMS (Table 2): monomer procyanidins [(+)-catechin and (−)-epicatechin] were iden-

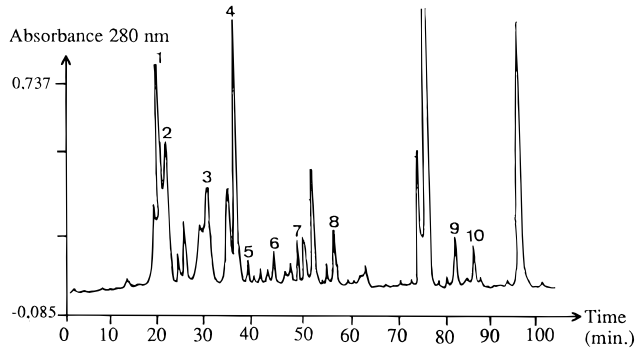

Figure 5. HPLC chromatogram of fraction C/D of a Cabernet sauvignon (1997) grape seed extract. Peaks: (1) dimer B_1; (2) dimer B_3; (3) dimer B_4; (4) dimer B_2; (5) dimer B_6; (6) dimer B_8; (7) trimer C_1; (8) dimer B_7; (9) dimer B_5; (10) dimer A_2.

tified in fraction B; dimer procyanidins in fractions C (B_1, B_2, B_3, B_4, B_5, and B_7) and D (B_6, B_8, and trimer C_1).

The wine phenolic content was divided into seven parts (Figure 4). The LSIMS analysis showed that fraction A contained phenolic acids, esters of phenolic acids, and monoglucoside anthocyanins; fraction B contained (+)-catechin and (−)-epicatechin; fractions C and D contained dimer procyanidins, trimer C_1, and monogalloyl epicatechin; fractions E−G contained trimer and tetramer procyanidins, monogalloyl procyanidins, etc.

Later, only fractions associated with (+)-catechin, (−)-epicatechin (fraction B) and dimers (B_1−B_8 and A_2) and trimer C_1 (fraction C/D) were collected and quantified by HPLC. Their quantitative determination was carried out with a co-injection of reference standards at different known concentrations. Repeat analyses of these reference solutions according to increasing concentrations demonstrated the reproducibility and linearity of these quantifications in a range going from 0 to 750 mg L^{-1}.

For example, Figure 5 represents the HPLC chromatogram of the seed extract. The quantitative results are reported in Table 3. In the case of seeds, the results in milligrams per gram of dry matter have been reduced to milligrams per liter of juice produced.

Effect of Procyanidins Isolated from Grape Seeds and Wine on Superoxide Anion Radicals Generated by an HPX/XOD System. A preparation of seed and wine tannins also scavenged the $O_2^{\bullet-}$ radical. Their concentration for 50% inhibition, and their real antioxidizing potentials (% $O_2^{\bullet-}$ trapped for the real concentration of tannins in grapes and wine, without dilution) are shown in Table 4.

The seed fractions (C/D) contain a high concentration of dimeric procyanidins (near 12 g L^{-1}, Table 3), which give them a strong antiradical potential. On the other hand, the wine dimeric fractions contain lower concentrations (close to 100 mg L^{-1}, Table 3), but they also act as significant scavengers toward $O_2^{\bullet-}$. As a rule, we noted that the antiradical action of procyanidins is strong for the strong concentrations (>1000 mg L^{-1}), then weakens when concentrations are lower, and may even become prooxidizing (in the case of A_2 and B_2), but increases again for the weaker concentrations (<200 mg L^{-1}). As shown in Figure 6, these results might explain the high antiradical potential of seeds (containing a high concentration of dimer procyanidins) and of wine (containing a low concentration of these molecules). It is interesting to note that not only the concentration effect but also the synergy effect between procyanidins may have suggested this possibility to us. Indeed, procyanidin distribution in seeds and wine ($B_2 > B_1 > B_4 > \ldots > B_5$) is such that the $O_2^{\bullet-}$ scavenging action of grape and wine is more efficient than that of only one procyanidin in the same concentration. In support of this theory is the finding that dimeric fractions of seed and wine extracts have a stronger $O_2^{\bullet-}$ scavenging activity (low EC_{50} values, cf. Table 4) than pure procyanidin solutions Table 1).

Effect of Procyanidins Isolated from Grape Seeds on Different Free Radicals: $O_2^{\bullet-}$ Generated by an Enzymatic Method (HPX/XOD), the Stable Free Radical DPPH$^\bullet$, and $^\bullet$OH Generated by Fenton's Rreaction. The effect of seed procyanidins (fraction C/D) on different free radicals was then examined. We already know that seed procyanidins dose-dependently scavenge $O_2^{\bullet-}$ generated by the HPX/XOD system (enzymatic method). In a second experiment, we have examined the ability of this procyanidic fraction to scavenge an other radical: the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH$^\bullet$), which is a stable radical (chemical method). In its radical form, DPPH$^\bullet$ absorbs at 515 nm but when reduced by an antioxidant (AH like phenolic compounds) or a radical species (R$^\bullet$), absorption ceases:

$$DPPH^\bullet + AH \rightarrow DPPH{-}H + A^\bullet$$

$$DPPH^\bullet + R^\bullet \rightarrow DPPH{-}R$$

Therefore, the absorption at 515 nm was proportional to the amount of residual DPPH$^\bullet$.

A comparison of $O_2^{\bullet-}$ radical inhibition [$EC_{50}(O_2^{\bullet-}) = 52\ \mu M\ L^{-1}$] by these tannins with DPPH$^\bullet$ inhibition [$EC_{50}(DPPH^\bullet) = 61\ \mu M\ L^{-1}$] suggested that seed procyanidins scavenge the DPPH radical. Indeed, the inhibitions of DPPH$^\bullet$ and $O_2^{\bullet-}$ by these tannins were much the same. Thus, the ability of these tannins to scavenge free radicals was proportional to their concentration but did not depend on the chemical nature of the free radicals.

Our results suggested that grape seed procyanidins may help to prevent and treat cell damage attributed to the reaction of active oxygen species. Flavonoids with an antioxidant activity have been widely studied, but direct $^\bullet$OH scavenging action by tannins on DNA have not, to our knowledge, been reported. We therefore decided to investigate the scavenging activity of seed procyanidins on DNA lesions induced by $^\bullet$OH generated by Fenton's reaction. To do this, we used a new assay called 3D (damaged DNA detection), a quick and easy method to assess DNA damage caused by free radicals.

The 3D can detect, along with the repair activity of DNA damage, all sorts of lesions and especially lesions induced by active oxygen species or free radicals.

The 3D assay procedure is summarized in Figure 7: (1) plasmid DNA adsorption in sensitized wells; (2) DNA damaging treatment; (3) incubation with protein extract that promoted repair synthesis; (4) recognition of incorporated digoxygenylated dUMP by anti-DIG antibody conjugated with alkaline phosphatase; (5) quantification of the light emitted due to dephosphorylation of the Lumi-Phos 530.

A dose−response curve of repair activity was obtained (Figure 8A) by incubation of DNA adsorbed in sensitized wells with H_2O_2 (5 mM) generating hydroxyl free radicals ($^\bullet$OH) in the presence of iron ($FeCl_2$, 1 μM)

Table 3. Concentration of Dimeric Procyanidins and Trimer C_1 in the Wine and the Seeds

	B_1	B_2	B_3	B_4	B_5	B_6	B_7	B_8	C_1	A_2	total
seeds, g L^{-1}	2.86	3.42	2.03	2.19	0.04	0.36	0.70	0.34	0.46	0.07	12.47
wine, mg L^{-1}	25.4	41.6	9.8	8.0	9.5	4.4	3.1	1.9	3.6	0.8	108.1

Table 4. Concentration Inducing a 50% Decrease of $O_2^{\bullet-}$ EC_{50} Values and Real Antioxidizing Potential (Percent $O_2^{\bullet-}$ Trapped for the Real Concentration of Tannins in Seeds and Wine)

	% $O_2^{\bullet-}$ trapped (real concn, without dilution)	EC_{50}[a] (mg L^{-1})	EC_{50}[a] (μM L^{-1})
seeds, fraction C/D	99 ± 3 (5)	30 ± 1	52 ± 2
wine, fraction C/D	96 ± 2 (5)	42 ± 2	73 ± 3

[a] Numbers represent means ± SD of results from (numbers in parentheses) different preparations.

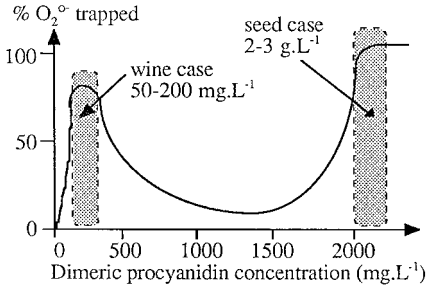

Figure 6. Schematic dose−response curve of $O_2^{\bullet-}$ scavenging effect of phenolic compounds in general.

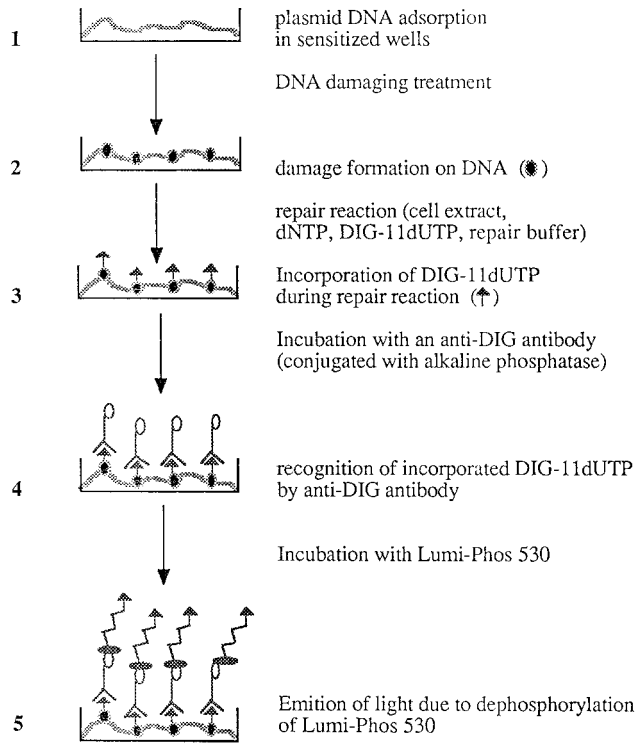

Figure 7. Schematic representation of the 3D assay.

according to Fenton's reaction ($H_2O_2 + Fe^{2+} \rightarrow {}^{\bullet}OH + {}^{-}OH + Fe^{3+}$). These radicals, in the presence of DNA induced some strand breaks, modifications, or loss of bases. The concomitant incubation of this system that generates an oxidizing agent ($^{\bullet}OH$) with a compound makes it possible to evaluate the antioxidizing potential of the latter. In this way, we tested the protective power of seed procyanidins on DNA (Figure 8B). Procyanidins extracted from grape seeds prove to be extremely

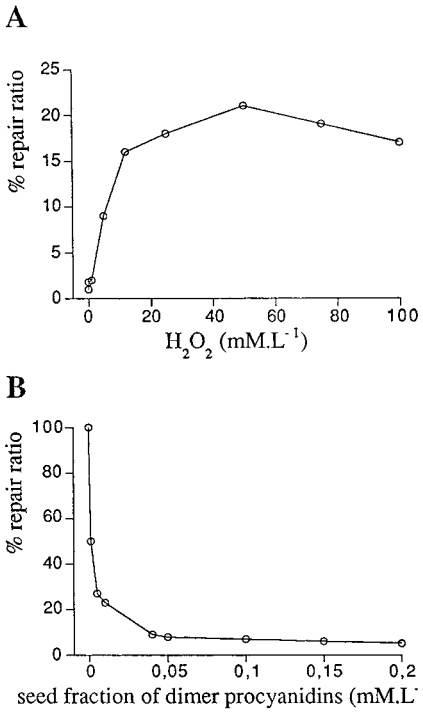

Figure 8. (A) Measurement of lesion intensity generated on DNA by hydroxyl radicals in the 3D assay. (B) Inhibitor effect of dimer procyanidin extracted from grape seeds on the production of oxydative lesions on DNA in the 3D assay.

effective in protecting DNA from radical attacks. The number of repaired lesions dropped significantly in the presence of these tannins, showing the inhibiting effect of dimer procyanidins on the lesion production of DNA.

CONCLUSION

There are a number of studies on the free radical scavenging activities of various antioxidants, including phenolic compounds. However, the physicochemical properties that determine the scavenging activity of these compounds are scarcely known. The results obtained here for $O_2^{\bullet-}$ scavenging effect of phenolic compounds indicate that the unit (−)-epicatechin is more efficient than the unit (+)-catechin and that inhibition of the $O_2^{\bullet-}$ radical tends to increase in the order of polymerization. Therefore, other physicochemical factors also govern the $O_2^{\bullet-}$ scavenging activity of phenolic compounds. For example, the antioxidizing potential of series A procyanidins is much weaker than that of series B procyanidins.

It is notewortly that not only the superoxide anion $O_2^{\bullet-}$ but also DPPH$^{\bullet}$ and $^{\bullet}OH$ are scavenged by tannins isolated from a natural source (grape seeds).

Moreover, our later results suggest that tannins can protect DNA from free radicals. They could act then as a deterrent against radical attacks on DNA. In this way, they may help to prevent and treat clinical disorders attributed to radicals.

Currently, we know that the regular consumption of grapes or wine provides phenolic compounds in sufficient quantity to protect DNA extracted from hepatic

cells against free radicals. However, what we do not know is whether these compounds are still active against free radicals after absorption and metabolization by hepatic cells. The binding of the compounds to certain enzymes, which would induce a complete or partial inhibition of their scavenging activities, may also have to be considered. To this end, we need to study the scavenging activity of phenolic compounds in vivo. At the present time, a study of the transfer process of polyphenols in hepatic cells is in progress.

ABREVIATIONS USED

$O_2^{\bullet -}$, superoxide anion radical; DPPH$^\bullet$, 1,1-diphenyl-2-picrylhydrazyl radical; $^\bullet$OH, hydroxyl radical; EC_{50}, efficient concentration for 50% inhibition; HPX, hypoxanthine; XOD, xanthine oxidase; 3D assay, damaged DNA detection assay; LPLC, low-pressure liquid chromatography; HPLC, high-performance liquid chromatography; LSIMS, liquid secondary ion mass spectrum; NBT, tetrazolium blue solution; T, sodium phosphate buffer; $C_{DPPH\bullet}$, initial DPPH$^\bullet$ concentration; Abs_{515nm}, absorbance at 515 nm; SEC, size exclusion chromatography.

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