JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Antioxidant Properties of Low Molecular Weight Phenols Present in the Mediterranean Diet

RAFFAELLA BRIANTE, FERDINANDO FEBBRAIO, AND ROBERTO NUCCI*

Institute of Protein Biochemistry, CNR Via Marconi, 10-80125 Naples, Italy

The antioxidant capacity of low molecular weight phenols found in olive fruit and in virgin olive oil has been investigated. The radical scavenging activity of some of the investigated phenols is higher than that of the most used antioxidants, and among them, 3,4- or 2,5-dihydroxyl phenols are also able to chelate copper ions leading to chelates that are only slightly active in the promotion of free radical reactions. The ability of tested phenols to reduce Cu(II) and their activity−structure relationships was also studied, showing that their reducing capacity is connected to the presence of a specific ligand of the reduced ion. The number of reduced ions per mole of phenol is lower than that calculated for some flavonols and isoflavones so exerting a lower prooxidant action. This fact may be important in vivo when free transition metal ions are involved in oxidation processes.

KEYWORDS: Phenols; radical scavenging capacity; prooxidant activity; chelating ability

INTRODUCTION

Free radicals are continuously generated in small amounts by normal processes of metabolism. Many of them serve useful physiological functions (*1*, *2*), but they can also damage the biomolecules when present in excess; they are implicated in the aetiology of several diseases and aging (*3*, *4*). For the balance of the physiological generation of free radicals, organisms have evolved a wide array of enzymatic and nonenzymatic endogenous antioxidant defenses (*5*, *6*). Nevertheless, in situations of increased free radical generation, the reinforcement of endogenous antioxidants may be particularly important in diminishing the cumulative effects of oxidatively damaged molecules.

A large body of epidemiological studies shows that the incidence of coronary heart disease (CHD) and certain cancers (breast and colon cancers) in the Mediterranean countries is low as compared with more northern European societies (*7*). It was suggested that this is largely due to the relatively safe and even protective dietary habits of this southern area where olive oil is the principal source of fat (*7*−*9*). The formulation of an antioxidant/atherosclerosis hypothesis stimulated experimental and epidemiological studies on the possible role of antioxidants, including olive oil phenols, in the protection from CHD observed in the Mediterranean area. Animal and in vitro studies suggest that the high concentration of phenolic antioxidants in extra virgin olive oil may contribute to the healthy nature of the Mediterranean diet (see reviews *10*−*12*).

On the other hand, the growing interest in the substitution of synthetic food antioxidants by natural ones has fostered research on vegetable sources and on the screening of raw materials for identifying new antioxidants (*13*). In fact, the oxidative deterioration of fats and oils in foods is responsible for rancid odors and flavors, with a consequent decrease in nutritional quality and safety caused by the formation of secondary, potentially toxic compounds. The addition of antioxidants is required to preserve flavor and color and to avoid vitamin destruction. Furthermore, oxidation reactions are not an exclusive concern for the food industry; antioxidants are widely believed to be necessary to prevent deterioration of other oxidable goods, such as cosmetics, pharmaceuticals, and plastics.

From this point of view, olive mill wastewaters (OMWW), produced in large quantities during the processing of olive oil, may be a powerful source of natural antioxidants. Low molecular weight phenols (**Figure 1**) identified in olive oils are also present in OMWW.

The antioxidant capacities of oleuropein, its aglycons, and hydroxytyrosol, the main phenols identified in *Olea europaea* L. tissues, are known (*10*−*12*), but few studies have been made on the antioxidant activities of other low molecular weight phenols present in olives and olive oil as well as in fruits, herbs, vegetables, cereals, and other plant materials characteristic of the Mediterranean diet.

In this work, we have evaluated the antioxidant properties of the main low molecular weight phenols identified in olive oil and also present in OMWW. In particular, their ability to scavenge free radicals by donating a hydrogen atom is investigated. Moreover, we have studied another antioxidant mechanism of these compounds, which may result from the interaction between phenols and metal ions (especially copper) leading to chelate generation that is only slightly active in the promotion of free radical reactions (*14*−*16*).

Phenolic compounds such as flavonoids have been reported to show prooxidant effects, which have been related with their iron and copper reducing activities. These reduced metals can

* To whom correspondence should be addressed. Tel and Fax: +39-081-7257300. E-mail: nucci@dafne.ibpe.na.cnr.it.

10.1021/jf034471r CCC: $25.00
Published on Web 10/14/2003

No.	Compounds	Substituents	Structures
	benzoic acids		
1	3-hydroxybenzoic acid	3–OH	
2	*p*-hydroxybenzoic acid	4–OH	
3	3,4-dihydroxybenzoic acid	3,4–OH	
4	gentisic acid	2,5–OH	
5	vanillic acid	3–OCH_3, 4–OH	
6	gallic acid	3,4,5–OH	
7	syringic acid	3,5–OCH_3, 4–OH	
	cinnamic acids		
8	cinnamic acid		
9	*o*-coumaric acid	2–OH	
10	*p*-coumaric acid	4–OH	
11	caffeic acid	3,4–OH	
12	ferulic acid	3–OCH_3, 4–OH	
13	sinapinic acid	3,5–OCH_3, 4–OH	
	phenylethanols		
14	tyrosol	4–OH	
15	dihydroxytyrosol	3,4–OH	
	phenylacetic acids		
16	*p*-hydroxyphenylacetic acid	4–OH	
17	3,4-dihydroxyphenylacetic acid	3,4–OH	
18	4-hydroxy-3-methoxyphenylacetic acid	3–OCH_3, 4–OH	
	complex phenols		
19	oleuropein		
20	mono-aldehydic elenolate oleuropein aglycon		
	non-polyphenol		
21	ascorbic acid		
	others		
22	trolox		
23	BHT		
24	α-tocopherol		

Figure 1. Structures of the studied low molecular weight phenolic compounds. In each table, the compounds are identified by arab numbers on the first column.

catalyze the production of hydroxyl radicals through the Fenton reaction (*17*, *18*) and lipid radicals through the decomposition of preformed lipid hydroperoxides (*19*). As a matter of fact, the ability of simple phenols to reduce copper ions and their activity−structure relationships were also investigated.

MATERIALS AND METHODS

Chemicals. Ferric chloride, copper(II) sulfate, and DMPD were from Fluka (Fluka Chimica, Milano, Italy); 3-hydroxybenzoic acid, *p*-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid (gentisic acid), 4-hydroxy-3-methoxybenzoic acid (vanillic acid), 3,4,5-trihydroxybenzoic acid (gallic acid), 3,5-methoxy-4-hydroxybenzoic acid (syringic acid), cinnamic acid, *o*-coumaric acid, *p*-coumaric acid, 3,4-dihydroxycinnamic acid (caffeic acid), 4-hydroxy-3-methoxycinnamic acid (ferulic acid), 4-hydroxy-3,5-methoxycinnamic acid (sinapinic acid), 2-*p*-hydroxyphenyl-ethanol (tyrosol), *p*-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, 3-methoxy-4-hydroxyphenylacetic acid, ascorbic acid, α-tocopherol, BHT, trolox, and BC were from Sigma (St. Louis, MO). Oleuropein was from Extrasynthèse (Genay, France). Oleuropein aglycone and hydroxytyrosol were obtained according to the procedure reported in ref *20*. All chemicals used were of analytical grade.

Measurement of Antioxidant Activity by DMPD Method. The radical hydrogen donor ability of antioxidants was measured colorimetrically using the DMPD method (*20*, *21*). At acidic pH (0.1 M sodium acetate buffer, pH 5.25) in the presence of an oxidant solution of 0.1 mM $FeCl_3$, DMPD (1.0 mM) made a stable red radical cation

($DMPD^{\bullet+}$), which was a useful reagent to investigate the radical scavenging activity. One milliliter of this solution was put down in a 1 cm light path quartz cuvette, and its absorbance at 505 nm at 25 °C was measured; the optical density that was obtained represented the uninhibited signal, which remained constant up to 12 h at room temperature. Antioxidant activity was determined by adding different concentrations of antioxidant in ethanol (from 10 to 60 μL) to 1 mL of the above $DMPD^{\bullet+}$ solution; absorbance at 505 nm after 15 min at 25 °C was revealed by a double beam Cary 1E thermostated spectrophotometer (Varian, Victoria, Australia) equipped with a Peltier temperature control.

The above reaction mechanism consisted of abstracting a hydrogen atom from a phenol donor to give the $DMPD^{+}$ and a phenoxyl radical. The reaction involved a color change producing a bleaching of the solution proportional to the antioxidant amounts (*21*). This reaction was fast, and its stable end point was taken as a measure of antioxidant activity. Ethanol in water for the dosage did not exceed 6.0%, and in these experimental conditions, the absorbance of the uninhibited radical cation solution did not change.

The antioxidant efficiency (AE) was calculated for each sample by considering the absorbance at 505 nm as a percentage of uninhibited radical cation solution absorbance, according to eq 1:

$$\mathrm{AE}\ (\%) = [1 - (A_{\mathrm{AO}}/A_0)] \times 100$$

where A_0 is the uninhibited radical cation absorbance and A_{AO} is the absorbance measured 15 min after the addition of antioxidant samples.

The concentration of antioxidant necessary to bleach the red radical cation by 50% (AE_{50}) was calculated by eq 2:

$$1/\mathrm{AE}\ (\%) = a + S_{\mathrm{AOI}} \times 1/\mu\mathrm{g}$$

where a and S_{AOI} are the intercept and the slope of the linear plot of 1/AE (%) values vs 1/μg of antioxidants in the reaction mixture, respectively. For each phenol, the radical scavenging activity (EC_{50}), which is the ratio of the AE_{50} value to the initial DMPD concentration, was determined. The EC_{50} describes the global activity of a compound toward DMPD, and to obtain more information on the antioxidant activity, we also made use of the inverse antioxidant power (AP_I) parameter calculated as $AP_I = S_{AOI}$ that described the antioxidant activity in function of the antioxidant concentration. So, when the EC_{50} and AP_I values were lower, the antioxidant capacity was greater.

Measurement of Cu(II) Reduction. Cu(I) formation was monitored using the specific Cu(I) chelator BC. On chelation, a characteristic increase in A_{480} was seen (*22*). Cu(II) (40 μM final concentration) was added to 400 μM BC in PBF in absence or in the presence of various amounts of antioxidant (from 5 to 50 μL), and the A_{480} was monitored as a function of time against the appropriate reference. Cu(I) was quantified using the absorption coefficient $\epsilon_{480} = 12\,540 \pm 500\ \mathrm{M^{-1}\,cm^{-1}}$ (*23*). Ethanol for the dosages did not exceed 5.0%, not influencing the absorbance of the Cu(I) chelate.

Copper Chelating Capacity. The metal chelating capability of the tested compounds was measured by $CuSO_4$. Solutions in PBF (10 mM, pH 7.4) containing 50 μM of the tested compounds were prepared in a quartz cuvette (1.0 mL final volume). The absorption spectra were recorded in the range of 200−800 nm at 120 nm/min with a spectral acquisition spacing of 0.1 nm. All of the compounds were soluble under these conditions. Scans with 25.0, 50.0, 75.0, and 100.0 μM $CuSO_4$ were taken after 5 s and 30 min and compared to the tested compound alone. A 2.5-fold EDTA concentration was added in order to verify the chelation reversibility (*16*, *24*).

RESULTS

Measurement of Antioxidant Activities. The obtained results are given in **Table 1**. Making reference to natural and synthetic antioxidants such as BHT and α-tocopherol, they were not tested by DMPD for their poor solubility in aqueous solution whereas ascorbic acid and trolox resulted in good radical scavengers.

Table 1. Radical Scavenging Activity (EC_{50}) and Inverse Antioxidant Power (AP_I) of the Tested Phenols Determined by the DMPD Method[a]

compd	AP_I	EC_{50}
1	90.6 ± 0.5	80.8 ± 12.2
2[b]	ND	at 13.8 mg/mL AE (%) = 14.7
3[c]	1.25 ± 0.02	$EC_{28} = 4.29 \pm 1.12$
4	0.059 ± 0.002	$(5.71 \pm 1.65) \times 10^{-2}$
5	12.8 ± 0.1	42.8 ± 4.9
6	0.0426 ± 0.0006	$(5.51 \pm 0.68) \times 10^{-2}$
7	0.124 ± 0.002	$(1.09 \pm 0.18) \times 10^{-1}$
8[b]	ND	at 0.44 mg/mL AE (%) = 0
9	7.59 ± 0.080	9.93 ± 1.40
10	1.46 ± 0.03	1.13 ± 0.15
11	0.0210 ± 0.0002	$(1.95 \pm 0.30) \times 10^{-2}$
12	0.0376 ± 0.0003	$(2.00 \pm 0.09) \times 10^{-2}$
13	0.0316 ± 0.0008	$(1.16 \pm 0.12) \times 10^{-2}$
14[b]	ND	at 13.8 mg/mL AE (%) = 0
15	0.286 ± 0.002	1.56 ± 0.16
16[c]	842.8 ± 8.8	$EC_{30} = (4.35 \pm 1.34) \times 10^2$
17	0.0443 ± 0.0006	$(3.44 \pm 0.32) \times 10^{-2}$
18[b]	ND	at 8.2 mg/mL AE (%) = 22.8
19	0.337 ± 0.005	$(1.04 \pm 0.09) \times 10^{-1}$
20	0.574 ± 0.006	$(1.37 \pm 0.16) \times 10^{-1}$
21	0.0575 ± 0.0011	$(2.99 \pm 0.30) \times 10^{-2}$
22	0.139 ± 0.004	$(6.61 \pm 0.87) \times 10^{-2}$

[a] Data are expressed as means ± standard deviation of at least four independent experiments. [b] The AP_I and EC_{50} values cannot be determined due to the low phenol solubility, and the calculated AE (%) value is referred to as the maximum phenol concentration soluble in the tested conditions. [c] The EC_{50} cannot be determined due to the short range of phenol solubility.

The investigated phenols were grouped in series (**Figure 1**), and marked differences in each phenol series were observed. In each series, the monosubstituted phenols (compounds **1**, **2**, **9**, **10**, **14**, and **16**) exhibited a very poor radical scavenging activity; on the contrary, a higher number of substituents (compounds **6**, **7**, **11**, **13**, **15**, and **17**), and in particular the 3,4- and the 2,5-dihydroxyl substitutions (compounds **3**, **4**, **11**, **15**, and **17**), increased the radical scavenging activity (**Table 1**). Nevertheless, the *o*-methylation of the 3-hydroxyl group caused a significant decrease of the antioxidant capacity in 3,4-substituted benzoic and phenylacetic acids (compounds **5** and **18**) whereas a slight decrease in the cinnamic acids series (compounds **12** and **13**) is observed. As concerns the substituent in the 1-position, a significant difference among the cinnamic acids **8**−**13**, which present a conjugated diene due to the monounsaturated alkylic group, in comparison to the other phenol series, must be emphasized. Only slight differences occurred on EC_{50} and AP_I between the compound **19** and its derivative **20**.

Measurement of Cu(II) Reduction. Aiming to test the ability of the different phenols series to reduce Cu(II), experiments were carried out according to eq 3.

$$\mathrm{Ph_{red}} + \mathrm{Cu(II)} \rightarrow \mathrm{Ph_{ox}} + \mathrm{Cu(I)}$$

The copper reduction was measured over periods of 3 and 30 min. **Table 2** shows the results of the production of cuprous ion by reaction between phenols and cupric ion in aqueous solution.

In benzoic and phenylacetic acids series, the increasing number of hydroxyls on the aromatic ring (compounds **6** and **17**) and the 2,5-dihydroxyl substitution (compound **4**) are important determinants for high Cu(II) reduction potency (**Table 2**), as supported by the evidence that the monohydroxyl phenols

Table 2. Reduction of Cu(II) by Phenols[a]

	Cu(II)/phenol molar ratio of						
	Cu(I) (μM) after 3 min of incubation			Cu(I) (μM) after 30 min of incubation			
compd	8:1	4:1	2:1	8:1	4:1	2:1	Cu(I)/phenol[b]
1	0.18 ± 0.12	0.36 ± 0.10	0.43 ± 0.10	0.82 ± 0.18	0.73 ± 0.18	0.69 ± 0.19	0.3 ± 0.2
2	0.23 ± 0.12	0.25 ± 0.11	0.46 ± 0.10	0.67 ± 0.19	0.50 ± 0.20	1.01 ± 0.16	0.1 ± 0.0
3	7.99 ± 0.50	18.4 ± 1.33	34.30 ± 2.60	14.81 ± 0.94	33.23 ± 2.41	33.04 ± 2.39	3.0 ± 0.5
4	20.47 ± 1.50	35.54 ± 2.70	35.54 ± 2.70	19.93 ± 1.35	32.13 ± 2.32	33.11 ± 2.40	3.4 ± 0.8
5	9.88 ± 0.65	20.28 ± 1.48	31.84 ± 2.40	11.51 ± 0.68	22.92 ± 1.58	32.68 ± 2.36	3.0 ± 0.2
6	22.34 ± 1.65	35.74 ± 2.72	35.81 ± 2.72	23.86 ± 1.66	33.76 ± 2.45	34.12 ± 2.49	5.4 ± 1.3
7	9.48 ± 0.62	20.43 ± 1.50	34.73 ± 2.64	10.13 ± 0.56	20.57 ± 1.40	32.90 ± 2.28	2.0 ± 0.2
8						0.60 ± 0.19	
9	5.25 ± 0.28	10.40 ± 0.70	17.44 ± 1.26	9.44 ± 0.51	16.41 ± 1.07	25.41 ± 1.78	1.4 ± 0.7
10	7.14 ± 0.43	13.56 ± 0.95	22.82 ± 1.68	9.54 ± 0.52	18.16 ± 1.21	28.26 ± 2.01	2.0 ± 0.2
11	5.06 ± 0.27	12.10 ± 0.83	28.26 ± 2.12	7.66 ± 0.37	18.73 ± 1.25	32.79 ± 2.36	1.7 ± 0.3
12	11.43 ± 0.78	24.21 ± 1.80	34.30 ± 2.6	12.80 ± 0.78	26.80 ± 1.89	33.16 ± 2.40	2.4 ± 0.4
13	14.64 ± 1.03	28.72 ± 2.16	36.34 ± 2.76	19.20 ± 1.29	32.81 ± 2.87	34.10 ± 2.48	3.7 ± 1.3
14	6.12 ± 0.35	11.72 ± 0.79	12.83 ± 0.89	9.7 ± 0.53	18.18 ± 1.21	16.52 ± 1.08	2.6 ± 0.4
15	4.17 ± 0.20	10.09 ± 0.67	14.05 ± 0.99	7.43 ± 0.35	17.72 ± 1.17	25.09 ± 1.76	2.4 ± 0.3
16	3.99 ± 0.18	7.71 ± 0.48	14.13 ± 0.99	6.25 ± 0.26	12.26 ± 0.74	21.92 ± 1.51	1.8 ± 0.2
17	18.54 ± 1.34	34.25 ± 2.60	34.92 ± 2.65	27.06 ± 1.92	33.74 ± 2.45	33.90 ± 2.46	4.2 ± 1.6
18	11.64 ± 0.79	22.81 ± 1.68	34.37 ± 2.61	13.63 ± 0.84	25.30 ± 1.77	32.78 ± 2.37	4.1 ± 0.4
19	9.85 ± 0.65	18.89 ± 1.45	34.93 ± 2.65	18.27 ± 1.21	32.80 ± 2.37	33.15 ± 2.40	3.9 ± 0.6
20	4.78 ± 0.25	10.43 ± 0.70	22.87 ± 1.69	9.66 ± 0.53	19.90 ± 1.34	33.99 ± 2.47	2.2 ± 0.1
21	4.52 ± 0.23	7.85 ± 0.49	14.12 ± 0.99	5.89 ± 0.23	10.52 ± 0.60	18.91 ± 1.26	1.0 ± 0.2
22	9.36 ± 0.61	23.25 ± 1.72	34.67 ± 2.63	9.10 ± 0.48	22.62 ± 1.56	32.72 ± 2.37	3.0 ± 0.2
23	5.73 ± 0.32	13.92 ± 0.98	33.77 ± 2.56	10.48 ± 0.59	22.96 ± 1.59	33.83 ± 2.77	2.8 ± 0.4
24	3.2 ± 1.12	7.20 ± 0.44	34.62 ± 2.63	4.88 ± 1.15	8.98 ± 0.47	33.14 ± 2.40	3.8 ± 0.3

[a] Data are reported as Cu(I) (μM) concentrations calculated after 3 and 30 min of incubation at different Cu(II)/phenol molar ratios. The initial concentration of Cu(II) was 40.0 μM. Results are expressed as means ($n = 4$) ± standard deviation. [b] Data are calculated after 30 min of incubation and expressed as Cu(I)/phenol molar ratios.

1, **2**, and **16** show the lower activity whereas a slight difference is observed between mono- and dihydroxyl-substituted cinnamic acids (compounds **9**−**11**). Contrary to that expected, dihydroxyphenylethanol (**15**) reveals a lower reducing activity than hydroxyphenylethanol (**14**).

The *o*-methylation of the hydroxyl group gives rise to different effects on Cu(II) reduction. Small differences between 3,4-substituted benzoic acids (compounds **3** and **5**) are noted whereas *o*-methylated cinnamic acids show an increased reducing capacity in the function of the *o*-methylation degree (compounds **11**−**13**). An opposite effect on the Cu(II) reduction is observed for phenylacetic (compounds **17** and **18**) and trisubstituted benzoic acids (compounds **6** and **7**). As concerns the substituent in the 1-position, phenylethanols (compounds **14** and **15**) and nonmethylated cinnamic acids (compounds **9** and **10**) show the lowest reducing capacity.

According to this test, ascorbic acid is a relatively poor reductant as compared to some tested phenols (**Table 2**). For the other reference antioxidants (compounds **22**−**24**), this capacity is strictly linked to their concentration; in fact, only at a 2:1 copper-to-phenol ratio do they quantitatively reduce the Cu(II) ion.

Copper Chelating Capacity. The direct interaction of the tested compounds with copper ion at pH 7.4 was assessed by UV/vis spectroscopy; the effect of stepwise increments of $CuSO_4$ concentration on the spectral characteristics of each compound is analyzed in terms of λ_{max} shift. The peak's positions expressed as λ_{max} in the phenol spectra in the absence and in the presence of different $CuSO_4$ concentrations are shown in **Table 3**.

The tested compounds exhibited characteristic bands in the range of 200−350 nm. The peak's positions in the spectra of compounds **2**, **5**, **7**−**10**, and **12** did not change by the addition of copper ions at different concentrations even after 30 min of incubation (**Table 3**), with an indication that these compounds did not form complexes with copper ions in the observed conditions.

The spectra of remaining compounds have shown differences in the presence of $CuSO_4$. In particular, the interactions of copper ions with compound **3** at different Cu(II)/phenol molar ratios produced a bathochromic shift in the band at 288 nm of about 20 nm while a hypsochromic shift (14 nm) in the 250 nm band is observed. On the addition of EDTA at 2.5-fold Cu(II) concentration, the original spectrum of compound **3** was recovered even after 30 min.

The bands at 259 and 212 nm of compound **4** showed bathochromic shifts of about 40 and 21 nm, respectively, but a residual band at 212 nm with a lower absorbance at 0.5:1 Cu(II)/phenol molar ratio was observed. On treatment with EDTA, the spectra returned to their original positions but the band at 212 nm had a lower absorbance. After 30 min in the presence of 2:1 Cu(II)/phenol molar ratio, very broad bands were noted and the addition of EDTA produced only a broad band at 263 nm. The compound **6** spectrum has displayed a similar behavior in the presence of copper ions (**Table 3**), but we did not note a residual band at lowest the Cu(II) concentration.

In the presence of caffeic acid (compound **11**), copper ions caused red shifts in the observed three bands at 311, 286, and 216 nm of about 32−36, 19, and 47 nm, respectively, with a residual broad band with lower absorbance persisting at 216−214 nm. EDTA addition regenerates the original spectra, but the absorbancies were lower; in particular, broad bands were noted for the sample incubated for 30 min in the presence of Cu(II). Finally, only small differences in the spectra of the other phenols (compounds **1** and **13**−**20**) in the presence of copper ions have been evidenced and indicated in the footnotes of **Table 3**.

Considering the reference antioxidants after the Cu(II) addition, spectra with very broad bands (compound **24**) and quite linear bands (compound **21**) appeared, while a precipitate is observed in the presence of BHT (compound **23**). A band shift of about 16 nm appears for compound **22** after 30 min in the presence of copper ion also after the EDTA addition.

Table 3. Peak's Positions, Expressed as λ_{max}, in the Phenol Spectra in the Absence and the Presence of Different Concentrations of Cu(II) after 5 s and 30 min of Incubation

	peak position (λ_{max} in nm)						
		Cu(II)/phenol molar ratio of					
	control	0.5:1 ($t = 5$ s)	1:1 ($t = 5$ s)	1.5:1 ($t = 5$ s)	2:1 ($t = 5$ s)	2:1 ($t = 30$ min)	with EDTA[a]
1[b]	287	288	288	288	288	290	288
2[c]	246	246	246	246	246	246	246
3	288, 250	307, 236	307, 236	308, 236	306, 236	307, 239	288, 250
4	259, 212	295, 232, 214	299, 233	299, 233	300, 232	broad bands	259[d], 212[d]
5	285, 251, 207	285, 251, 207	285, 251, 206	285, 251, 207	285, 251, 207	285, 251, 207	285, 251, 207
6	258, 211	296, 233	300, 233	300, 233	300, 233	broad bands	258[d], 211[d]
7[e]	261, 210	261, 210	261, 210	261, 210	261, 210	261, 210	261, 210
8	269	269	269	269	268	268	269
9	312, 268, 213	312, 269, 213	312, 269, 212	312, 270, 313	312, 270, 314	312, 270, 313	312, 269, 313
10[b]	285	285	285	285	285	285	285
11[f]	311, 286, 216	343, 304, 263, 214	347, 304, 263, 216	345, 305, 263, 217	346, 305, 263, 216	broad bands	311, 286, 214
12	310, 286, 215	310, 286, 215	310, 286, 215	310, 286, 215	310, 286, 215	310, 286, 215	310, 286, 215
13[g]	306, 229	306, 228	306, 229	306, 229	306, 227	333, 238	335[h], 239[h]
14[b]	276	276	276	276	276	277	276
15[i]	280	282	282	283	282	broad band	280
16[c]	277, 224	277, 224	276, 224	274, 224	275, 224	276, 224	277, 224
17[i]	281	282	283	283	282	282	281, 280[h]
18	280	280	280	280	280	278	280
19	280, 231	281, 232	282, 233	282, 232	281, 232	279, 233	280[j], 231[j]
20[k]	280, 229	281, 230	281, 229	281, 229	282, 230	280, 230	280, 230
21[l]	266	277 broad band	-	-	-	-	-
22	289	289	290	290	289	273	274[h,m]
23	278	279[n]					278[k]
24	299	299 broad band	294 broad band	292 broad band	292 broad band	233 broad band	236[m,h]

[a] The EDTA concentration was 2.5-fold Cu(II), and the values are referred to as the EDTA addition to the sample at $t = 5$ s. [b] After the Cu(II) addition, the Abs values were lower and remained lower after EDTA addition. [c] After Cu(II) addition, the Abs values were higher and remained higher after EDTA addition. [d] For the samples at $t = 30$, after EDTA addition, a very broad band at 263 nm was revealed. [e] The peak at 210 nm has a lower Abs in the presence of Cu(II). [f] EDTA addition regenerates the original spectrum with lower Abs values for the samples at $t = 5$ s, while broad bands were observed after 30 min. [g] For the samples at $t = 5$ s, the EDTA addition produced a slight bathocromic shift in the 306 nm band of about 1.5–2 nm. [h] The value is referred to EDTA addition to the sample incubated for 30 min in the presence of Cu(II). [i] After Cu(II) addition at about 305 nm appeared a small shoulder, which disappeared after the EDTA addition. [j] For the samples at $t = 30$ min, the EDTA addition produced a slight bathocromic shift in the two bands (282 and 231 nm). [k] After Cu(II) addition, the Abs values were higher and slightly decreased after EDTA addition. [l] After Cu(II) addition, linear spectra appeared. [m] For the samples at $t = 5$ s, the EDTA addition produced a slight bathocromic shift of about 1.5–2 nm. [n] A precipitate was observed in the presence of higher Cu(II) concentrations.

DISCUSSION

Phenolic compounds can be active as antioxidants by a number of potential pathways. The most important is likely to be by free radical scavenging in which the phenol can break the free radical chain reaction. The presence of different substituents in the phenol backbone structures modulates their antioxidant property, in particular their hydrogen-donating capacity. The DMPD test provides information on this capacity of the studied compounds. The results illustrate that in each investigated series the antioxidant activity increases with the number of hydroxyls on the aromatic ring (**Table 1**). Radical scavenging occurs by reducing the active oxygen species and/or lipid peroxyl radicals by means of hydrogen atom donation from the free hydroxyls, giving rise to phenoxyl radicals (*25*). Furthermore, as demonstrated for flavonoids (*26*), the *ortho*-dihydroxyl structure (catechol) and a 2′,3′-double bond in conjugation with a 4′-carboxyl function enhance the radical scavenging capacity. As a consequence, cinnamic acid derivatives **9**−**13** are good hydrogen donors (**Table 1**).

As concerns the presence of methoxyl groups, our data suggest that the methylation of the 3- and 5-hydroxyl group decreases the antioxidant activity of phenolic compounds. The AP_1 values calculated for compounds **5**, **7**, and **18** were larger then those of the corresponding di- and trihydroxylated phenols **3**, **6**, and **17**, respectively, whereas only a slight increase is observed between the antioxidant capacities of caffeic acid **11** and its 3-methoxyl derivative compound **12**. Furthermore, in aqueous solution, the electron-donating properties of the methoxyl substituents in the ortho-position, with respect to a hydroxyl group on the aromatic ring, confer more effective antioxidant activity if compared to that of monohydroxylated phenols. The antioxidant capacities of these compounds is as follows: **7** > **5** ≫ **2** in benzoic acids series and **13** ≈ **12** ≫ **10** in cinnamic acids series (**Table 1**).

Molecules with *ortho*-dihydroxyl and/or *para*-hydroxyl functionalities are characterized by high antioxidant activity (*27*), but it is necessary to underline that the electronic and steric effects of substituents near the phenolic hydroxyl group may be of importance in governing the hydrogen-donating capacity of monohydroxyl phenols. Electron-donating substituents in the ortho-position tend to weaken the O−H bond of phenol and provide extra stability to the phenoxyl radical. Furthermore, replacement of the −OH substituent by $-OCH_3$ increased the liposolubility of phenols (*28*); therefore, these compounds could act as lipid soluble free radical scavengers.

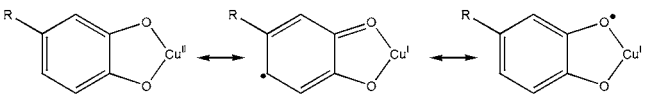
Interestingly, the DMPD assays show that a large number of radical cations per molecule of phenol was reduced. It seems that for some phenols the number of electrons involved in their oxidation is higher than that expected from the number of hydroxyl groups. On this matter, it has been suggested that phenolic compounds, when they are oxidized, generally undergo polymerization reactions, which can reproduce oxidable −OH moieties in phenol polymeric products explaining the higher radical scavenging activities of the studied phenols (*29*).

Another possible mechanism for the antioxidative activity of phenolic compounds is the chelation of the metal ions in

oxidation systems using a transition metal ion such as copper or iron. Transition metals are strongly implicated in the production of highly reactive hydroxyl radicals by the superoxide driven Fenton reaction as well as in the direct reductive decomposition of lipid hydroperoxides to provide alkoxyl and lipid peroxyl radicals as propagation radicals (*30*). Thus, metal chelation by phenols could be considered as prevention means of the lipid peroxidation either by sequestering metal ions into inert complexes unable to decompose H_2O_2 or by restricting the access of metal ions toward lipid hydroperoxides, which are consistently produced in living cells (*31*, *32*).

The protonated phenolic group is not a particularly good ligand for metal cations, but once deprotonated, an oxygen center is generated that possesses a high charge density, a so-called "hard" ligand. Although the pK_a value of most phenols is in the region of 9.0−10.0, in the presence of suitable cations such as Fe(III) or Cu(II), the proton is displaced at much lower pH values (5.0−8.0). Thus, metal chelation by phenolic compounds can occur at physiological pH (*33*).

For chelation, bidentate ligands behave as much more powerful metal cations scavengers than monodentate ligands; as a consequence, phenolic compounds with a catechol structure bind Fe(III) tightly at pH 7.0, whereas phenol does not (*33*). Our results on the metal chelating ability at physiological pH of some phenols confirm this hypothesis. On the basis of spectrophotometric studies in the presence of copper ions (**Table 3**), the formation of metal−phenol chelates is followed by the appearance of new peaks only in the spectra of phenols containing the *o*-3,4-dihydroxy structure (compounds **3**, **6**, **11**, **15**, and **17**), but only for compound **3**, the formation of a chelate was reversible even after 30 min. Following up the addition of Cu(II) ions, spectral changes occur on copper ion binding to the above-mentioned compounds, although the peaks shift is much less pronounced for compounds **15** and **17** than for **6** and **11** (**Table 3**). After 30 min in the presence of copper ions, by titrating out the bound ions, new spectra appear, showing that these compounds have not reverted to their original forms before Cu(II) ion interaction, being oxidized (*16*). Our data on the Cu(II) reduction point out that these compounds with a catechol structure are susceptible to oxidation (**Table 2**); nevertheless, we observe a reversible formation of a chelate after few seconds of incubation in the presence of Cu(II) ion (**Table 3**). Metal−catechol complexes are able to undergo intramolecular electron transfer reactions according to eq 4.



The equilibrium positions of such reactions are influenced by the presence of other ligands, and catechol complexes of copper show such ligand dependence (*33*). We suppose that in the presence of a specific Cu(I) chelator, such as BC, the Cu(II) ions are fastly reduced, as demonstrated by the high concentration of produced Cu(I) ions (see **Table 2**). Otherwise, in absence of a specific Cu(I) ligand, Cu(II)−phenol complexes with different stabilities can be observed (see **Table 3**).

The reduction studies also show that monohydroxyl and mono- and bimethoxyl phenols are able to reduce Cu(II) ion but their spectra displayed only small modifications after the addition of copper ions at physiological pH. Also, in these cases, the presence of a specific Cu(I) chelator may promote the Cu(II) reduction reaction. The data on the chelating and reducing capacities of the reference antioxidants reinforce this hypothesis. As matter of fact, the absorption spectra of compounds **21**−**24**, which do not chelate but reduce copper ion, are irreversibly modified by Cu(II) ion addiction.

Considering that the number of reduced copper ions per molecule of phenol (**Table 2**) is lower than that calculated for some flavonols and isoflavones (*34*), phenols could exert lower prooxidant effects on promoting Fenton or Haber−Weiss reactions. Cuprous ion is more reactive toward hydroperoxide than the cupric ion; hence, a reductant, which reduces Cu(II) to Cu(I), may well act as a prooxidant (*24*).

CONCLUSION

Oxidative stress is involved in the pathology of cancer, atherosclerosis, malaria, and rheumatoid arthritis and could play a role in neurodegenerative diseases and aging processes (*10*−*13*). On the other hand, the protection by fruits and vegetables against several diseases has been attributed to various antioxidants and vitamins. Dietary phenolic compounds have generally been considered as nonnutrients, and their possible benefit to human health has only recently been considered.

In this work, the capacity to be active in vitro, as antioxidants, of simple phenolic acids and other phenolic compounds usually found in olive fruit and in virgin olive oil has been investigated. In particular, the radical scavenging activity of some phenols is higher than that of the most used antioxidant ascorbic acid and trolox. Compounds **7**, **10**, **12**, **13**, **19**, and **20** behave as powerful scavengers of stable free radical cation $DMPD^{\bullet+}$. Furthermore, they do not chelate copper ion, suggesting that they may exert their antioxidant activity primarily by scavenging free radical differently from phenols with a 3,4- or 2,5-dihydroxyl substituents, which are also able to chelate copper ions.

As concerns the prooxidant activity, our studies show that the reducing capacity of simple phenols is connected to the presence of a specific ligand of the reduced ion. This statement may be important in vivo when free transition metal ions are involved in oxidation processes. In the healthy human body, metal ions appear largely sequestered in forms that are unable to catalyze free radical reaction (*35*). However, injury to tissues may release iron or copper (*36*) and catalytic metal ions have been measured in atherosclerotic lesions (*37*). On this matter, further investigations into the antioxidant and prooxidant activities by biophenols present in the Mediterranean diet as well as into their absorption and bioavailability are required.

ABBREVIATIONS

DMPD, *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride; BHT, 2,6-di-*tert*-butyl-4-methylphenol; trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; BC, bathocuproine disulfonate.

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Received for review May 7, 2003. Revised manuscript received September 12, 2003. Accepted September 16, 2003.

JF034471R