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Grape and Grape Seed Extract Capacities at Protecting LDL against Oxidation Generated by Cu^{2+}, AAPH or SIN-1 and at Decreasing Superoxide THP-1 Cell Production. A Comparison to other Extracts or Compounds

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A large body of evidence supports the key role of oxidized low-density lipoprotein in atherosclerosis. The aim of this study was to compare the capacity of natural polyphenols (PP) from *Vitis vinifera* and *Olea europea* at protecting LDL against oxidation brought about by Cu^{2+}, oxygen-centered radical-generating AAPH, or peroxynitrite-generating SIN-1 *in vitro* systems, or at impairing superoxide production in promonocyte cells (THP-1) conveniently differentiated into adherent macrophages. PP were either from the whole grape (fraction A) containing mainly procyanidins, (epi)-catechin and anthocyanins, or from grape seed extracts (fractions B and C) consisting of tannins and procyanidin oligomers with a higher content in B than in C, or from a grape skin extract (fraction D) consisting mainly of anthocyanins, or from a hydrosoluble olive mill wastewater PP extract (fraction E) containing hydroxytyrosol and oleuropein. Chlorogenic acid (F) and catechin (G) were taken as archetypes of PP preventing oxidation partly as copper scavenger and as radical scavenger only, respectively. All grape fractions were efficient towards Cu^{2+} system (equally or more efficient than F), whereas they were rather poorly efficient towards AAPH and SIN-1 (less efficient than G but as efficient as F). Among the PP fractions, B was the most effective at protecting LDL in the SIN-1 system and at impairing THP-1 superoxide production. Taken together, these data suggest that the PP fraction from grape seed rich in procyanidins achieves the best compromise between the direct and indirect (i.e. cell-mediated) types of action in protecting LDL against oxidation, strengthening the need for improving the knowledge of its bioavailability in humans.

Keywords: Catechin; Procyanidin; Chlorogenic acid; LDL; THP-1

Abbreviations: LDL, low-density lipoprotein; IFNγ, interferon γ; SOD, superoxide dismutase; DTPA, diethylenetriamine pentacetic acid; PMA, phorbol myristate acetate; AAPH, 2-2′-azobis-(2-amidinopropane) hydrochloride; SIN-1, 3-morpholinosydnonimine; PBS, phosphate buffer salt

INTRODUCTION

A large body of evidence supports the key role of oxidized low-density lipoprotein (ox-LDL) in the early (inflammatory)[1] and more advanced[2] stages of the atherosclerotic lesion. Ox-LDL is cytotoxic to endothelial cells and chemotactic for monocytes, decreases the mobility of macrophages partly due to its capacity at increasing the extra-cellular matrix synthesis, promotes platelet aggregation, endothelial cell adhesiveness towards monocytes[3,4] and vascular smooth muscle cell proliferation.[2] The main lipophilic antioxidant located inside the LDL particle—vitamin E (α-tocopherol)—reduces *in vitro* ox-LDL formation,[5] and is also able to impair the monocyte/macrophage superoxide anion production[6,7] partly responsible for LDL oxidation.[8] This supports the idea that antioxidant-rich diets delay the development of atherosclerotic lesions by preventing LDL oxidation. This is the rationale for which we (and other groups) are

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interested in looking for natural products present in foods in a more or less concentred form in order to assess their potential antioxidant capacities at diminishing LDL oxidation susceptibility and/or depressing superoxide cell production.

Polyphenols (PP) are known to be antioxidant *in vitro* or to protect other antioxidants in the biological fluids and LDL *in vivo*.[9,10,11,12] Some of them are also known to be able to depress superoxide production in a promonocyte cell line.[13] They are present in cereals, (fresh) fruits and vegetables, olive oil and wine, all components of the Mediterranean-style diet. Other foods or beverages are also rich in PP. This is the case for legumes and non-alcoholic fruit juices.[14] Several epidemiological studies have shown a negative correlation between PP consumption and the frequency of cardiovascular disease.[15,16] In addition, in spite of a theoretical higher risk of coronary heart disease due to a high saturated fat intake and an elevated cholesterolemia,[17] some populations present a lower risk[18] which could be attributed to a higher consumption of PP in the form of vegetables, fruits or wine.[18,19]

The purpose of the present work was to compare antioxidant properties of several natural polyphenolic compounds under *in vitro* conditions. This was carried out on the basis of (1) their ability to protect LDL against oxidative modifications brought about by several oxidation models, (2) their ability to impair the cell production of superoxide anion, which is recognized as one of the main reactive species (RS) leading to LDL oxidation. *In vivo* interactions between these compounds and the intestinal microflora or systemic metabolic reactions are not taken into account here. We are fully aware that such interactions, which account for the essential notion of bioavailability, play an important role in determining the actual (patho)physiological action of PP *in vivo*. However, the present *in vitro* preliminary screening is thought to be of great interest to direct future human intervention studies aiming at improving atherosclerosis prevention by food. This may be particularly true when diversified approaches are used mimicking different types of *in vivo*-exhibited oxidative stress.

It is presently found that PP from the whole grape, different fractions of grape or from olive mill wastewaters possessed: (1) different antioxidant activity profiles towards three oxidation systems and (2) different depressing effects on the superoxide cell production, supporting that the nature of PP and the mixture of them greatly contribute to antioxidative effects.

MATERIALS AND METHODS

Chemicals

PP in the dry powder form were from the whole grape (fraction A), from grape seed extracts (fractions B and C), from a grape skin extract (fraction D) and from a hydrosoluble olive mill wastewater PP extract (fraction E) (Table I). They were prepared by La Gardonnenque (Cruviers-Lascours, France) which get clearance to sell them under the trade-marks reported in Table I. The fractions were obtained after aqueous extraction excluding any organic solvent and always stored under inert atmosphere. They were characterized by their PP content[20] and their PP composition. The fraction D was already used as a colorant in food industry. The main representative PP were catechin/epicatechin, procyanidin dimers and oligomers and anthocyanins for A, the same without anthocyanins for B and C (42.9 and 31.0% procyanidin oligomers, respectively, with a mean monomer number of 4 instead of 5 for A), anthocyanins for D and hydroxytyrosol or hydroxytyrosol-containing oleuropein for E. Chlorogenic acid and catechin were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). AAPH, 2-2′-azobis(2-amidinopropane) hydrochloride was from Biovalley (Conches, France). SIN-1, 3-morpholinosydnonimine was from Molecular Probes Inc (Leiden, Holland). The THP-1 cell line was a gift from Dr J. Dornan (University of Montpellier). Retinoic acid, IFNγ, phorbol myristate acetate (PMA) and lucigenin (10, 10′-dimethyl-9,9′-bisacridinium dinitrate) were purchased from Sigma Chemicals (St Quentin Fallavier, France). Nutritive media and fetal calf serum (FCS) were from Bio Media (Boussens, France). 1α,25-dihydroxycholecalciferol was a gift from Hoffmann-La Roche SA (Basel, Switzerland). ATP bioluminescent reagents (the ViaLight HS kit) were purchased from Lumitech Ltd (Nottingham, Great Britain).

Preparation, Biochemical Parameters and Oxidizability of LDL

Human plasma was collected and LDL preparation was carried out as previously indicated.[9] Apoprotein B (apoB) was determined by immunonephelometry using a Turbitimer apparatus (Behring, Rueil-Malmaison, France). The protein content was assessed using the Markwell's modification of the Lowry's method using bovine serum albumin (BSA) as a standard.[24] Total cholesterol and triglycerides were measured by automatized enzymatic methods proposed by Bio-Merieux (Marcy-l'Etoile, France).[25] LDL α- and γ-tocopherol were extracted and measured by means of

TABLE I Characteristics of polyphenol mixtures under investigation

Polyphenols	A	B	C	D	E
Nature of extract (trade-mark)	Whole grape (exGrape® total)	*Vitis vinifera* seed (exGrape® seed 40)	*Vitis vinifera* seed (exGrape® seed 30)	*Vitis vinifera* skin (exGrape® skin)	Olive mill wastewaters
PP content*	1.75 ± 0.21	2.75 ± 0.07	2.55 ± 0.07	0.90 ± 0.14	1.15 ± 0.21
Anthocyanins†	1.97	–	–	1.62	–
Catechin/epicatechin†	3.54	7.33	4.48	–	–
Epicatechin-3-gallate†	0.10	0.38	1.17	–	–
Procyanidin dimers†	4.99	6.56	5.36	–	–
Procyanidin B2-3′-gallate†	1.29	0.46	0.23	–	–
Oligomer procyanidin compounds‡	20.1	42.9	31.0	–	–
mean polymerisation index¶	5.1	4.3	4.2	0.46	–
Gallic acid†	0.26	0.02	0.62	–	305/38
Hydroxytyrosol/Tyrosol§	–	–	–	–	1004
Oleuropein§	–	–	–	–	

*Expressed as catechin, μmol/mg; the fractions correspond to 50, 80, 74, 26 and 33% expressed as weight percent, respectively. †weight percent; they have been determined by HPLC (see Ref. [21]) and authentic standards, in particular: delphinidin-3-glucoside, cyanidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside, malvidin-3-glucoside (M3G), acetyl-M3G, paracoumaroyl-M3G for anthocyanins; dimers B1, B2, B3, B4 for procyanidins. ‡Using the vanillin method (see Ref. [22]), expressed as catechin equivalent in weight percent. ¶Using the thiolyse procedure (see Ref. [23]). §mg/kg of powder.

a fluorescent detector (model LS-3B fluorescence spectrometer, Perkin–Elmer, 290 and 330 nm for excitation and emission wave lengths, respectively) after HPLC separation.[9] An Uptisphere 5μHDO C18 column (250 × 4.6 μm Interchim, Montluçon, France) was employed for separation using a mobile phase consisting of a water/acetonitrile/acetic acid mixture (0.5:99.4:0.1, v/v/v) at a 1 ml/min flow rate for 16 min. Oxidation of isolated LDL in the presence of copper (Cu^{2+}) or AAPH was performed as previously described.[9] The PBS solution of AAPH was made just before use. Briefly, LDL (1 μM apoB) was preincubated for 1 h at 37°C under N_2 in the presence of various concentrations of the products to be tested, and oxidized either by 5 μM Cu^{2+} after 10 fold dilution in oxygenated PBS (10 mM phosphate buffer, 150 mM NaCl, pH 7.4) or by 2.5 mM AAPH after 20 fold dilution in the same buffer. LDL oxidizability was assessed by measuring the CD absorbance at 234 nm for Cu^{2+}-oxidation of LDL (0.1 μM apoB) and at 245 nm for AAPH-oxidation of LDL (0.05 μM apoB). The expression of the anti-oxidant capacities of natural products used the notion of specific antioxidant activity (SAA) thoroughly described in a previous paper.[9] Briefly, T_{lag} was plotted versus increasing concentrations of the product under investigation. We obtain a linear relationship $y = \alpha x + 100$, where y represents the "relative" T_{lag} (designated by $rT_{lag[P]} = [T_{lag[P]}/T_{lag[0]}] \times 100$ for a given concentration of product [P], $T_{lag[0]}$ being the T_{lag} in the absence of added product), whereas α represents the coefficient of regression of the linear relationship. The coefficient α is equivalent to the expression $[rT_{lag[P]} - 100]/[P]$ considered here as the SAA for one given product and expressed in a specific antioxidant unit (μM^{-1} or $1\ mg^{-1}$).

The SIN-1 stock solution was in PBS, stored at −20°C and spectrophotometrically verified before use. LDL SIN-1 (21 μM)-mediated oxidation was carried out as in the Cu^{2+} oxidation system, excepted that PBS was not previously oxygenated. In order to avoid unwanted oxidations by metal ions during LDL SIN-1 oxidation, DTPA (10 μM) was added to the milieu. LDL (0.1 μM apoB) oxidizability was assessed by measuring the CD production as for the Cu^{2+} oxidation conditions. The results were given as the concentration of products leading to diminish by 50% the CD formation at $t = 5$ h (IC_{50}). In order to homogenize the modes of expression of the results obtained in the three oxidation systems, we also expressed the LDL oxidizability in Cu^{2+} and AAPH systems as the concentration needed to double the rT_{lag} ($C_{2T_{lag}}$). The loss of polyunsaturated fatty acids during coincubation of LDL and SIN-1 (21 μM) was determined after lipid extraction, fatty acid trans-esterification and a gas chromatography procedure leading to a complete profile of LDL fatty acids.[25]

Cell Culture and Superoxide Production Assessment

Cells were cultured in RPMI 1640 with glutamine (2 mmol/l), 10% heat-inactivated FCS, penicillin (100 U) and streptomycin (0.1 μg/ml) in flasks (75 cm^2, Corning Costar Corporation). Cells were maintained at a density inferior to 1.0×10^6 cells/ml in a humidified incubator (5% CO_2) at 37°C, with a complete change of medium twice a week. The viability of cells (>95%) was determined by trypan blue exclusion. For differentiation into adherent macrophages, they were seeded at a density of 6.0×10^4 for 200 μl of culture medium in wells (96-well microplate, Corning Costar Corporation) and incubated for 72 h in the presence of 1 mM retinoic acid, 0.1 μM vitamin D3 and 100 units/ml IFNγ. Once differentiated, cells were placed in the same medium with the substances to be tested for 24 h and then in the medium containing 0.1 μM PMA only. Lucigenin-enhanced $O_2^{\bullet -}$ bioluminescence was assessed at 37°C by means of a microplate luminometer Microlumat*plus* (EGG Berthold, Evry, France) controlled by a personnal computer equipped with the Winglow software. The luminescence signal was counted for 10 s every 10 min until 90 min after PMA addition. The 100% values corresponded to the control conditions, i.e. the $O_2^{\bullet -}$ bioluminescence in the presence of PMA without previous phenolics co-incubation. The control medium contained the solvent vehicle (alcohol) at a concentration equivalent to that added with substances to be tested. Finally, the cell amounts were assessed in each well by using the bioluminescence due to cell lysis-released ATP in the presence of luciferin-luciferase system. Briefly, 100 μl of culture medium and 100 μl of ATP releasing agent were added to the cell-containing wells and incubated at room temperature for 5 min. Then, 20 μl of the luciferin-luciferase reagent were added to the wells and the luminescence was immediately recorded within a 10 s time period at 30°C using the microplate luminometer. An ATP standard solution (0.1 μM) was used for quantification purposes.

Statistic Analysis

Data are means ± SEM, from at least three separate experiments, unless special mention. The statistical differences were evaluated using the analysis of variance (ANOVA) (StatView™, Alsyd, Meylan, France) and the significance ($p \leq 0.05$) of each difference was evaluated by the Fisher's test.

RESULTS

Figure 1 shows the typical linear relationship between rT_{lag} and increasing concentrations of catechin taken as a model of PP and how this relationship was used for assessing SAA. Figure 2 shows the typical timecourse of absorbance at 234 nm accounting for CD production with the three oxidation systems. In order to confirm that the increased absorbance with SIN-1 was really due to fatty acid oxidation (whose the first step implies CD formation) we have shown the increase in CD production and the simultaneous decrease in two polyunsaturated fatty acids present in LDL: arachidonic acid (20:4n-6) and docosahexaenoic acid (22:6n-3) (Fig. 3).

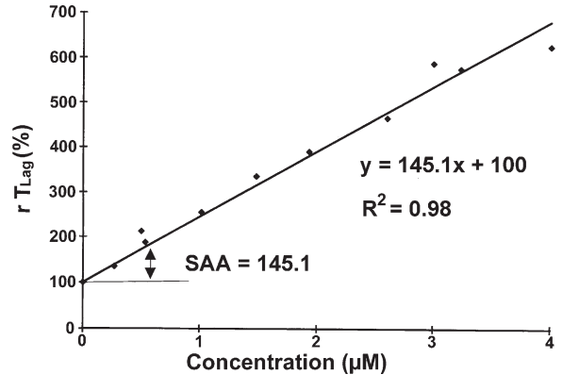


FIGURE 1 A typical assessment of catechin specific antioxidant capacity (SAA) plotting the relative lag time of CD production in LDL submitted to a Cu^{2+} oxidation versus increasing concentrations of catechin.

Since the chemical characteristics—specifically the tocopherol content—of LDL deeply influence its oxidation resistance, they were reported in Table II. LDL exhibited an exceptionally high α-tocopherol content, and a rather high protein content as accounted for by the ratio total-protein/apoB of 1.7 in accordance with our LDL isolation procedure.[22]

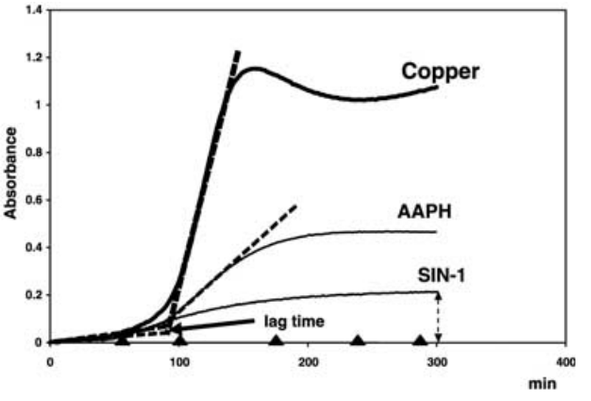


FIGURE 2 Typical time courses of CD production in LDL exposed to the following oxidation-generating systems: copper (Cu^{2+}/molecular oxygen), 2-2′-azobis(2-amidinopropane) hydrochloride (AAPH) and 3-morpholinosydnonimine (SIN-1). Lag time of production was measured for the first two one, and production during a 5 h time period for the last one. ▲Indicate the values of absorbance obtained when 3.5-mg/l superoxide dismutase was added to SIN-1.

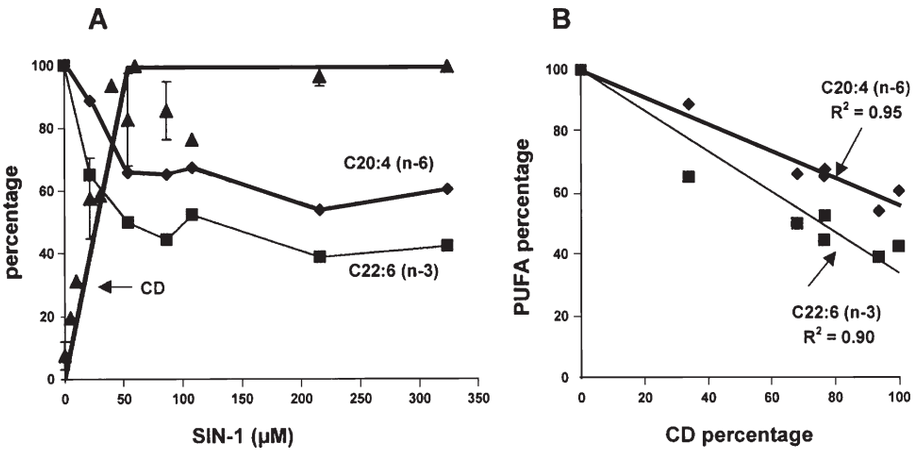


FIGURE 3 Relationship between the CD production due to coincubation of LDL with SIN-1 and the loss of polyunsaturated fatty acids: arachidonic acid (20:4n-6) and docosahexaenoic acid (22:6n-3).

Figure 4A shows the modification of the absorbance profile of LDL and SIN-1 during their co-incubation in the presence or absence of catechin taken as a PP model. It clearly appears a more pronounced shoulder with time at a wavelength close to 230 nm. The spectrum of difference (Fig. 4B) allows us to show that the largest profile changes were due to SIN-1 and catechin, and that they took place at 234 nm. The second peak of absorbance (at 273 nm) resulted from the shift (see Fig. 5) of the maximal absorbance of SIN-1 from 292 (curve A) to 278 nm (curve B) during breakdown into superoxide and nitric oxide. Since superoxide dismutase (SOD) is able to catalyze the disproportionation of $O_2^{\bullet -}$, it has been verified that CD formation was only observed in the absence of SOD (and not in the presence, see Fig. 2), indicating that nitric oxide was not involved in the changes due to the SIN-1 oxidation system. This leads to conclude that enhanced 234 nm absorbance was most likely due to $ONOO^-$, which is known to be rapidly generated from the reaction of superoxide with nitric oxide. Thermally inactivated SOD did not exert the action provided by native SOD (not shown). It has also been found that the maximal antioxidative PP protection was obtained at low SIN-1 concentration (21 μM), with a CD-production depressing effect superior to 80%. This is the reason why we chose this concentration in the following part of the study.

TABLE II Chemical composition of one particle of LDL

α-Tocopherol	12.7 ± 1.1*
γ-Tocopherol	0.5 ± 0.1*
Triglycerides	464.8 ± 32.5*
Total cholesterol	2415.4 ± 559.7*
Total protein	1.7 ± 0.3†

*Expressed as mol/mol of apoB. †Expressed as g/g of apoB.

Figure 6 shows the inverse linear relationship between the SIN-1 oxidation system CD production and the increasing concentrations of PP from the different origins. Table III shows the values of SAA and $C_{2T_{lag}}$ established with the copper and AAPH oxidation systems. They have to be compared to those of IC_{50} provided by the SIN-1 oxidation system and deriving from the curves of Fig. 6. Considering the PP fractions, the results shown in Table III established that fraction B exhibited a higher protective effect than fractions A, D and E in the SIN-1 system, whereas this effect did not differ from that of fraction C. Catechin and chlorogenic acid had clearly similar effects and they were more potent than any aforementioned fraction. In the Cu^{2+} system, the fractions A, B, C and D showed the same antioxidative protective effect and a higher effect than that of the most poorly efficient catechin, whereas only fraction D was more efficient than chlorogenic acid and fraction E was as poorly efficient as catechin. In the AAPH system, the fractions A, B, C and D showed the same protective effect, as it was the case in the Cu^{2+} system, but their efficiencies were low as compared with that of the most efficient catechin, and they were as low as that of the most poorly efficient chlorogenic acid. Fraction E exhibited the same poor effect as chlorogenic acid and was less efficient than fractions A and B.

Figure 7 shows the antioxidant capacities of the phenolic products expressed on a massic basis. Regardless of the oxidation system, the fraction B was found to be the most efficient. However, the capacity of fraction C was similar to that of B with copper and SIN-1.

Table IV illustrates the capacity of the different preparations at impairing the superoxide production in a cellular model of differentiated promonocytes. The most efficient one was the fraction B (almost 40% inhibition; the fraction C was lacking for this part of

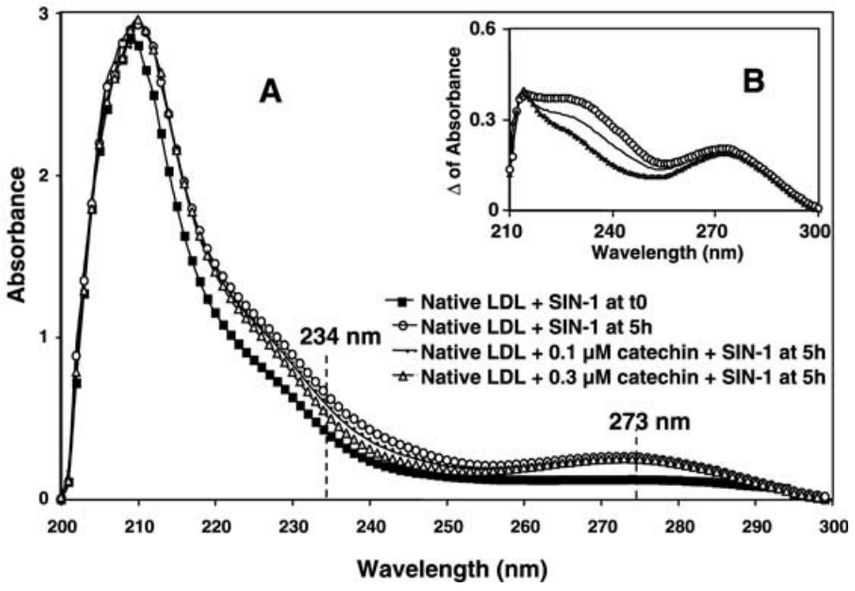


FIGURE 4 Absorbance profiles of 21-μM SIN-1 in the presence of LDL (0.1 μM apoB). A: spectrums for LDL + SIN-1 as a function of time and the presence or absence of catechin. Curve B: differences of spectrums, the t_0 spectrum being subtracted in all cases.

the study). It is worth mentioning that efficiency was higher than that found for chlorogenic acid, whereas catechin effect was not significant and vitamin E showed a slight (but non significant) enhancing effect on the superoxide production (see footnote of Table IV).

DISCUSSION

PP mixtures were presently tested and compared to catechin and chlorogenic acid. We found that grape seed and skin PP-extracts originating from *Vitis vinifera* (fractions B, C and D) protected more efficiently LDL against oxidation generated by cell-free systems than the PP extract originating from olive wastewaters (fraction E). This was particularly true with the Cu^{2+} system (Table III). More specifically, grape fractions A and B were more efficient than fraction E with the Cu^{2+} and AAPH systems, and fraction B was more efficient than any other PP fractions with the SIN-1 system. For comparison purposes, it is interesting that the same determinations with an extract from *Lycopersicon esculentum* rich in lycopene (molar equivalence calculated on the basis of lycopene content) gave the following values of SAA: $20.2 \pm 1.2\,\mu M^{-1}$ with Cu^{2+}, $10.4 \pm 2.6\,\mu M^{-1}$ with AAPH and an

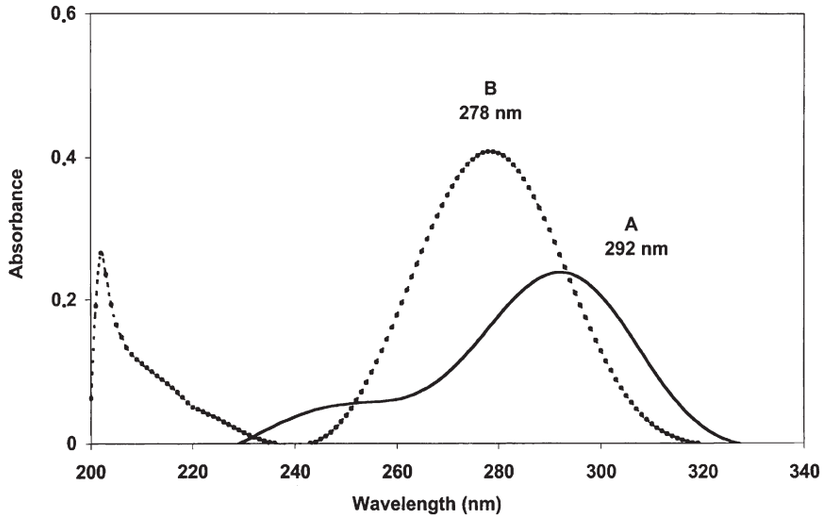


FIGURE 5 Shift in the spectrum of 21-μM SIN-1 as a function of time. Spectrums of native SIN-1 at t_0 (curve A) and after 5-h incubation (curve B) in the same buffer as that used when co-incubated in the presence of LDL (Fig. 2).

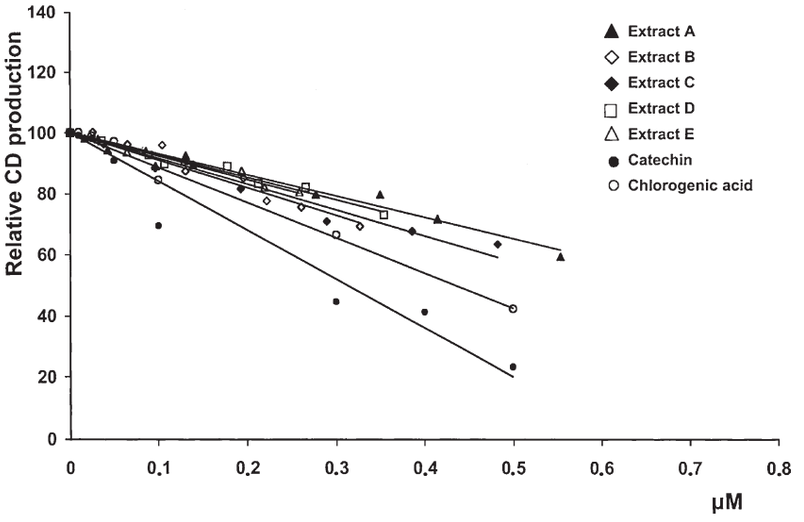


FIGURE 6 The CD production of LDL (0.1 μM apoB) in the presence of 21 μM SIN-1 as a function of increasing concentrations of polyphenols to be tested. LDL was incubated without (the 100 control) or with polyphenols at 37°C. The 5-h CD production was measured at 234 nm. Data are the means of three separate experiments. Standard deviations are not represented for clarifying purposes. Correlation coefficients were superior to 0.9.

$IC_{50} > 40\,\mu M$ with SIN-1, emphasizing that the PP fractions presently tested were much more efficient. Additionally, fraction B which probably showed the best combination of antioxidant properties when the three cell-free oxidation systems were considered showed also the highest ability to impair the macrophage-generated superoxide production, illustrating its prominent cell-mediated action.

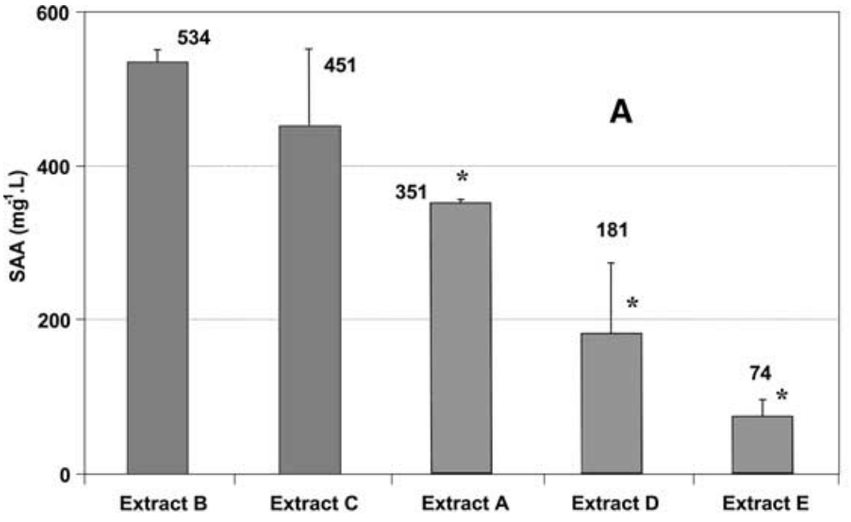
It is to stress that the results we presently comment were expressed on a molecular basis. Therefore, they account for intrinsic properties of PP or interactive effects between mixture components rather than for PP concentrations in the mixtures. Accordingly, the results support that synergistic actions between oligomers and monomers of (epi)catechin may take place. Synergistic actions have frequently been suggested between lipophilic and hydrophilic (phenolic) antioxidants [26,27], but not between PP.

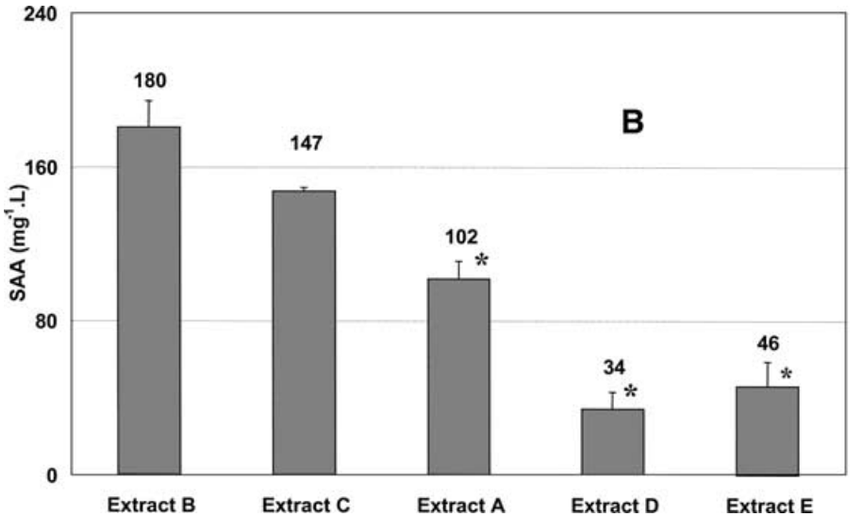
More interestingly, scavenger and modulating cell properties of a tea-PP fraction towards nitrogen RS have been found.[28] It is worth comparing such combined properties to those presently found towards oxygen RS. PP are indeed revealing that they can interfere with cell mechanims involved in anti-apoptotic, anti-inflammatory and anti-carcinogenic processes.[29–34] We have also reported that a hydroxytyrosol-enriched olive mill wastwater extract[13] was able to impair the superoxide production in the cell model THP-1. The present results were in accordance with these results. However, one question is the rationale for which the fraction B exhibited the most efficient down-regulating action on superoxide production. We can only speculate that this effect is mediated by one or different steps involved in cell signaling.[13] Most likely procyanidolic compounds, whose content was high in fraction B, do not enter the cell. In order to explain their cell effect, it can be hypothesized that an early plasma-membrane step exists involving specific receptor(s). Interestingly, consistent data exist in favor of inhibiting properties of complex PP towards tyrosine kinase receptors.[35,36]

TABLE III Antioxidant capacities of natural phenolic products assessed by copper (Cu^{2+}), AAPH or SIN-1 LDL-oxidation system

	Copper		AAPH		
Products	SAA* (μM^{-1})	$C_{2T_{lag}}$† (μM)	SAA (μM^{-1})	$C_{2T_{lag}}$ (μM)	SIN-1 IC_{50}‡ (μM)
A	212.8 ± 8.5*^{ab}	0.47 ± 0.02	63.6 ± 5.5^{a}	1.58 ± 0.13	0.72 ± 0.08^{a}
B	190.6 ± 3.3^{ab}	0.53 ± 0.01	64.4 ± 4.8^{a}	1.55 ± 0.08	0.55 ± 0.03^{b}
C	175.5 ± 20.8^{ab}	0.59 ± 0.07	56.6 ± 0.8^{ab}	1.77 ± 0.02	0.60 ± 0.07^{ab}
D	225.8 ± 66.4^{a}	0.43 ± 0.09	42.8 ± 10.6^{ab}	2.43 ± 0.56	0.70 ± 0.01^{a}
E	63.9 ± 10.7^{c}	1.26 ± 0.41	39.8 ± 10.9^{b}	2.63 ± 0.39	0.69 ± 0.11^{a}
Catechin	34.6 ± 4.8^{c}	2.89 ± 0.70	156.2 ± 19.2^{c}	0.64 ± 0.08	0.32 ± 0.02^{c}
Chlorogenic acid	55.5 ± 7.0^{b}	0.64 ± 0.05	49.8 ± 8.0^{ab}	2.01 ± 0.32	0.44 ± 0.04^{c}

Data are the means of three separate experiments. Correlation coefficients of the linear equation $rT_{lag} = SAA[P] + 100$, used to assess SAAP are ≥0.9. *Values with the same letters as exposant are not significantly different at $p \leq 0.05$. *Specific antioxidant activity. †Concentration required to increase the lag time by 100%. ‡Concentration required to reduce the conjugated diene production by 50%.





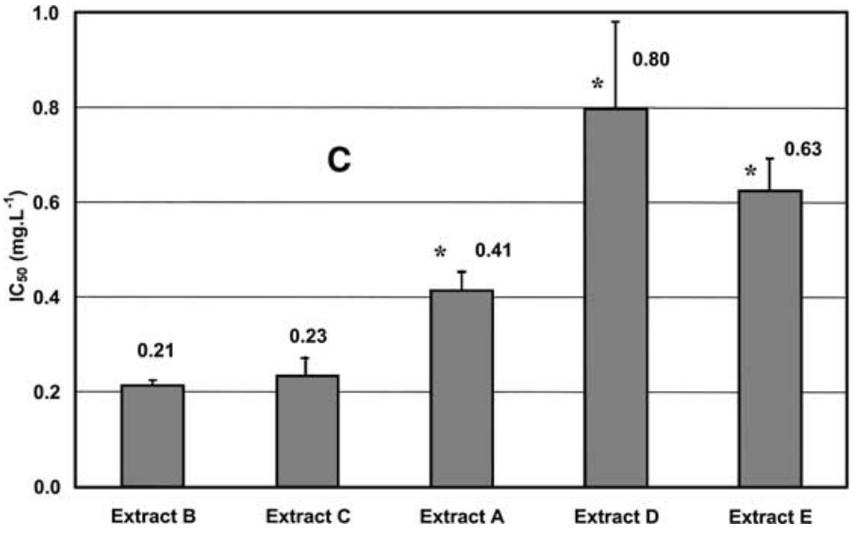


TABLE IV Effects of increasing concentrations of natural products on the cell production of $O_2^{\bullet -}$-lucigenin bioluminescence by 0.1 μM PMA-stimulated THP-1 cells once differentiated into macrophages

Products (μM)		RLU $O_2^{\bullet -}$ (%)	RLU $O_2^{\bullet -}$/RLU ATP (%) [*,†]	Inhibition (%)[‡]
A	4.0	77.6 ± 4.8	92.2 ± 5.2	7.8 ± 0.4
	16.3	68.4 ± 3.2	76.8 ± 2.1	23.2 ± 0.6
	32.5	63.8 ± 2.4	77.2 ± 4.6	22.8 ± 1.3
B	3.9	82.0 ± 3.7	78.3 ± 4.3	21.7 ± 1.2
	15.4	67.3 ± 4.4	66.4 ± 1.4	33.6 ± 0.7
	30.8	59.0 ± 3.2	60.3 ± 3.2	39.7 ± 2.1
Vitamin E	33.5	93.0 ± 4.8	94.6 ± 5.7	5.4 ± 0.3
	50.3	102. ± 2.8	104.0 ± 2.8	–
	67.0	104.7 ± 1.6	108.2 ± 6.6	–
	134.0	107.8 ± 2.2	112.5 ± 5.5	–
Catechin	5.0	78.5 ± 3.3	88.4 ± 2.2	11.6 ± 0.3
	20.0	77.6 ± 3.4	89.9 ± 5.7	10.1 ± 0.6
	40.0	76.1 ± 3.4	88.6 ± 0.1	11.4 ± 0.3
Chlorogenic acid	5.0	75.9 ± 3.3	84.8 ± 6.9	15.2 ± 1.2
	20.0	78.9 ± 5.2	87.8 ± 7.2	12.2 ± 1.0
	40.0	70.9 ± 3.2	81.0 ± 5.4	19.0 ± 1.3

Results were expressed as RLUs (Relative Light Units). The 100% value corresponded to the value of the control. Values are means ± SEM of six or three separate experiments. [*]The $O_2^{\bullet -}$ bioluminescence was reported to the ATP cell production as assessed by a luminescence procedure (see "Materials and Methods" section) which is directly indicative of cell number. [†]The 100% values corresponded to the absolute values as follows (mean ± SEM): 0.55 ± 0.13 for A and B ($n = 6$), 0.23 ± 0.03 for vitamin E ($n = 3$), 0.69 ± 0.17 for catechin and chlorogenic acid ($n = 6$). ANOVA test for absolute values of RLU $O_2^{\bullet -}$/RLU ATP in response to increasing concentrations: $p \leq 0.05$ except in the case of catechin and vitamin E. [‡]Percentage inhibition calculated from the bioluminescence reported to the ATP cell production.

These receptors generally stimulate mitogen-activated protein kinases which could be involved in NADPH oxidase activation[37] leading to an increased superoxide production. The superoxide-production modulating tyrosine kinase receptor hypothesis needs however to be verified in the case of monocyte-macrophage. Complex interactions between these receptors and procyanidin oligomers would deserve a special interest.

Among the cell-free oxidant systems, we chose those mimicking pathophysiological oxidation processes taking place in the vascular compartment.[38] Besides the well-known but controversial (from a pathophysiological point of view, see below) Cu^{2+}-induced peroxidation system, two other oxidant systems were relevant: AAPH and SIN-1, generating the alk-oxyl/-peroxyl radicals (R–O•/R–OO•)[39] and the peroxynitrite anion ($ONOO^-$),[40] respectively. The Cu^{2+} and AAPH systems correspond to a mono-electronic oxidation process and their physiological significance has already been discussed.[9] Briefly, the Cu^{2+}-oxidation system implies a Cu^{2+} interaction with an LDL low-affinity binding site, the formation of the reduced form Cu^+ which in turn leads to lipid peroxide formation. This reduced form could also interact with one specific site of ceruloplasmin, a protein which has been authentified in the LDL we usually prepared and has been found to be pro-oxidant towards LDL.[25] Contrasting with the Cu^{2+}-oxidation system, AAPH is able to directly produce radicals in the aqueous phase and thus to trigger lipid peroxidation independently of transition metals.[25,41] In this respect, chlorogenic acid and catechin could be considered as the archetypes of substances preventing LDL oxidation via copper-dependent and copper-independent processes, respectively.[9] The first one acts as a copper chelating agent (but also as a radical scavenger), whereas the second one acts only as a radical scavenger. The SIN-1, $ONOO^-$-generating system corresponds to a di-electronic process, in which $ONOO^-$ formation results from the simultaneous release of nitric oxide and superoxide anion by the 3-morpholinosydnonimine molecule, and the subsequent immediate reaction between them.[42] This mimicks their simultaneous massive productions in pathological conditions, for example in endothelial dysfunction taking place in hypercholesterolemia, hypertension and diabetes.[43] It is clear from the present findings (see Table III) that all the fractions tested were specifically efficient as Cu^{2+} scavengers (fraction E except), and as efficient in particular as chlorogenic acid. The anthocyanin-containing fractions A and D tended to be the most active and it is worth mentioning that fraction D—containing mainly anthocyanins—was significantly more efficient than chlorogenic acid, which is in accordance with the efficient capacity of anthocyanins at complexing divalent metal ions.[44] However, fraction D contained other PP which remain to be described,

FIGURE 7 Specific antioxidant activities (SAA) or IC_{50} of polyphenols in the presence of LDL submitted to an oxidative stress due to the following oxidation systems: Cu^{2+} (A), AAPH (B) and SIN-1 (C). Data were expressed as SAA for Cu^{2+} and AAPH, and as IC_{50} for SIN-1. Values are means ± SD of three separate experiments. * Antioxidant capacities significantly lower than that of extract B. For *y*-axis units, see "Materials and Methods" section.

preventing to establish any closer structure–function relationship.

Although little is known about the structure-chelating function relationship in flavanols, we can also speculate[45] that the Cu^{2+}-chelate formation which tended to be higher with fraction A as compared with the other two catechin-PP containing fractions B and C was due to the presence of 3′-gallate substitution in procyanidin B2. On the other hand, all the fractions tested were rather poorly efficient as radical scavengers (contrasting to that extent with catechin).

Regarding the protective effect of fractions towards the SIN-1 oxidation system, it is worth noting that we did measure the protection against peroxynitrite oxidation since active SOD (and not the form inactivated by boiling) completely abolished the CD production.[42] It is also of interest to keep in mind that SIN-1 leads to the formation of RS with a hydroxyl radical-like reactivity.[46] As for AAPH, the SIN-1 system implies peroxidation whose initiation occurs independently of the transition metals and of the prerequisite of the lipid peroxide formation.[47] Both systems only implies hydrophilic RS. It is of interest, therefore, that antioxidant capacities towards SIN-1 and AAPH were similarly low for all PP fractions as compared with catechin, which is purely a radical scavenger, and were rather closer to the transition-metal chelating and radical scavenging chlorogenic acid. Of interest also is the fact that the most efficient PP fraction towards $ONOO^-$-generating system was the fraction B which was that with the highest procyanidin oligomer content. It is worthwhile to compare this result with those showing a protection against $ONOO^-$ by cocoa procyanidins.[48]

It is well established that the oxygen-centred radicals generated by AAPH are representative of radicals potentially generated in plasma. In contrast, using a free metal ion such as Cu^{2+} raises the issue of the extremely low level of this form in plasma and interstitial fluids which in turn leads to the question of its relevance *in vivo*. Our opinion is that the role of one ceruloplasmin-linked copper ion in LDL oxidation we have already mentioned and the presence of ceruloplasmin in plasma, atherosclerotic lesions (as a monocytic cell-derived protein) and LDL particle[25] make plausible a significant role of Cu^{2+} in oxidation processes involved in pathophysiological mechanisms. This leads us, therefore, to consider that the action of the fractions presently tested as copper chelators is likely of interest in preventing the formation of the atherogenic ox-LDL *in vivo*. In this context, fractions A, B, C and D could be considered as equally relevant.

Taken together the present results suggest that the polyphenolic fractions from grape, and in particular that from grape seed rich in procyanidin oligomers tend to presently exhibit the best compromise between the abilities to protect LDL both directly against oxidation, regardless of the oxidation-generating process, and indirectly through a cell-mediated oxidation process. It is crucial now to get further information on how and to what extent this type of highly efficient polyphenolic compounds could come to the space surrounding the cells *in vivo*. In fact, as already mentioned in the "Introduction" section, we are now fully aware that all metabolic reaction taking place in the digestive tract, in the cell or in the extracellular space have to be taken into account regarding the PP (patho)physiological actions. One emerging notion is that some bioderivatives (those methylated in particular, in progress in our laboratory and see Ref. [49]) could be more efficient than the parent compounds. Taken with recent findings considering that procyanidins could be decomposed in lower units (dimers in particular),[50] these findings are going to rapidly improve our understanding of the catechin-PP scavenger and cell actions.

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