Principal Phenolic Phytochemicals in Selected California Wines and Their Antioxidant Activity in Inhibiting Oxidation of Human Low-Density Lipoproteins

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The phenolic antioxidant phytochemicals in wines have been implicated for the lower rates of cardiac disease mortality among people drinking wine regularly in certain European populations. The activities of 20 selected California wines in inhibiting the copper-catalyzed oxidation of human low-density lipoproteins (LDL) were determined. This antioxidant activity was related to the major phenolic compounds and not to resveratrol analyzed in wines by HPLC and GC–MS. The relative inhibition of LDL oxidation varied from 46 to 100% with the red wines and from 3 to 6% with the white wines. When compared at the same total phenol concentration, the inhibition of LDL oxidation varied from 37 to 65% with the red wines and from 27 to 46% with the white wines. The relative antioxidant activity correlated with total phenol contents of wines ($r = 0.94$) and with the concentrations of gallic acid ($r = 0.92$), catechin ($r = 0.76$), myricetin ($r = 0.70$), quercetin ($r = 0.68$), caffeic acid ($r = 0.63$), rutin ($r = 0.50$), epicatechin ($r = 0.45$), cyanidin ($r = 0.43$), and malvidin 3-glucoside ($r = 0.38$). Therefore, the activity of wines to protect LDL from oxidation appeared to be distributed widely among the principal phenolic compounds.

Keywords: *Antioxidants; phenolic compounds; wine; LDL oxidation; phytochemicals; lipoproteins; atherosclerosis; cholesterol*

INTRODUCTION

Atherosclerosis and coronary heart disease are associated with elevated levels of cholesterol in low-density lipoproteins (LDL) circulating in blood. There is accumulating evidence that oxidation of LDL may play a significant role in atherosclerosis (Steinberg et al., 1989; Esterbauer et al., 1992). This oxidation has been shown to lead to modification of the lysine residues of apolipoprotein B (Steinbrecher, 1987). Other studies have demonstrated that antioxidants can reduce the yield of oxidized LDL particles formed in vitro (Esterbauer et al., 1991) and can inhibit experimental atherosclerosis in cholesterol-treated rabbits (Björkhem, 1991; Sparrow, 1992). Several epidemiologic studies have shown that increased consumption of phenolic antioxidants, including vitamin E (Stampfer et al., 1993) and quercetin in food products (Hertog et al., 1993), correlates with reduced coronary heart disease (CHD).

Renaud and Lorgeril (1992) demonstrated that in contrast to most countries where a high saturated fat intake was positively correlated to CHD mortality, in certain parts of France CHD mortality was low despite a relatively high fat intake. Consumption of wine was one dietary factor that could partly explain this low mortality from CHD. This epidemiological evidence thus suggested that the intake of wine may counteract the effect of a high fat diet in reducing the incidence of CHD and was referred to as the "French Paradox". The phenolic components in red wine were shown to exert potent antioxidant activity in inhibiting the oxidation of human LDL in vitro (Frankel et al., 1993a; Kanner et al., 1994). The antioxidant properties of phenolic compounds in red wine in retarding atherogenesis were proposed as an explanation for the French Paradox. In addition, phenolic compounds in plant foods may also be effective in reducing thrombosis, a fatal event in a large proportion of deaths from CHD (Kinsella et al., 1993). The hypothesis that dietary phenolic compounds could protect LDL from oxidation is supported by a previous study showing that when catechin, the principal monomeric phenolic compound found in wine, is administered orally in human subjects, over half was absorbed and excreted in the urine (Hackett et al., 1983). This study showed also that unchanged catechin was present in the blood.

In the present study we evaluated the antioxidant activities of 20 commercial wines in inhibiting human LDL oxidation in vitro. This antioxidant activity was evaluated in terms of the principal phenolic compounds and resveratrol analyzed by HPLC and GC–MS.

MATERIALS AND METHODS

Wines. The different types of red and white wines obtained from various commercial wineries in California are listed in Table 1.

Inhibition of LDL Oxidation. LDL was prepared from the plasma of three normolipidemic volunteers by sequential ultracentrifugation (Orr et al., 1991) and exhaustively dialyzed. The inhibition of Cu^{2+}-catalyzed oxidation of LDL was determined by static headspace gas chromatography (GC) of hexanal produced by oxidation of the $n-6$ polyunsaturated fatty acids in LDL (Frankel et al., 1992). For each evaluation wines were diluted with distilled water and standardized to 10 μM total phenol expressed as gallic acid equivalents (GAE). Results of duplicate analyses were expressed as percent inhibition = $[(C - S)/C] \times 100$, where C = hexanal formed in control and S = hexanal formed in sample. Relative percent

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Table 1. California Wines Evaluated for Antioxidant Properties

wine[a]	type	abbrev	year
1R	Cabernet Sauvignon	CS	1989
2R	Merlot	Mer	1990
3R	Cabernet Sauvignon	CS	1987
4R	Zinfandel	Zin	1990
5R	Cabernet Sauvignon	CS	1987
6R	Petite Sirah	PS	1987
7R	Petite Sirah	PS	1989
8R	Cabernet Sauvignon	CS	1987
9R	Pinot Noir	PN	1992
10R	Cabernet Sauvignon	CS	1987
11R	Petite Sirah	PS	1990
12R	Merlot	Mer	1991
13R	Cabernet Sauvignon	CS	1991
14R	Cabernet Sauvignon	CS	1989
15W	Sauvignon Blanc	SB	1990
16W	Sauvignon Blanc	SB	1991
17W	Chardonnay	CH	1990
18W	Chardonnay	CH	1991
19W	White Zinfandel	WZ	1992
20W	White Zinfandel	WZ	1991

[a] Wine numbers; R, red; W, white.

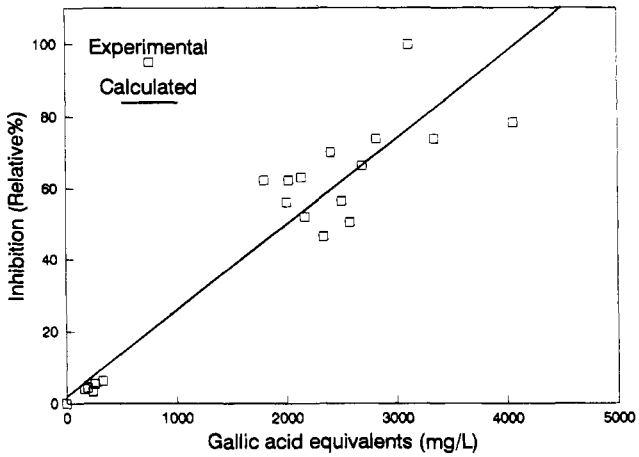

Figure 1. Relative percent inhibition of LDL oxidation by wines vs total phenol content as gallic acid equivalents. (Relative percent inhibition was calculated by multiplying the values of percent inhibition at 10 μM total phenol by the dilution factor used in the headspace analyses and by taking the highest value as 100%.) Correlation coefficient, $r = 0.94$. Regression equation, calculated $Y = 0.024X + 1.904$.

inhibition was calculated by multiplying the values of percent inhibition at 10 μM total phenol by the dilution factor used in the headspace analyses and by taking the highest value as 100% (Figure 1).

Analyses of Phenolic Compounds. Total phenols were analyzed according to the Folin–Ciocalteu method (Singleton and Rossi, 1965), calibrating against gallic acid standards and expressing the results as GAE. Individual phenolic components of wine (gallic acid, catechin, caffeic acid, epicatechin, cyanidin, malvidin 3-glucoside, myricetin, and quercetin) were analyzed according to a previously reported HPLC method (Lamuela-Raventos and Waterhouse, 1993, 1994), modified by using a buffer at pH of 1.5 during the entire separation.

Resveratrol. The resveratrol analysis was carried out according to an unpublished procedure that relies on a solid-supported liquid–liquid extraction. The wine is diluted with water to reduce the concentration of ethanol in the aqueous phase, which is suspended on diatomaceous earth. The immobilized aqueous droplets are extracted by the organic phase when the first portion of ethyl acetate is added to the cartridge between ethyl acetate additions. The second portion of ethyl acetate displaces the first, which is eluted from the cartidge with its extracted resveratrol. After the extraction, the ethyl acetate is very wet, and if not dried, water droplets appear when the solvent is evaporated. The evaporation step provides approximately a 10-fold concentration effect from the wine. A calibration curve was made by spiking several wine samples and was linear from 10 to 5000 ppb.

Wine samples were removed from corked bottles by syringe, injecting approximately 10 mL of nitrogen gas into the bottle and removing the same quantity of wine, which was placed in a small Erlenmeyer flask; 4 mL of this wine was placed in a test tube and diluted with 4 mL of water. 2-Naphthol internal standard (100 μL of an ethyl acetate solution, to achieve 10 ppb) was then added. For spiking purposes, 50–100 μL samples of a resveratrol standard solution in ethyl acetate were added when desired. This solution was then added to 10 mL diatomaceous earth cartridges (EM Merck Extrelut). After exactly 5 min, 12 mL of ethyl acetate was added to the cartridges. After another 5 min, another 12 mL of ethyl acetate was added and solvent then eluted from the cartridge, approximately 12 mL over 2 min. This solution was dried with sodium sulfate, and 5 mL was then transferred by syringe to a 20 mL scintillation vial for evaporation in a centrifugal vacuum evaporator (Savant). After 40 min, the vial was removed with approximately 200 μL of solvent remaining. The solution was transferred to a 300 μL vial for GC analysis, 50 μL of bis(trimethylsilyl)trifluoroacetamide (BSTFA) added, and the vial capped and heated at 70 °C for 15 min. The samples were then analyzed by GC–MS on an HP 5890/5970 gas chromatograph using a 30 m, 0.25 mm, 0.25 μm capillary column DB-5 (J&W Scientific, Folsom, CA), monitoring at mass 216 for 2-naphthol and at mass 444 for *cis*- and *trans*-resveratrol. The column temperature was initially 100 °C for 1 min, then heated to 290 °C at 10 °C/min, and maintained at that temperature for 15 min. The results are expressed as total *cis*- and *trans*-resveratrol, assuming that the *cis* isomer has the same mass spectrometer response as the *trans* isomer. By this method the recovery of resveratrol was 96%, and the coefficient of variation was 4%.

RESULTS

The amounts of phenolic materials vary considerably in different types of wine, depending on the grape variety, environmental factors in the vineyard, and wine processing techniques. The concentrations of total phenol as determined by the Folin–Ciocalteu method varied from 1800 to 4059 mg/L GAE, averaging 2567 mg/L GAE, for the red wines and from 165 to 331 mg/L, averaging 239 mg/L GAE, for the white wines (Table 2). These levels reflect the effects of different fermentation methods used for the two wine types.

The levels of total phenol in wines determined according to the Folin–Ciocalteu method are not absolute measurements of the amounts of phenolic materials but are in fact based on their chemical reducing capacity relative to an equivalent reducing capacity of gallic acid. Various phenolic compounds have different responses in this assay (Singleton and Rossi, 1965). The molar response of this method is roughly proportional to the number of phenolic hydroxyl groups in a given substrate, but the reducing capacity is enhanced when two phenolic hydroxy groups are oriented ortho or para. Since these structural features of phenolic compounds are reportedly also responsible for antioxidant activity in fat systems (Mehta and Seshadri, 1959; Letan, 1966), measurements of total phenol in wines may be related to their antioxidant activities.

Because there was such a large variation in the amount of phenolic material, the LDL oxidation assay for antioxidant activity was carried out at the same concentration of total phenol equivalent to 10 μM GAE, as determined by the Folin–Ciocalteu assay. At the same total phenol concentration, the inhibition of LDL oxidation varied from 37 to 65% with the red wines and from 27 to 46% with the white wines (Table 2). This

Table 2. Inhibition of LDL Oxidation and Phenolic Composition of Tested Wines[a]

wine	type	GAE, mg/L	% In at 10 μM GAE[b]	dilution factor	mg/L									RV, μg/L
					Gal	Cat	Caf	Epicat	Cyan	Malv	Rutin	Myr	Quer	
1R	CS	3340	42	39.3	126	141	5.7	25	3.4	9.7	6.8	6.5	3.2	2249
2R	Mer	1800	65	29.4	91	180	6.0	90	7.2	19.9	5.0	11.6	2.1	1651
3R	CS	2500	43	29.3	103	182	7.0	106	0.3	0.9	9.8	17.9	7.8	1927
4R	Zin	2000	53	23.5	90	245	5.7	85	0.0	10.6	3.2	6.7	3.5	608
5R	CS	2400	55	28.2	65	229	7.1	73	0.6	0.0	26.1	13.4	8.4	x
6R	PS	4059	37	47.7	107	390	8.2	60	6.7	5	31.7	9.6	13.2	338
7R	PS	3114	61	36.6	106	160	8.4	39	2.6	37.4	2.4	17.8	7.6	2243
8R	CS	2572	37	30.2	85	159	6.9	37	0.0	6.1	9.0	8.2	17.1	1156
9R	Pn	2816	50	33.1	110	120	4.7	50	0.0	33.6	4.4	4.4	12.9	977
10R	CS	2688	47	31.6	103	152	4.9	74	0.0	1.2	2.7	5.8	10.0	x
11R	PS	2020	58	23.8	86	145	4.8	100	3.3	68.5	8.3	5.9	3.2	993
12R	Mer	2133	56	25.1	81	256	8.1	154	5.6	32.9	2.9	0.0	6.8	2969
13R	CS	2164	45	25.4	71	176	9.6	162	9.5	90.2	2.4	0.0	8.4	1749
14R	CS	2334	38	27.4	108	144	12.8	95	0.0	12.6	12.9	11.2	3.9	1460
15W	SB	165	46	1.9	6	32	1.3	9	0.0	0.0	0.0	0.0	0.0	x
16W	SB	193	43	2.3	11	16	2.1	6	0.0	0.0	0.0	0.0	0.0	x
17W	Ch	240	25	2.8	9	34	3.5	27	0.0	0.0	0.0	0.0	0.0	16
18W	Ch	259	39	3.1	6	46	3.5	60	0.0	0.0	0.0	0.0	0.0	0
19W	WZ	243	27	2.9	6	43	3.6	17	0.0	3.5	0.0	0.0	0.0	64
20W	WZ	331	35	3.9	4	38	2.6	9	0.0	2.7	0.0	0.0	0.0	28
av		1869	45		69	144	5.8	64	2.0	16.7	6.4	5.9	5.4	1152
av red		2567	49		95	191	7.1	82	2.8	23.5	9.1	8.5	7.7	1527
av white		239	36		7	35	2.8	21	0.0	1.0	0.0	0.0	0.0	27

[a] GAE, gallic acid equivalent; Gal, gallic acid; Cat, catechin; Caf, caffeic acid; Epi, epicatechin; Cyan, cyanidin; Malv, malvidin 3-glucoside; Myr, myricetin; Quer, quercetin; RV, resveratrol; x, not analyzed. See Table 1 for abbreviations. [b] Inhibition of LDL oxidation at 10 μM GAE (See Materials and Methods).

difference implies that the predominant phenolic compounds in red wines are comparatively more potent antioxidants than those in white wines when evaluated by the LDL assay at the same GAE equivalent concentration. However, when the antioxidant activities of the wines were compared on the basis of their total phenol contents, the relative inhibition of LDL oxidation varied from 46 to 100% with the red wines and from 3 to 6% with white wines (Figure 1). A correlation coefficient r of 0.94 ($p < 0.001$) was calculated between relative percent inhibition of LDL oxidation and gallic acid equivalents.

The HPLC analyses of the major monomeric phenolic constituents show that the concentration of catechin ranged from 120 to 390 mg/L, averaging 191 mg/L, in the red wines and from 16 to 46 mg/L, averaging 35 mg/L, in the white wines (Table 2). Epicatechin levels in most red wines were lower than the catechin levels. The average level of epicatechin in red wines was about 40% that of catechin. In the red Cabernet Sauvignon wine 13R, however, the catechin and epicatechin levels were nearly equal. In white wines the average level of epicatechin was 60% that of catechin. In one white Chardonnay wine, 18W, the concentration of epicatechin was higher than that of catechin.

Gallic acid was the next most abundant monomeric phenolic determined by our HPLC assay, and the levels of this component ranged from 65 to 126 mg/L, averaging 95 mg/L, in red wines and from 4 to 11 mg/L, averaging 7 mg/L, in white wines. This 10-fold difference would be expected since gallic acid is principally formed by hydrolysis of flavonoid gallate esters, which are largely absent in white wines due to the lack of skin extraction. Caffeic acid varied from 5 to 13 mg/L in red wines and from 1 to 4 mg/L in white wines. Caffeic acid is a hydrolysis product of the tartrate ester. A recent study suggests that caffeic acid levels are dependent on a sun-exposure-dependent induction of caftaric acid hydrolysis (Price et al., 1994).

The levels of the two anthocyanins analyzed, cyanidin (0–7 mg/L) and malvidin 3-glucoside (0–90 mg/L), were found as expected in relatively high concentrations in the red wines because they are the major contributors of color. These anthocyanins were absent in most of the white wines, except for about 3 mg/L in the white zinfandels, which are blush with a small amount of red color. Smaller amounts of the following compounds were found only in red wines, including the flavonols rutin (2–32 mg/L), myricetin (0–18 mg/L), and quercetin (2–17 mg/L), as expected since these materials are found in grape skin.

Resveratrol levels averaged 1527 μg/L in the red wines and 27 μg/L in the whites and followed the same pattern established in other studies (Goldberg et al., 1994; Lamuela-Raventos and Waterhouse, 1993). The small number of wines analyzed does not allow for significant comparisons between grape varieties. Since resveratrol is a compound that is induced by fungal infections, its presence and levels can be extremely variable.

Correlation of the relative LDL antioxidant activity with the concentration of individual phenolic constituents gave the following r values in decreasing order: 0.92 ($p < 0.001$) for gallic acid, 0.76 ($p < 0.001$) for catechin, 0.70 ($p < 0.001$) for myricetin, 0.68 ($p < 0.001$) for quercetin, 0.63 ($p < 0.002$) for caffeic acid, 0.50 ($p < 0.02$) for rutin, 0.48 ($p < 0.02$) for epicatechin, 0.43 ($p < 0.05$) for cyanidin, and 0.38 ($p < 0.1$) for malvidin 3-glucoside. Thus, the capacity to protect LDL from oxidation appears to be distributed widely among a large number of phenolic constituents in wine. On the other hand, the resveratrol content had no relation to this antioxidant activity (Table 2).

DISCUSSION

To account for the antioxidant activities of the individual phenolic compounds found in wines, we may consider structural factors such as the number of phenolic hydroxyl or methoxyl groups, flavone hydroxyl and keto groups, free carboxyl acid groups, and other structural features. The dihydroxylation in both rings

and in the 3-position in catechin, myricetin, quercetin, and epicatechin is required for antioxidant activity reported in various lipid systems (Pratt and Hudson, 1990). Although the phenolic compounds have similar chemical properties, their reducing capacity is not a very precise predictor of their antioxidant activity. In the LDL oxidation assay and other tests for antioxidant activity, the system is typically heterogeneous and physical properties, such as lipophilicity, solubility, and partition between the aqueous and lipid phases of LDL can become important in determining antioxidant activity (Frankel, 1993; Frankel et al., 1994).

In different wines we need to consider possible effects of synergism and antagonism in complex mixtures of these phenolic compounds. On the basis of previous studies both the physical and chemical properties of individual phenolic antioxidants strongly affect their potency (Pryor et al., 1988, 1993). More research is needed to evaluate individual components from different wines and from different vintages to gain a better understanding of the interactions of phenolic compounds and their changes on aging.

Resveratrol has attracted much attention because it is an active ingredient of the oriental folk medicine kojokon, which is reported to have a multitude of therapeutic uses (Kimura et al., 1985). The presence of resveratrol in wine has been suggested as a possible explanation for the French Paradox without any direct evidence for its beneficial effect (Siemann and Creasy, 1992). However, we found that resveratrol was less potent than either epicatechin or quercetin as an antioxidant in inhibiting human LDL oxidation (Frankel et al., 1993b). Since resveratrol constitutes approximately 0.1% of the phenolic material in a typical red wine, the oxidation of LDL would not be expected to be significantly affected by this compound. Therefore, the health significance of resveratrol relative to the other antioxidants is an open question at present.

All phenolic compounds that are absorbed could have a health impact. Thus, unless resveratrol is absorbed from wine to the exclusion of other phenolics, it would not be expected to have much effect in preventing LDL oxidation. Previous work has shown that large doses of catechin are absorbed from the gut, but the catechin found in blood is largely transformed into glucuronides (Hackett et al., 1983). On the other hand, quercetin is reported not to be absorbed by humans (Gugler et al., 1975). Most recently, Maxwell et al. (1994) reported that ingestion of a red Bordeaux wine was associated with a rapid increase in "antioxidant activity" in the serum of 10 human volunteers. Although this study suggests that the antioxidant phenolic compounds in red wine are absorbed and remain active after absorption, the antioxidant assay used in this study was not specific. This assay was based on suppression by antioxidants of chemiluminescence, which may be confounded by many factors, especially in the preparation of serum, where polyphenolic compounds may be destroyed by coagulation.

This study showed that the antioxidant activity of different commercial wines toward LDL oxidation is not a property of a single phenolic compound and that this activity is widely distributed among the phenolic phytochemical constituents. The wide distribution of activity suggests that it will be important to determine the response of individual phenolic constituents to the LDL oxidation assay to better understand which chemical and physical factors are most significant in determining antioxidant potency. A measure of the human absorption of these compounds is also necessary to assess their potential health effects.

ABBREVIATIONS USED

BSTFA, bis(trimethylsilyl)trifluoroacetamide; CHD, coronary heart disease; GC–MS, gas chromatography–mass spectrometry; GAE, gallic acid equivalents; HPLC, high-performance liquid chromatography; LDL, low-density lipoproteins; r, correlation coefficient.

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