JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

FEBRUARY 1998
VOLUME 46, NUMBER 2

Antioxidant Activity of Different Phenolic Fractions Separated from an Italian Red Wine

Andrea Ghiselli,*,† Mirella Nardini,† Alessandro Baldi,‡ and Cristina Scaccini†

Istituto Nazionale della Nutrizione, Via Ardeatina 543, 00178 Roma, Italy, and Dipartimento di Scienze Farmaceutiche, University of Florence, Via Gino Capponi 9, 50121 Firenze, Italy

Using liquid/liquid extraction, three fractions were obtained from an Italian red wine containing single polyphenolic subfractions: (1) phenolic acids and quercetin-3-glucuronide, (2) catechins and quercetin-3-glucoside, and (3) anthocyanins. Beside the scavenging capacity of the different fractions against hydroxyl and peroxyl radicals, the in vitro inhibition of low density lipoprotein oxidation and platelet aggregation (two main events in the pathogenesis of atherosclerosis) were tested. The antioxidant activity of the fractions has been compared with that of the original red wine before and after dealcoholization. The anthocyanin fraction was the most effective both in scavenging reactive oxygen species and in inhibiting lipoprotein oxidation and platelet aggregation. This higher activity can be explained by both its high concentration in red wine and its antioxidant efficiency, which, at least for peroxyl radical scavenging, was three times as high as that of the other two fractions. Our results suggest that anthocyanins could be the key component in red wine in light of the protection against cardiovascular diseases, although this hypothesis needs *in vivo* evidence.

Keywords: *Red wine; antioxidants; phenols; oxidation; low density lipoprotein; platelet aggregation*

INTRODUCTION

Several epidemiological studies have shown that increased dietary intake of natural phenolic antioxidants correlates with reduced coronary heart disease (Hertog et al., 1993; Stampfer et al., 1993). This helps to explain the "French paradox", i.e., the lower rate of cardiac disease mortality among populations regularly drinking red wine in spite of their high intake of saturated fatty acids (Renaud and de Lorgeril, 1992). In fact, red wines contain phenolic compounds, a large number of which may act as antioxidants, with mechanisms involving both free radical scavenging and metal chelation (Chimi et al., 1991; Iwahashi et al., 1990; Frankel, 1993; Nardini et al., 1995).

Consolidated scientific evidence supports the hypothesis of the involvement of oxidatively modified LDL in the pathogenesis of atherosclerosis (Steinberg et al., 1989; Esterbauer et al., 1992; Berliner and Heinecke, 1996; Parthasarathy and Rankin, 1992; Berliner et al., 1995). Nevertheless, the precise mechanism of the *in vivo* changes in structural and biochemical properties of LDL leading to the increased affinity for the scavenger receptor of macrophages is still not completely explained.

The susceptibility of LDL to oxidative modification is modulated by its fatty acid composition and by the plasma level of antioxidants (Esterbauer et al., 1992). A number of naturally occurring antioxidants have been found to strengthen the resistance of LDL to oxidative modification *in vitro* and *in vivo* (Nardini et al., 1995; Jialal and Scaccini, 1992; Jialal and Grundy, 1992; Frei, 1991; Jialal et al., 1991; Stocker et al., 1991; De Whalley et al., 1990; Nardini et al., 1997).

The positive effect of red wine on the modulation of human LDL resistance against oxidative modification

* Corresponding author (tel, +396 5032412; fax, +396 5031592; e-mail, ghiselli@inn.ingrm.it).
† Istituto Nazionale della Nutrizione.
‡ University of Florence.

S0021-8561(97)00486-X CCC: $15.00
Published on Web 01/23/1998

has been demonstrated *in vitro* (Frankel et al., 1993, 1995), while data *ex vivo* after wine supplementation are controversial (Fuhrman et al., 1995; de Rijke et al., 1995; Sharpe et al., 1995), probably due to different experimental conditions. Fuhrman et al. (1995) found that chronic red wine consumption (400 mL/day) reduced the susceptibility of LDL to undergo lipid peroxidation catalyzed by copper. On the contrary, the consumption of 200 mL/day red wine (Sharpe et al., 1995) as well as that of 550 mL/day partially dealcoholized (3.5%) red wine (de Rijke et al., 1995) did not give the same results. The discrepancies could be accounted for by a decreased intestinal absorption of polyphenols in the absence of alcohol (Ruf et al., 1995) or by the different quantities of wine consumed. Moreover, also the concentration of the metal catalyst is different in the three studies ranging from 10 μM (Fuhrman et al., 1995) to 38 μM (de Rijke et al., 1995).

Besides lipoprotein oxidative modification, other events are involved in the pathogenesis and progression of cardiovascular disease, such as an increased free radical production and a platelet hyperfunction (Berliner et al., 1995).

In this study, we investigated the *in vitro* effect of an Italian red wine (Chianti, produced using Sangiovese R10 clone) on the modulation of LDL resistance to oxidative modification and platelet function. To discriminate the action of the various phenols in wine, different classes of polyphenols were extracted from the same wine. After qualitative and quantitative analysis, the antioxidant capacity of the different fractions was tested. The scavenging of hydroxyl ($OH^{\bullet}$) and peroxyl radicals ($ROO^{\bullet}$) by the red wine and of its phenolic fractions was also studied.

In each phase of the study, the quantitative relation among the different classes of polyphenols was maintained constant (1:500 with respect to the original wine) to discriminate the relative contribution of each fraction to the total antioxidant activity of the original wine.

METHODS

Isolation and Characterization of Polyphenolic Fractions from Red Wine. Wine was derived from grapes grown in an experimental field in the Chianti Classico area (Rocca di Castagnoli, Siena, Italy) and belonging to the same cultivar and the same clone (Sangiovese R10). The wine, produced under controlled fermentation conditions, was stored in a stainless steel container for 6 months. The wine was then stored in bottle for 12 months.

Liquid/liquid extraction methods were performed to obtain several fractions containing different classes of polyphenolic compounds (Figure 1). Alcohol removal was obtained by under-vacuum treatment (at 25 °C and 30 mbar). Special attention was paid to control the evaporation process, monitoring the pH and the volume of the solution to avoid complexation and precipitation processes. The dealcoholized wine (100 mL) was first extracted with ethyl acetate (three times with 100 mL of EtOAc each), obtaining an aqueous residue (containing all the anthocyanic compounds) and an organic phase (containing flavonols, phenolic acids, and catechins). Two further EtOAc (three times with 100 mL of EtOAc each) extractions were performed on the organic phase evaporated and redissolved in water at pH 7.0. The first EtOAc extract contained the subclass of the catechins and several flavonols (mainly quercetin-3-*O*-glucoside). The aqueous residue from this extraction was adjusted at pH 2.0 and extracted again with EtOAc (three times with 100 mL of EtOAc each) to obtain a fraction containing phenolic acids and quercetin-3-*O*-glucuronide.

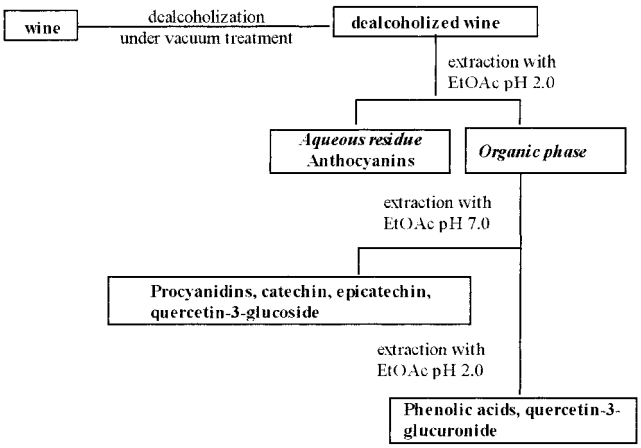

Figure 1. Scheme of the phases of the polyphenolic fractions extraction.

The recovery of polyphenolic compounds in the aqueous residue and EtOAc extracts has been evaluated using a "like-wine" solution at pH 3.3 in which we put several standards at the closer expected concentrations in wine. The mix was composed by malvidin-3-glucoside, cyanidin-3-glucoside, catechin, epicatechin, almost all the phenolic acids, quercetin, and quercetin-3-glucoside. By application of the extraction method, the recovery was close to the 100% for each standard.

To separate and identify each compound, HPLC analysis with diode array detector (DAD) was performed on each fraction, applying chromatographic conditions previously described (Baldi et al., 1993). In brief, the HPLC/DAD analyses were performed by means of a liquid chromatograph HP 1090A equipped with a diode array detector HP 1040 A, managed by a HP 9000 workstation (Hewlett-Packard Palo Alto, CA).

Anthocyanins were separated on an Aquapore RP300 (Brownlee Lab, Santa Clara, CA) column 7 μm 250 × 4.6 mm, using the following mobile phases: Solution A, H_2O/HCOOH (93:7); solution B, H_2O/CH_3CN/MeOH/HCOOH (47:23:23:7) in a three-step linear gradient starting from 0 up to 50% of solution B within 60 min. The flow rate was 1.5 mL/min. Chromatograms were acquired at 535-330-310-280 nm.

Phenolic acids, flavonols, and catechins were separated using a LiChrosorb RP18 (Merck), 5 μm 125 × 4.6 mm, using the following mobile phases: solution A, CH_3COOH 5% in water; solution B, CH_3CN in a two-step linear gradient starting from 100% solution A up to 80% solution B in 50 min. The flow rate was 1.2 mL/min. Chromatograms were acquired at 360-330-310-280 nm.

The unknown compounds were identified by HPLC coupled with mass spectrometry (HPLC/MS) (Baldi et al., 1995), using a Perkin Elmer LC-250 with a UV−VIS detector PE LC-95 equipped with a PE API/Sciex ion spray interface and a triple-quadrupole mass spectrometer Sciex Taga 6000E (Perkin-Elmer & Co GmbH, Uberlingen, Germany). Chromatograms were recorded at 280 nm. A valve was used to split the flow in the two different systems: API interface/mass spectrometer and UV−VIS detector. A 1:25 ratio was chosen to have a 60 μL/min flow rate in the interface. The splitter was connected with the ion spray interface via a fused-silica capillary (length 30 cm, 100 μm i.d.). Ion spray voltage was 3 kV, and gas pressure in the sprayer was 80 psi. Due to the chemical characteristics of the anthocyanins (analyzed in the flavilium form), the mass spectrometer was operated in the positive ion mode. Negative ion mode was chosen for catechins, phenolic acids, and flavonols. Spectra were acquired between 200 and 700 m/z, using a 0.1 kDa resolution. The flow rate was 1.5 mL/min. Chromatograms were acquired at 535, 330, 310, and 280 nm.

After the characterization, a quantitative analysis on the identified compounds was performed by HPLC (Baldi et al., 1993). The wine fractions were further analyzed spectrophotometrically to measure the total amount of polyphenols

Table 1. List of Quantified Compounds in Wine (mg/L)[a]

free anthocyanins	191 mg	phenolic acids	494 mg
delphinidin-3-*O*-glucoside	22 mg	caffeic acid	17 mg
cyanidin-3-*O*-glucoside	38 mg	*p*-coumaric acid	22 mg
petunidin-3-*O*-glucoside	18 mg	ferulic acid	19 mg
peonidin-3-*O*-glucoside	32 mg	vanillic acid	8 mg
malvidin-3-*O*-glucoside	69 mg	*p*-hydroxybenzoic acid	20 mg
malvidin-3-(6-*O*-*p*-coumaroyl)glucoside	12 mg	gallic acid	320 mg
flavonols	65.3 mg	protocatechuic acid	88 mg
quercetin-3-glucoside	21 mg	catechins and procyanidins	440 mg
quercetin-3-glucuronide	19 mg	(+)-catechin	145 mg
quercetin	5 mg	(−)-epicatechin	128 mg
myricetin-3-glucoside	2.3 mg	procyanidin B3	66 mg
kaempferol-3-glucoside	12 mg	procyanidin B1	29 mg
kaempferol	6 mg	procyanidin B6	12 mg
hydroxycinnamoyltartaric acids	334 mg	procyanidin B2	30 mg
caffeoyltartaric acid	178 mg	procyanidin B7	18 mg
p-coumaroyltartaric acid	139 mg	procyanidin C1	12 mg
feruloyltartaric acid	27 mg		

[a] Free anthocyanins are expressed as malvidin-3-glucoside for the malvidin and petunidin groups; delphinidin-3-glucoside for the delphinidin and cyanidin group; peonidin for the peonodin group. Phenolic acids are expressed as gallic acid. Hydroxycinnamoyltartaric acids are expressed as caffeic acid. Procyanidins and catechins are expressed as (+)-catechin. Data are based on HPLC analysis.

(Singleton and Esau, 1969). Data were expressed as (+)-catechin, gallic acid, or malvidin-3-glucoside equivalents.

Free Radical Scavenging. *Hydroxyl Radical.* The scavenging of the hydroxyl radical ($OH^{\bullet}$) was measured by the deoxyribose method (Halliwell et al., 1987). One hundred microliters of diluted sample (1:10, pH 7.4, see Table 2 for the total phenolic concentration in the undiluted samples) was added to 690 μL of 10 mM phosphate buffer (PBS) at pH 7.4 containing 2.5 mM 2-deoxyribose. One hundred microliters of 1.0 mM iron ammonium sulfate premixed with 1.04 mM EDTA was added. Samples were kept in a water bath at 37 °C, the reaction was started by adding 100 μL of 1.0 mM ascorbic acid and 10 μL of 0.1 M H_2O_2. Samples were maintained at 37 °C for 10 min, and then 1.0 mL of cold 2.8% trichloroacetic acid was added followed by 0.5 mL of 1% thiobarbituric acid. Samples were boiled for 8 min and cooled, and the absorbance was measured at 532 nm (Winterbourn and Sutton, 1986).

Peroxyl Radical. The influence of the wine fractions on the peroxyl radical trapping was measured as previously described (Ghiselli et al., 1995), using an azo initiator to generate a constant flow of peroxyl radicals. In brief, a 15 nM *R*-phycoerythrin (R-PE) solution in 75 mM phosphate buffer at pH 7.0 was incubated at 37 °C with 4 mM 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) in the presence or absence of the wine fractions (80 μL of a 1:200 dilution, pH 7.0, see Table 2 for the total phenolic concentration in the undiluted samples). The delay of loss of R-PE fluorescence (Ts) induced by the samples was continuously monitored in a Perkin-Elmer LS-5 luminescence spectrofluorometer. The delay induced by a known amount of trolox (Tt), the water-soluble analogue of vitamin E, was used to quantify the results according to the equation

$$\text{sample antioxidant activity} = Ts \times [\text{trolox}]/Tt$$

Data are expressed as trolox equivalents (mmol of trolox having a scavenging activity corresponding to 1 L of wine or to the single phenolic fractions as contained in 1 L of wine).

Inhibition of Oxidative Modification of Human LDL. *LDL Preparation and Oxidation.* Human LDL (d 1.019−1.063 g/mL) was isolated from fasting plasma collected in EDTA (1 mg/mL) by sequential ultracentrifugation in salt solutions, according to Havel et al. (1955), using a Beckman T-100 benchtop ultracentrifuge (T-100.3 rotor). LDL solution was flushed with N_2, stored at 4 °C, and used within 1 week from the preparation. Protein was measured by the method of Lowry et al. (1951), using bovine serum albumin as standard.

For oxidation experiments, LDL was dialyzed in the dark for 24 h at 4 °C against three changes of 1 L each of 0.01 M phosphate-buffered saline (PBS) 0.15 M NaCl, pH 7.4.

Table 2. Polyphenolic Compounds in Red Wine and in Red Wine Fractions

wine/wine fractions	mg/L	wt %[a]	mM
wine	3250[b]	-	11.2[b]
dealcoholized wine	2940[b]	100	10.1[b]
ethyl acetate extract pH 7.0 (procyanidins, catechin, epicatechin, quercetin-3-glucoside)	510[b]	17.5	1.7[b]
ethyl acetate extract pH 2.0 (phenolic acids, quercetin-3-glucuronide)	390[c]	13.3	2.3[c]
anthocyanins (aqueous residue)	1980[d]	67.4	4.0[d]

[a] wt % respect to dealcoholized wine. [b] (+)-Catechin equivalents. [c] Gallic acid equivalents. [d] Malvidin-3-glucoside equivalents.

Dialyzed LDL (200 μg protein/mL) was oxidized in PBS at 37 °C for 4 h in the presence of 5 μM $CuCl_2$ or 4 mM AAPH. The oxidation of LDL was performed in the presence and in the absence of the wine fractions. The dilution factor was 1:500, with respect to the whole wine (see Table 2 for the total phenolic concentration in the undiluted samples).

Oxidation was stopped by refrigeration and the addition of 3 mM EDTA and 100 μM BHT. The oxidative modifications of LDL were evaluated through the measure of conjugated dienes formation, lipid hydroperoxide production, tryptophan residues, electrophoretic mobility, and α-tocopherol.

The kinetics of conjugated diene formation (CD) was followed by continuously monitoring the absorbance at 234 nm, using a Beckman DU 70 spectrophotometer thermostated at 37 °C. Lipid hydroperoxides were measured iodometrically at different time points, according to El Saadani et al. (1989). Tryptophan residues were determined by the measure of intrinsic fluorescence in cetyltrimethyl ammonium bromide (Reyftmann et al., 1990). LDL electrophoresis was performed at pH 8.6 in 0.05 M barbital buffer on 0.5% agarose gels, using a Beckman Paragon apparatus. The gels were stained with Sudan B black. The increased electrophoretic mobility of LDL was expressed relative to the mobility (REM) of native LDL. α-Tocopherol was measured, after extraction, by reversed-phase HPLC with fluorescence detection (Bieri et al., 1979).

Platelet Function. Blood samples were obtained from normal human volunteers, after an overnight fasting, in a vacutainer containing 129 mM sodium citrate as the anticoagulant. Platelets were separated by centrifugation at 150*g* for 10 min. Platelets were washed three times in PBS, pH 7.4, containing 5 mM EDTA and resuspended in EDTA-free PBS. Ten microliters of each sample was then added to 490 μL of PBS containing 3×10^8 platelets and 1.5 mg/mL of fibrinogen. Platelet aggregation was induced by 2 μM adenosine diphosphate, and samples were incubated for 5 min at 37 °C. The reaction was stopped by adding 500 μL of cold

Table 3. Free Radical Scavenging Activity of Different Polyphenolic Fractions from Red Wine

wine/wine fractions	ROO• trapped[a] mM trolox equiv	OH• [b] % inhibition
wine	7.8 (0.71)	48.6
wine (dealcoholized)	3.4 (0.34)	9.2 (100)
procyanidins, catechin, etc.	0.2 (0.12)	2.7 (29)
phenolic acids, etc.	0.5 (0.22)	1.4 (15)
anthocyanins	2.4 (0.60)	3.9 (42)

[a] Millimoles of trolox having the equivalent antioxidant capacity to 1 L of wine (see Table 2 for the total phenolic concentration in the undiluted samples) or to the single phenolic fractions as contained in 1 L of wine. Values in parentheses are the ratio between the antioxidant capacity of the fraction (mM trolox equiv) and their millimolar concentration (see Table 2) and represent the molar efficiency of the compounds in the fraction. [b] % inhibition of OH• (dilution factor 1:500, see Table 2 for the total phenolic concentration in the undiluted samples). Values in parentheses are the percent contribution of the individual fraction to the scavenging activity of dealcoholized wine (taken as 100%).

trichloroacetic acid (20% w/v). The extent of platelet aggregation was measured as malondialdehyde (MDA) production (Violi et al., 1988). In fact, the formation of MDA, a metabolite of prostaglandin endoperoxides, is a marker of platelet aggregation (Smith et al., 1976). The results are expressed as percent inhibition of platelet aggregation. The dilution factor was 1:500, with respect to the wine before alcohol removal and fractions separation (see Table 2 for the total phenolic concentration in the undiluted samples).

RESULTS AND DISCUSSION

The application of the liquid/liquid extraction method (Figure 1) allowed us to separate wine phenolic fractions containing compounds with similar characteristics. HPLC/DAD and HPLC/MS analyses were applied to identify the compounds shown in Table 1. Besides gallic acid, caffeoyltartaric acid, *p*-coumaroyltartaric acid, epicatechin, and catechin were the major components of wine. Phenolic acids and quercetin-3-glucuronide were the main component of the EtOAc extract at pH 2.0. Some procyanidins, catechin, epicatechin, and quercetin-3-glucoside were found in the EtOAc extract at pH 7.0. Kaempferol-3-glucoside, myricetin-3-glucoside, and traces of aglicons of quercetin and kaempferol were also found. Several anthocyanins were identified in the aqueous residue after the EtOAc extraction, malvidin-3-glucoside being the most represented.

The concentration of phenolic compounds in red wine, dealcoholized red wine, and in the above described wine fractions based on spectrophotometric data are reported in Table 2. Results are expressed as equivalents of the most representative polyphenol in the fraction. The data relative to whole and dealcoholized wine were consistent with literature (Bakker et al., 1986), and the anthocyanin fraction accounted for ~70% (w/w) of the total phenols in red wine.

In Table 3, the scavenging activity of wine and wine fractions toward peroxyl and hydroxyl radicals is reported. As expected, wine and dealcoholized wine were the most efficient against both radicals; however, the dealcoholization caused a 50% loss of the peroxyl scavenging activity and a strong decrease (about 80%) of the hydroxyl radical scavenging. While the latter result was expected, as ethanol is reported to be a powerful hydroxyl radical scavenger (Gutteridge et al., 1984), the same is not true for ROO•. Moreover, the addition of ethanol (up to 0.5% v/v of the final volume) in the reaction mixture does not interfere with the

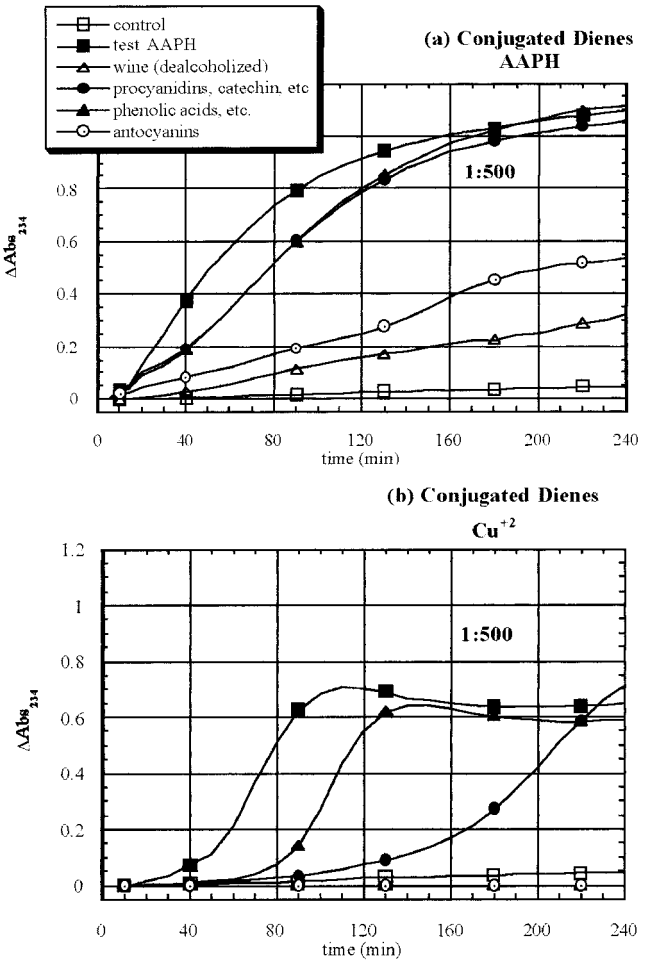

Figure 2. Effect of dealcoholized wine and of the different polyphenolic fractions on AAPH-induced (a) and Cu^{2+}-mediated (b) oxidative modification of LDL, as measured by kinetics of conjugated dienes formation. LDL (50 μg/mL) was incubated at 37 °C for 6 h with 5 μM Cu^{2+} or with 4 mM AAPH in the presence of the samples (1:500 dilution, respect to the original wine). In these experimental conditions, the phenolic concentration in the samples was wine dealcoholized = 20 μM catechin equivalents; procyanidins, catechin, etc. = 3.4 μM catechin equivalents; phenolic acids, etc. = 4.6 μM gallic acid equivalents; anthocyanins = 8.0 μM malvidin-3-glucoside equivalents. Absorbance was continuously monitored at 234 nm.

measure of peroxyl radical scavenging activity in our test conditions (data not shown). Therefore, we can reasonably exclude any antioxidant action of alcohol in our assay, since the final concentration of ethanol from wine in the reaction mixture was 0.025% v/v. The loss of activity against ROO• after dealcoholization could be linked to some polymerization processes during the evaporation due to change of polyphenols solubility and pH of the solution.

Among the wine fractions (Table 3), anthocyanins were effective against both radicals. The procyanidins−catechin fraction exerted a certain inhibition only with regard to OH• production (29%), while the phenolic acid fraction exhibited a slight inhibition in both cases.

The anthocyanins contained in 1 L of wine had a ROO• trapping capacity corresponding to 2.4 mM trolox equivalents (31% and 70% of the capacity of wine before and after dealcoholization), while the inhibition of OH• represented 42% of that obtained with the dealcoholized wine.

Table 4. Inhibitory Effect of Phenolic Fractions from Red Wine on Oxidative Modification of Human LDL by 4 mM AAPH and 5 μM Cu^{2+} [a]

	lipid hydroperoxide (nmol/mg of protein) 4 h		tryptophan residues (%) 4 h		REM[b] 4 h		vitamin E residue (%) 2 h	
	AAPH	Cu^{2+}	AAPH	Cu^{2+}	AAPH	Cu^{2+}	AAPH	Cu^{2+}
control LDL	2	1	100	100	1	1	100	100
oxidized LDL	442	752	46	44	2.4	2.9	3	6
+ wine (dealcoholized)	279	28	84	100	1.3	1.4	64	96
+ procyanidins, catechin, etc.	426	801	46	41	2.3	2.9	2	2
+ phenolic acids, etc.	397	830	45	44	2.4	3.2	3	1
+ anthocyanins	279	5	68	80	1.6	1.6	40	77

[a] LDL (200 μg of protein/mL) was incubated at 37 °C for 4 h with 5 μM Cu^{2+} or 4 mM AAPH in the presence and absence of phenolic fractions from red wine (dilution factor 1:500). In these experimental conditions, the phenolic concentration in the samples was as follows: wine dealcoholized = 20 μM catechin equivalents; procyanidins, catechin, etc. = 3.4 μM catechin equivalents; phenolic acids, etc. = 4.6 μM gallic acid equivalents; anthocyanins = 8.0 μM malvidin-3-glucoside equivalents. [b] Electrophoretic mobility, relative to native LDL.

The high efficiency in scavenging peroxyl radicals exhibited by the anthocyanins is not only due to their relative abundance in red wine. In fact, when the scavenging of peroxyl radical was expressed as molar efficiency (mM trolox equivalents/mM phenolic concentration) anthocyanins had an antioxidant efficiency (0.60) higher than those of the dealcoholized wine (0.34), phenolic acid (0.22), and catechin (0.12) fractions.

The modulation by dealcoholized red wine and its phenolic fractions of LDL resistance to oxidative modification was tested using two oxidative systems: the thermal decomposition of the azo compound AAPH and the classical copper-catalyzed oxidation (Figure 2 and Table 4).

The principal difference between the two systems is that, while AAPH produces peroxyl radicals at constant rate through its spontaneous thermal decomposition, Cu^{2+}-catalyzed LDL oxidation depends on the reduction of the metal ion probably through the reaction with endogenous lipid hydroperoxides, with lipid hydroperoxyl radicals production (Esterbauer et al., 1992; Gebicki et al., 1991; Patel et al., 1997; Thomas and Jackson, 1991). The reduced copper (Cu^{+}) in its turn decomposes preexisting peroxides, producing alkoxyl radicals.

Therefore, the inhibition of the oxidation by AAPH represents the simple scavenging of peroxyl radicals. The inhibition of the Cu^{2+}-catalyzed oxidation represents the association of both chelation of metal ions and scavenging of different free radicals.

Dealcoholized wine and anthocyanins consistently delayed the conjugated dienes formation (Figure 2). In copper-catalyzed oxidation, procyanidins and catechins also retarded CD formation, but this inhibitory effect was no more detectable in the other parameters tested after 2−4 h incubation (Table 4). Only dealcoholized wine and anthocyanins were able to inhibit the oxidative damage to LDL, using both the oxidizing systems. Noteworthy was the sparing effect on alpha tocopherol. Moreover, the inhibitory effect of dealcoholized wine and anthocyanins was stronger when Cu^{2+} was used than with AAPH.

These results, in partial disagreement with other studies in which the inhibitory effect of single phenolic antioxidants has been studied, can be explained by our choice to maintain the concentration of the different classes of wine phenols proportional to their concentration in the original wine. For example, (i) the final concentration of the hydroxycinnamic acid derivatives (caffeic and ferulic acid) in the assay conditions used in this study was less than 0.3 μM, while in a previous study (Nardini et al., 1995) we found that they were able to exert an effective inhibitory action at concentration above 1 μM; (ii) catechin, epicatechin, and procyanidins were ≈3.5 μM (61% as catechin and epicatechin), while catechin is reported to inhibit copper-catalyzed LDL oxidation at a concentration above 15 μM (Mangiapane et al., 1992).

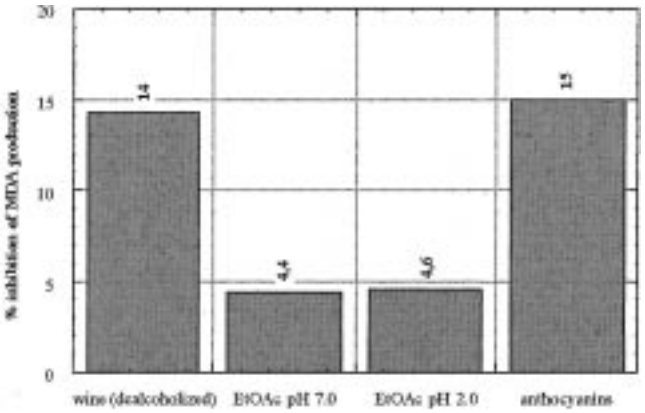

Figure 3. Percent inhibition of platelet aggregation by dealcoholized wine and by the different polyphenolic fractions. Platelets (3×10^8 cells) were incubated at 37 °C with 2 μM adenosine diphosphate and 1.5 mg/mL fibrinogen for 10 min, in the presence and absence of wine and wine fractions (dilution factor 1:500). In these experimental conditions, the phenolic concentration in the samples was wine dealcoholized = 20 μM catechin equivalents; procyanidins, catechin, etc. = 3.4 μM catechin equivalents; phenolic acids, etc. = 4.6 μM gallic acid equivalents; anthocyanins = 8.0 μM malvidin-3-glucoside equivalents. MDA production was used as an indicator of platelet aggregation.

The inhibition of platelet function provided similar indications (Figure 3). In fact, both wine (dealcoholized) and anthocyanins suppressed platelet aggregation more efficiently than the two ethyl acetate extracts (around 14% and 4%, respectively). The effect of wine phenols on platelet function can be related both to the scavenging of reactive oxygen species generated in the first phases of platelet aggregation and necessary to complete the aggregation process (Leo et al., 1997) and to the inhibition of cyclooxygenase, the key enzyme for thromboxane and malondialdehyde production (Smith et al., 1976).

CONCLUSIONS

The antioxidant capacity of wine, dealcoholized wine, and relative polyphenolic fractions extracted from wine was measured in different systems simulating different events involved in the pathogenesis of atherosclerosis. In spite of the strong loss in antioxidant capacity after dealcoholization, wine and its fractions still maintained

an appreciable free radical scavenging activity, inhibiting LDL oxidative modification and platelet aggregation.

The protective effect of wine is mainly due to the anthocyanic fraction (quantitatively the more abundant phenolic subclass in red wine); however, our results do not exclude the possibility of a synergistic action among the different classes of polyphenols.

A number of epidemiological evidences focus on the non-alcohol component of wine as "protective factor" in regard to human health (Klatsky and Armstrong, 1993; Grønbæk et al., 1995). Our results suggest that anthocyanins, characteristic of red wine, could be an important contributor to protective action. However, no data are still available on their bioavailability and metabolic fate, and further research is necessary to state their action in vivo.

ABBREVIATIONS USED

AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; BHT, buthylated hydroxytoluene; CD, conjugated dienes; EDTA, ethylenediaminetetraacetic acid; EtOAc, ethyl acetate; HPLC/DAD, high-performance liquid chromatography/diode array detector; HPLC/MS, high-performance liquid chromatography/mass spectrometer; LDL, low density lipoprotein; MDA, malondialdehyde; $OH^{\bullet}$, hydroxyl radical; PBS, phosphate-buffered saline; R-PE, R-phycoerythrin; REM, relative electrophoretic mobility; $ROO^{\bullet}$, peroxyl radicals.

LITERATURE CITED

Bakker, J.; Preston, N. W.; Timberlake, C. F. The determination of anthocyanins in aging red wines: comparison of HPLC and spectral methods *Am. J. Enol. Vitic.* **1986**, *37*, 121−126.

Baldi, A.; Romani, A.; Mulinacci, N.; Vincieri, F. F. Composes phenoliques dans les cepages de Toscane de *Vitis vinifera* L. *J. Int. Vigne Vin.* **1993**, *27*, (3), 201−215.

Baldi, A.; Romani A.; Mulinacci N.; Vincieri F. F.; Casetta B. HPLC/MS application to anthocyanins of *Vitis vinifera* L. *J. Agric. Food Chem.* **1995**, *48*, 2104−2109.

Berliner, J. A.; Heinecke, J. W. The role of oxidized lipoprotein in atherosclerosis. *Free Radical Biol. Med.* **1996**, *20*, 707−727.

Berliner, J. A.; Navab, M.; Fogelman, A. M.; Frank, J. S.; Demer, L. L.; Edwards, P. A.; Watson, A. D.; Lusis, A. J. Atherosclerosis: Basic mechanisms: oxidation, inflammation and genetics. *Circulation* **1995**, *91*, 2488−2496.

Bieri, J. G.; Talliver, G. T.; Catignani, L. G. Simultaneous determination of alpha-tocopherol and retinol in plasma or red cells by high pressure liquid chromatography. *Am. J. Clin. Nutr.* **1979**, *32*, 2143−2149.

Chimi, H.; Cillard, J.; Cillard, P.; Rahmani, M. Peroxyl and Hydroxyl radical scavenging activity of some natural phenolic antioxidant. *J. Am. Oil Chem. Soc.* **1991**, *68*, 307−312.

de Rijke, Y. B.; Demacker, P. N. M.; Assen, N. A.; Sloots, L. M.; Katan, M. B.; Stalenhoef, A. F. H. Red wine consumption does not affect oxidizability of low-density lipoproteins in volunteers. *Am. J. Clin. Nutr.* **1995**, *63*, 329−334.

De Whalley, C. V.; Rankin, S.; Hoult, J. R. A.; Jessup, W.; Leake, D. S. Flavonoids inhibit the oxidative modification of LDL by macrophages. *Biochem. Pharmacol.* **1990**, *39*, 1743−1750.

El-Saadani, M.; Esterbauer, H.; El-Sayed, M.; Goher, M.; Nasser, A. Y.; Jurgens, G. A spectrophotometric assay for lipid peroxides in serum lipoproteins using a commercially available reagent. *J. Lipid Res.* **1989**, *30*, 627−630.

Esterbauer, H.; Gebiki, J.; Puhl, H.; Jurgens, G. The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free Radical Biol. Med.* **1992**, *13*, 341−390.

Frankel, E. N.; Kanner, J.; German, J. B.; Parks, E.; Kinsella, J. E. Inhibition of human LDL oxidation by phenolic substances in red wine. *Lancet* **1993**, *341*, 454−457.

Frankel, E. N.; Waterhouse, A. L.; Teissedre, P. L. Principal phenolic phytochemicals in selected California wines and their antioxidant activity in inhibiting oxidation of human low density lipoproteins. *J. Agric. Food Chem.* **1995**, *43*, 890−94.

Frei, B. Ascorbic acid protects lipid in human plasma and LDL against oxidative damage. *Am. J. Clin. Nutr.* **1991**, *54*, 1113S−1118S.

Fuhrman, B.; Lavy, A.; Aviram, M. Consumption of red wine with meals reduces the susceptibility of human plasma and low density lipoprotein to lipid peroxidation. *Am. J. Clin. Nutr.* **1995**, *61*, 549−554.

Gebicki, J. M.; Jurgens, G.; Esterbauer, H. Oxidation of low-density lipoprotein in vitro. In *Oxidative Stress: Oxidants and Antioxidants*; Sies, H., Ed.; Academic Press: London and San Diego, 1991; pp 371−397.

Ghiselli, A.; Serafini, M.; Maiani, G.; Azzini, E.; Ferro-Luzzi, A. A fluorescence-based method for measuring total plasma antioxidant capability. *Free Radical Biol. Med.* **1995**, *18*, 29−36.

Grønbæk, M.; Deis, A.; Sørensen, T. I. A.; Becker, U.; Schnohr, P.; Jensen, G. Mortality associated with moderate intake of wine, beer or spirits. *Br. Med. J.* **1995**, *310*, 1165−1169.

Gutteridge, J. M. C. Reactivity of hydroxyl and hydroxyl-like radicals discriminated by release of thiobarbituric acid-reactive material from deoxy sugars, nucleosides and benzoate. *Biochem. J.* **1984**, *224*, 761−767.

Halliwell, B.; Gutteridge, J. M. C.; Aruoma, O. I. The deoxyribose method: A simple "test-tube" assay for determination of rate constants for reactions of hydroxyl radicals. *Anal. Biochem.* **1987**, *165*, 215−219.

Havel, R. J.; Eder, H. A.; Bragdon, J. H. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **1955**, *34*, 1345−1353.

Hertog, M. G. L.; Feskens, E. J. M.; Hollman, P. C. H.; Katan, M. B.; Kornhout, D. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *Lancet* **1993**, *342*, 1007−1011.

Iwahashi, H.; Ishii, T.; Sugata, R.; Kido, R. The effects of caffeic acid and its related catechols on hydroxyl radical formation by 3-hydroxyanthranilic acid, ferric chloride, hydrogen peroxide. *Arch. Biochem. Biophys.* **1990**, *276*, 242−247.

Jialal, I.; Grundy, S. M. The effect of dietary supplementation with alpha-tocopherol in the oxidative modification of LDL. *J. Lipid Res.* **1992**, *33*, 899−906.

Jialal, I.; Scaccini, C. Antioxidants and atherosclerosis. *Curr. Opin. Lipidol.* **1992**, *3*, 324−328.

Jialal, I.; Norkus, E. P.; Cristol, L.; Grundy, S. M. Beta carotene inhibits the oxidative modification of LDL. *Biochim. Biophys. Acta* **1991**, *1086*, 134−138.

Klatsky, A. L.; Armstrong, M. A. Alcoholic beverage choice and risk of coronary artery disease mortality: do red wine drinkers fare best? *Am. J. Cardiol.* **1993**, *71*, 467−479.

Leo, R.; Praticò, D.; Iuliano, L.; Pulcinelli, F. M.; Ghiselli, A.; Pignatelli ,P.; Colavita, A. R.; FitzGerald, G. A.; Violi, F. Platelet activation by superoxide anion and hydroxyl radicals intrinsically generated by platelets that had undergone anoxia and then reoxygenated. *Circulation* **1997**, *95*, 885−891.

Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurements with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.

Mangiapane, H.; Thompson, J.; Salter, A.; Brown, S.; Duncan Bell, G.; White, D. A. The inhibition of the oxidation of low density lipoprotein by (+)-catechin, a naturally occurring flavonoid. *Biochem. Pharmacol.* **1992**, *43*, 445−450.

Nardini, M.; D'Aquino, M.; Tomassi, G.; Gentili, V.; DiFelice, M.; Scaccini, C. Inhibition of human low density lipoprotein oxidation by caffeic acid and other hydroxycinnamic acid derivatives. *Free Radical Biol. Med.* **1995**, *19*, 541−552.

Nardini, M.; Natella, F.; Gentili, V.; Di Felice, M.; Scaccini, C. Effect of Caffeic acid dietary supplementation on the antioxidant defense system in rat: an in vivo study. *Arch. Biochem. Biophys.* **1997**, *342*, 157−160.

Parthasarathy, S.; Rankin, S. M. Role of oxidized low density lipoprotein in atherogenesis. *Prog. Lipid. Res.* **1992**, *127*, 143−147.

Patel, R. P.; Svistunenko, D.; Wilson, M. T.; Darley-Usmar, V. M. Reduction of Cu(II) by lipid hydroperoxides: implications for the copper-dependent oxidation of low-density lipoprotein. *Biochem. J.* **1997**, *322*, 425−433.

Renaud, S.; de Lorgeril, M. Wine, alcohol, platelet and the French Pararox for coronary heart disese. *Lancet* **1992**, *339*, 1523−1526.

Reyftmann, J. P.; Santus, R.; Maziere, J. C.; Morliere, P.; Salmon, S.; Candide, C.; Maziere, C.; Haigle, J. Sensitivity of tryptophan and related compounds to oxidation induced by lipid autoperoxidation. Application to human serum low- and high-density lipoproteins. *Biochim. Biophys. Acta* **1990**, *1042*, 159−167.

Ruf, J. C.; Berger, J. L.; Renaud, S. Platelet rebound effect of alcohol withdrawn and wine drinking in rats. Relation to tannins and lipid peroxidation. *Arterioscler. Thromb. Vasc. Biol.* **1995**, *15*, 140−144.

Sharpe, P. C.; McGrath, L.; McClean, E.; Young, I. S.; Archbold, G. P. Effect of red wine consumption on lipoprotein (a) and other risk factors for atherosclerosis. *Q. J. Med.* **1995**, *82*, 101−108.

Singleton, V. L.; Esau, P. *Phenolic substances in grapes and wines and their significance*; Academic Press: New York, 1969.

Smith, J. B.; Ingerman, C. M.; Silver, M. J. Malondialdehyde formation as an indicator of prostaglandin production by human platelets. *J. Lab. Clin. Med.* **1976**, *88*, 167−172.

Stampfer, M. J.; Hennekens, C. H.; Manson, J. E.; Colditz, G. A.; Rosner, B.; Willet, W. C. Vitamin E consumptionand the risk of coronary disease in women. *N. Engl. J. Med.* **1993**, *328*, 1444−1449.

Steinberg, D.; Parthasarathy, S.; Carew, T. E.; Khoo, J. C.; Witztum, J. L. Beyond cholesterol. Modifications of low density lipoprotein that increases its atherogenicity. *N. Engl. J. Med.* **1989**, *320*, 915−924.

Stocker, R.; Bowry, V. W.; Frei, B. Ubiquinol-10 protects human LDL more efficiently against lipid peroxidation than does alpha tocopherol. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*,1646−1650.

Thomas, C. E.; Jackson, R. L. Lipid hydroperoxide involvement in copper-dependent and independent oxidation of low density lipoprotein. *J. Pharmacol. Exp. Ther.* **1991**, *256*, 1182−1188.

Violi, F.; Ghiselli, A.; Iuliano, L.; Alessandri, C.; Cordova, C.; Balsano, F. Influence of hydroxyl radical scavengers on platelet function. *Haemostasis* **1988**, *18*, 91−98.

Winterbourn, C. C.; Sutton, H. C. Iron and xanthine oxidase catalyze formation of an oxidant species distinguishable from OH: comparison with the Haber-Weiss reaction. *Arch. Biochem. Biophys.* **1986**, *244*, 27−34.

Received for review June 11, 1997. Revised manuscript received October 21, 1997. Accepted October 30, 1997.[⊗]

JF970486B

[⊗] Abstract published in *Advance ACS Abstracts,* December 15, 1997.