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Lipophilic Hydroxytyrosyl Esters. Antioxidant Activity in Lipid Matrices and Biological Systems

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Antioxidant activities of lipophilic hydroxytyrosyl acetate, palmitate, oleate, and linoleate were compared with those of hydroxytyrosol, α-tocopherol, and butylhydroxytoluene (BHT) in both glyceridic matrix and biological systems. Aliquots of a glyceridic matrix spiked with various concentrations of antioxidant were subjected to accelerated oxidation in a Rancimat apparatus operated at 90 °C. The relationships between induction time (IT) and antioxidant concentration (mmol/kg) presented by hydroxytyrosol and hydroxytyrosyl acetate, palmitate, oleate, and linoleate were similar. Hydroxytyrosol and its esters showed greater antioxidant activity than α-tocopherol or BHT. We also evaluated the capacity of hydroxytyrosyl esters to protect proteins and lipids against oxidation caused by peroxyl radicals, using a brain homogenate as an ex vivo model. All tested compounds showed a protective effect in these systems, which was greater in preventing the generation of carbonyl groups in protein than of malondialdehyde in lipid. Inclusion of a lipophilic chain in the hydroxytyrosol molecule enhanced its antioxidant capacities in this biological model.

KEYWORDS: Hydroxytyrosol; hydroxytyrosyl acetate; hydroxytyrosyl fatty esters; antioxidant; NMR; Rancimat; immunoblot analysis; malondialdehyde; lipid peroxidation; protein oxidation

INTRODUCTION

Various epidemiological studies have attributed a lower incidence of degenerative pathologies, including coronary heart disease and cancer, to the Mediterranean diet, which is largely vegetarian in nature and includes the consumption of large quantities of olive oil (*1*−*8*). Converging evidence indicates that the beneficial effects of olive oil are related not only to the high oleic acid content but also to the high level of phenolic compounds absent in seed oils (*9*−*11*).

Hydroxytyrosol and tyrosol and their secoiridoid derivatives are the main components of the phenolic fraction of virgin olive oil (VOO) which have been shown to delay LDL (low-density lipoprotein) oxidation (*12*−*14*). Other biological properties of the phenolic components of olive oil include antimicrobial (*15*, *16*), hypotensive, hypoglycemic, and platelet antiaggregation (*17*), and antiinflammatory (*18*) activities, inhibition of several lipoxygenases (*19*, *20*), and apoptosis induction in HL-60 cells (*21*). The antioxidant activity of hydroxytyrosol and its derivatives has already been well established (*22*−*24*). The presence of these natural antioxidants in virgin olive oil prevents its oxidative deterioration; consequently, addition of other antioxidants is not permitted.

Synthetic phenolic antioxidants are added to other fats and oils, and to other foods, to prevent the decrease in nutritional quality and safety caused by the appearance of nondesirable flavors and colors and/or the formation of other compounds from oxidation of lipids (*25*), some of which may be potentially toxic.

The growing interest in the substitution of synthetic food antioxidants by natural ones (*26*) has fostered research on vegetable sources and on the screening of raw materials for identifying new antioxidants (*27*). The solubility of natural antioxidants in aqueous media is good, but such hydrophilic nature might be a constraint for practical applications in hydrophobic/lipid media. With the aim of extending the catalog of hydrophobic antioxidants, syntheses of several new lipophilic antioxidants, such as poly(lauroyl-(+)-catechin)s (*28*), lipophilic ascorbic acid analogues (*29*), and lipophilic clovamide derivatives (*30*), among others, have recently been published.

Secoiridoid hydroxytyrosyl derivatives, with antioxidant activity similar to that of free hydroxytyrosol (*31*) but of more

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Table 1. ^{1}H NMR Data (500.13 MHz, DMSO-d_6, 303 K) for Compounds **1**–**8**[a]

	1	2	3	4	5	6	7	8
				Phenethyl Unit				
1′	3.49 (td) ($J_{1,2}$ = 7.2) (J_{1OH} = 5.2)	4.10 (t) ($J_{1,2}$ = 7.1)	4.10 (t) ($J_{1,2}$ = 7.0)	4.10 (t) ($J_{1,2}$ = 7.0)	4.10 (t) ($J_{1,2}$ = 7.0)	4.10 (t) ($J_{1,2}$ = 7.0)	4.10 (t) ($J_{1,2}$ = 7.0)	4.10 (t) ($J_{1,2}$ = 7.0)
2′	2.52 (t)	2.68 (t)	2.67 (t)	2.67 (t)	2.67 (t)	2.67 (t)	2.67 (t)	2.67(t)
4′	6.57 (d) ($J_{4,8}$ = 2.0)	6.60 (d) ($J_{4,8}$ = 2.1)	6.59 (d) ($J_{4,8}$ = 2.1)	6.59 (d) ($J_{4,5}$ = 2.1)	6.59 (d) ($J_{4,8}$ = 2.1)	6.59 (d) ($J_{4,5}$ = 2.1)	6.59 (d) ($J_{4,5}$ = 2.1)	6.59 (d) ($J_{4,5}$ = 2.0)
7′	6.60 (d) ($J_{7,8}$ = 8.0)	6.63 (d) ($J_{7,8}$ = 8.0)	6.62 (d) ($J_{7,8}$ = 7.9)	6.62 (d) ($J_{7,8}$ = 8.0)	6.62 (d) ($J_{7,8}$ = 7.9)	6.61 (d) ($J_{7,8}$ = 8.0)	6.62 (d) ($J_{7,8}$ = 8.0)	6.62 (d) ($J_{7,8}$ = 8.0)
8′	6.42 (dd)	6.45 (dd)	6.45 (dd)	6.44 (dd)	6.44 (dd)	6.44 (dd)	6.44 (dd)	6.44 (dd)
				Acyl Chain				
2		1.97 (s)	2.23 (t)	2.23 (t)	2.23 (t)	2.23 (t)	2.23 (t)	2.23 (t)
3			1.51 (m) (^3J = 7.3)	1.47 (m) (^3J = 7.3)	1.47 (m) (^3J = 7.4)	1.47 (m) (^3J = 7.3)	1.47 (m) (^3J = 7.3)	1.47 (m) (^3J = 7.3)
3			1.51 (m)	1.47 (m)	1.47 (m)	1.47 (m)	1.47 (m)	1.47 (m)
3			1.51 (m) (^3J = 7.3)	1.47 (m)	1.47 (m)	1.47 (m)	1.47 (m)	1.47 (m)
4			0.84(t) (^3J = 7.3)	1.22 (m)	1.22 (m)	1.22 (m)	1.22 (m)	1.22 (m)
5				1.22 (m)	1.22 (m)	1.22 (m)	1.22 (m)	1.22 (m)
6				1.22 (m)	1.22 (m)	1.22 (m)	1.22 (m)	1.22 (m)
7				1.22 (m)	1.22 (m)	1.22 (m)	1.25 (m)	1.25 (m)
8				1.22 (m)	1.22 (m)	1.22 (m)	1.97 (q) (^3J = 6.4)	2.00 (q) (^3J = 6.9)
9				1.22 (m)	1.22 (m)	1.22 (m)	5.31 (m)	5.31 (m)
10				1.22 (m)	1.22 (m)	1.22 (m)	5.31 (m)	5.31 (m)
11				1.22 (m)	1.22 (m)	1.22 (m)	1.97 (q) (^3J = 6.4)	2.72(t) (^3J = 5.5)
12				0.84(t) (^3J = 7.0)	1.22 (m)	1.22 (m)	1.25(m)	5.31(m)
13					1.22 (m)	1.22 (m)	1.22 (m)	5.31(m)
14					1.22 (m)	1.22 (m)	1.22 (m)	2.00 (m)
15					1.22 (m)	1.22 (m)	1.22 (m)	1.25 (m)
16					0.84(t) (^3J = 7.0)	1.22 (m)	1.22 (m)	1.22 (m)
17						1.22 (m)	1.22 (m)	1.22 (m)
18						0.84(t) (^3J = 7.0)	0.84(t) (^3J = 7.0)	0.84(t) (^3J = 7.0)

[a] Chemical shifts (δ, ppm) and coupling constants (J, Hz).

hydrophobic character, are well-known to be present in olive oils; however, they have a bitter taste (*32*, *33*) and their isolation from olive oils is very difficult, so therefore, they are not profitable. Hydroxytyrosyl acetate (*34*−*36*), another phenolic compound identified in VOO, presents antioxidant capacity similar to that of free hydroxytyrosol (*23*, *24*), it has no bitter taste, and it is more soluble in lipophilic phases due to the presence of the ester group. Synthesis of hydroxytyrosyl acetate has already been described (*23*, *37*), but the procedures are laborious and require special manipulation at critical steps of the reaction, and/or the yields obtained are very low.

Considering that esterification of polyphenols increases their hydrophobic nature, it is conceivable that aliphatic chains can be used as a tool to alter physical properties such as the solubility, miscibility, and activity of antioxidants in oil. In this regard, methods for the preparation of hydroxytyrosyl esters by reaction of hydroxytyrosol with various acylating agents have been described. The esterification with free acids (*38*) and transesterification with methyl or ethyl esters (*39*) are chemoselective reactions yielding the products in good yields. However, the selectivity of the reaction with acyl chlorides depends on the operating conditions and type of catalyst (*40*).

In the present work, the antioxidant activity of hydroxytyrosyl esters in lipid matrices is assessed by the Rancimat method. Their capacity to prevent protein and lipid oxidation in biological tissues is studied in brain homogenates. A comparison with natural (free hydroxytyrosol, α-tocopherol) and synthetic (butylhydroxytoluene, BHT) antioxidants is also presented.

MATERIALS AND METHODS

Materials. Cumene hydroperoxide (CH), α-tocopherol, BHT, and 2,4-dinitrophenylhydrazine (DNPH) were obtained from Sigma (Madrid, Spain). Electrophoresis reagents were from Bio-Rad Laboratories (Hercules, CA). Hydroxytyrosol was synthesized from 3,4-dihydroxyphenylacetic acid (Sigma) by reduction with $LiAlH_4$ (*37*). NMR spectra were recorded on a Bruker AMX-500 spectrophotometer operating at 500.13 MHz (^{1}H) and 125.75 MHz (^{13}C). Chemical shifts are given in parts per million with the residual solvent signals (2.49 ppm for ^{1}H and 39.5 ppm for ^{13}C) as references. Samples were dissolved (10 mg/mL) in hexadeuterated methyl sulfoxide (DMSO-d_6), and spectra were recorded at 303 K. Elemental analyses were made on a Leco CHNS-932 apparatus. High-resolution CI and FAB mass spectra were obtained on a Micromass AUTOSPECQ spectrometer.

Preparation of Hydroxytyrosyl Esters. Hydroxytyrosyl esters were obtained from hydroxytyrosol (**1**) by a procedure under patent (*39*). Briefly, to a solution of hydroxytyrosol (50 mg) in ethyl or methyl ester (acetate, butyrate, palmitate, stearate, oleate, or linoleate) (2 mL) was added *p*-toluenesulfonic acid (5 mg), and the mixture was stirred for 24 h. The resulting suspension was washed with a $NaHCO_3$-saturated solution, dried with Na_2SO_4, and purified by column chromatography to yield pure hydroxytyrosyl ester.

*Data for hydroxytyrosyl acetate (**2**)*: 86% yield; NMR data (see **Tables 1** and **2**); HRMS (CI) *m*/*z* calcd for $C_{10}H_{13}O_4$ $[M + H]^+$ 197.081384, found 197.080136 (6.3 ppm). Anal. Calcd for $C_{10}H_{12}O_4$: C, 76.59; H, 10.64. Found: C, 76.32; H, 10.91.

Table 2. ^{13}C NMR Chemical Shifts (ppm) (125.76 MHz, DMSO-d_6, 303 K) for Compounds **1**–**8**

	1	2[a]	3	4	5	6	7	8
					Phenethyl Unit			
1′	62.5	64.6	64.5	64.5	64.5	64.5	64.5	64.5
2′	38.4	33.7	33.7	33.7	33.7	33.7	33.7	33.7
3′	130.1	128.5	128.5	128.5	128.5	128.5	128.5	128.5
4′	116.2	116.1	116.1	116.1	116.1	116.1	116.1	116.1
5′	144.8	145.0	145.0	145.0	145.0	145.0	145.0	145.0
6′	143.2	143.7	143.7	143.7	143.7	143.7	143.7	143.7
7′	115.3	115.5	115.4	115.4	115.4	115.4	115.4	115.4
8′	119.3	119.4	119.4	119.4	119.4	119.4	119.4	119.4
					Acyl Chain			
1		170.2	172.6	172.8	172.8	172.8	172.7	172.7
2		20.6	35.3	33.5	33.5	33.5	33.5	33.5
3			17.8	24.4	24.4	24.4	24.4	24.4
4			13.3	29.0–28.3	29.0–28.3	29.0–28.3	28.5–28.3	28.5–28.3
5				29.0–28.3	29.0–28.3	29.0–28.3	28.5–28.3	28.5–28.3
6				29.0–28.3	29.0–28.3	29.0–28.3	28.5–28.3	28.5–28.3
7				29.0–28.3	29.0–28.3	29.0–28.3	28.7	28.7
8				29.0–28.3	29.0–28.3	29.0–28.3	26.5	26.5
9				29.0–28.3	29.0–28.3	29.0–28.3	129.6	129.7
10				31.2	29.0–28.3	29.0–28.3	129.6	127.7
11				22.0	29.0–28.3	29.0–28.3	26.5	25.1
12				13.9	29.0–28.3	29.0–28.3	29.0	127.7
13					29.0–28.3	29.0–28.3	28.5–28.3	129.7
14					31.2	29.0–28.3	28.5–28.3	26.5
15					22.0	29.0–28.3	28.5–28.3	28.9
16					13.9	31.2	31.2	30.8
17						22.0	22.0	21.9
18						13.9	13.8	13.8

[a] Data obtained for **2** were similar to those from the literature (*35*), but remained unassigned in ref *35*.

*Data for hydroxytyrosyl butyrate (**3**)*: 65% yield; NMR data (see **Tables 1** and **2**); HRMS (CI) *m*/*z* calcd for $C_{12}H_{16}O_4$ $[M]^+$ 224.104859, found 224.104273 (2.6 ppm).

*Data for hydroxytyrosyl laurate (**4**)*: 74% yield; NMR data (see **Tables 1** and **2**); HRMS (CI) *m*/*z* calcd for $C_{20}H_{32}O_4$ $[M]^+$ 336.230060, found 336.229674 (1.1 ppm).

*Data for hydroxytyrosyl palmitate (**5**)*: 62% yield; NMR data (see **Tables 1** and **2**); HRMS (FAB) *m*/*z* calcd for $C_{24}H_{40}O_4Na$ $[M + Na]^+$ 415.282430, found 415.283191 (1.8 ppm).

*Data for hydroxytyrosyl stearate (**6**)*: 68% yield; NMR data (see **Tables 1** and **2**); HRMS (CI) *m*/*z* calcd for $C_{26}H_{44}O_4$ $[M]^+$ 420.323960, found 420.320565 (8.1 ppm).

*Data for hydroxytyrosyl oleate (**7**)*: 76% yield; NMR data (see **Tables 1** and **2**); HRMS (CI) *m*/*z* calcd for $C_{26}H_{43}O_4$ $[M + H]^+$ 419.316135, found 419.315427 (1.7 ppm). Anal. Calcd for $C_{26}H_{42}O_4 \cdot {}^1/_2H_2O$: C, 73.07; H, 10.07. Found: C, 73.58; H, 10.48.

*Data for hydroxytyrosyl linoleate (**8**)*: 65% yield; NMR data (see **Tables 1** and **2**); HRMS (CI) *m*/*z* calcd for $C_{26}H_{41}O_4$ $[M + H]^+$ 417.300485, found 417.298737 (4.2 ppm).

Evaluation of Antioxidant Capacity by the Rancimat Method. A lipid matrix was obtained from virgin olive oil by purification through a column packed with alumina (*41*). The fatty acid composition of the matrix was C16:0 (18.4%), C16:1 (2.5%), C18:0 (1.8%), C18:1 (57.2%), C18:2 (17.9%), and C18:3 (0.8%). Aliquots of this glyceridic matrix were spiked with the antioxidants and then subjected to accelerated oxidation in a Rancimat apparatus, model CH 9100 (Metrohm Co., Basel, Switzerland). A flow of air (20 L/h) was bubbled successively through the oil heated at 90 °C and cold water. In this process, the volatile oxidation products were stripped from the oil and dissolved in the water, increasing the water conductivity. The time taken until there is a sharp increase of conductivity is termed the induction time (IT) and is expressed in hours.

Preparation of Tissue Homogenates. Wistar rats (3 months old) were used in all the experiments. Animals were handled according to guidelines established by the Animal House of the University of Sevilla. They were housed in a pathogen-free environment in groups of three or four in a temperature- and light-controlled room, with free access to food and water. Rats were decapitated, and the brain was rapidly removed. The brain was homogenized in 3 volumes of homogenization buffer: 50 mM Tris/HCl, 1 mM NaCl, and 0.25 M sucrose, pH 7.5. A cocktail of protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 50 mM EDTA, chymostatin, leupeptin, and pepstatin, 1 μg/mL of each) was added to the incubation mixture to prevent proteolysis. The homogenates were centrifuged at 1000*g*. All operations were performed at 0−4 °C.

Treatment with Oxidant. Brain homogenates (4 mg of proteins/mL) were preincubated at 37 °C for 30 min in homogenization buffer, in both the absence and the presence of the appropriate dose of the antioxidants. Aliquots (5 μL) of stock solutions of each antioxidant in methanol were added to 1 mL of brain homogenate to reach a final concentration of 1 or 5 mM. Oxidation was initiated by adding CH to a final concentration of 2 mM. After an additional incubation for 30 min at 37 °C, 10% (w/v) ice-cold TCA was added, and the samples were centrifuged in an Eppendorf centrifuge for 5 min at 8000*g*. The supernatant was used for malondialdehyde (MDA) quantification.

Immunoblot Analysis of Protein Carbonyls. A protein carbonyl profile was performed as described previously (*42*). Briefly, proteins—dissolved in 6% (w/v) SDS—were mixed with an equal volume of DNPH solution and incubated at room temperature. The solution was neutralized and prepared for loading onto SDS gels by addition of 2 M Tris/HCl. Samples were separated by SDS−polyacrylamide (12%) gel electrophoresis and transferred to a nitrocellulose membrane. The membranes were then incubated with a mouse anti-DNPH antibody at a dilution of 1:1500 for 1 h at 4 °C. After extensive washing, the membranes were incubated with peroxidase-conjugated mouse anti-rabbit IgG (Boehringer Mannheim) at a dilution of 1:5000 for 2 h and washed with blocking buffer. The oxidized proteins were detected by chemiluminescence (Boehringer Mannheim), and immunoblots were quantified using an image analysis program (ImageQuant for Windows 2000, Amersham Bioscience) which allows the measurement of the optical density, directly correlated to the amount of carbonyl groups in the sample.

Determination of Malondialdehyde. An aliquot (0.5 mL) of the supernatant obtained after the treatment with oxidant was used for MDA determination (*43*). Samples were treated with TBA reagent (20 mM TBA in 50% (v/v) glacial acetic acid) and then heated at 100 °C for 1 h. After a cooling period, butanol was added, the organic layer was

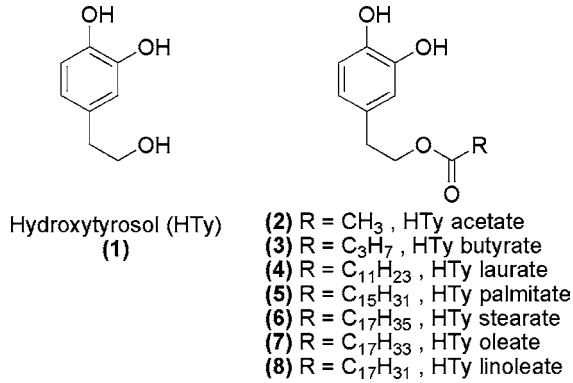

Figure 1. Structures of synthesized compounds.

removed, and end-point fluorescence was measured at $\lambda_{ex} = 515$ nm and $\lambda_{em} = 585$ nm.

Statistical Analysis. Statistical analyses of the data were performed using the SAS system (*44*). Statistical differences between means were determined using a one-way ANOVA. p values of <0.05 were considered significant.

RESULTS AND DISCUSSION

Preparation and Characterization of Hydroxytyrosyl Esters. Previous methods described for the preparation of hydroxytyrosyl acetate (*23*, *37*) are difficult or low-yielding or both. Moreover, with these procedures only hydroxytyrosyl acetate could be obtained. Our methodology, which is under patent protection (*39*), enables us to prepare compounds **2**−**8** (**Figure 1**) easily in good to excellent yields. Synthesized hydroxytyrosyl esters were characterized by NMR and MS spectroscopy. NMR chemical shifts (**Tables 1** and **2**) were unequivocally assigned by comparison with data from starting materials. As can be seen in **Table 1**, there are differences of 0.61 and 0.15 ppm, respectively, for the chemical shifts (δ) of $H_{1'}$ and $H_{2'}$ with respect to the same values in free **1**. For aromatic protons this difference is very weak, but still appreciable (0.02 ppm for each). Similar deshielding effects can be observed from ^{13}C data (Table 2), although the expected β-shielding effect is evident for $C_{2'}$ in each ester.

Representative hydroxytyrosyl esters with a short acylic chain (**2**) and saturated (**5**), monounsaturated (**7**), and polyunsaturated (**8**) fatty chains were chosen to evaluate their antioxidant capacities and were compared with those shown by α-tocopherol and BHT under the same conditions.

Antioxidant Activity in Lipid Matrices. Antioxidant capacity was evaluated by measuring the oxidative stability of purified glyceridic matrices spiked with the antioxidants, using the Rancimat method. This accelerated method is widely used to compare the oxidative stability of fats and oils and to check the effect of minor compounds (*45*). A temperature of 90 °C was used to minimize BHT loss by evaporation. Values of induction time—the time taken until volatile oxidation products are produced—obtained for different antioxidant concentrations are shown in **Figure 2**. **1**, **2**, **5**, **7**, and **8** showed similar antioxidant activities per millimole of substance, indicating that esterification does not affect the activity of hydroxytyrosol. The antioxidant actions of hydroxytyrosol and hydroxytyrosyl esters were more significant than that of α-tocopherol. The effect of BHT was the lowest. For all antioxidants, the experimental results fulfill a sigmoidal function; that is, at low concentrations there is a linear relationship of positive slope between induction time and concentration, whereas at high concentrations the induction time remains practically constant (*24*).

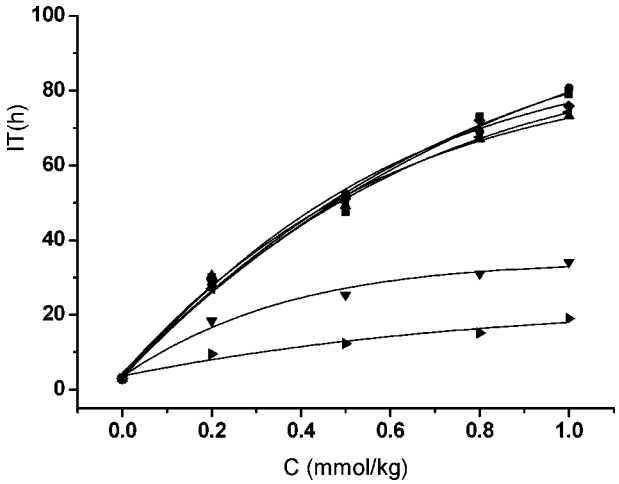

Figure 2. Induction times (ITs) of lipid matrices spiked with hydroxytyrosol (■), hydroxytyrosyl acetate (●), hydroxytyrosyl palmitate (▲), hydroxytyrosyl oleate (◆), hydroxytyrosyl linoleate (sideways triangle pointing to the left), α-tocopherol (▼), or BHT (sideways triangle pointing to the right).

These results are in agreement with the fact that, in bulk oils, the antioxidant activity of phenolic compounds is related to the structure of the phenolic moiety of the molecule, in such a way that *o*-diphenols show much greater activity than phenols having a single hydroxyl group (*24*).

Antioxidant Activity in Brain Homogenates. The chemical approach (Rancimat data) shows that hydroxytyrosyl esters have a promising antioxidant activity in fatty material, similar to that of free hydroxytyrosol. To find out how specific this protection is, we have also evaluated the capacity of hydroxytyrosyl esters to protect proteins and lipids against damage (oxidation) caused by the peroxyl radical, using a brain homogenate as an ex vivo model in which all the biomolecules (proteins, lipids, etc.) are present in an emulsified form. We have used this model because brain tissue is very vulnerable to oxidative damage due to its relative lack of antioxidant enzymes, such as catalase and glutathione peroxidase, and its high abundance of oxidizable substrates (*46*, *47*). We used cumene hydroperoxide (CH) as a lipid-oxidizing agent because it has been used to assess the effects of free radicals and reactive oxygen intermediates on various biological molecules (*47*, *48*). In the present study, the potential protective effect of hydroxytyrosyl esters against the damage caused by CH has been evaluated by measuring the MDA and carbonyl group content in a brain extract, as an assessment of oxidative damage to lipid and protein, respectively. These results have been compared with those for free hydroxytyrosol, α-tocopherol, and BHT.

Lipid Peroxidation. Aldehydes are always produced as products of lipid peroxidation when lipid hydroperoxides break down in biological systems, with MDA as the most abundant aldehyde (*43*). **Figure 3** shows the effect of CH on brain homogenate lipids. CH produced a significant increase in the MDA content in the absence of a protective substance. However, the inclusion of hydroxytyrosol, hydroxytyrosyl esters, or classical antioxidants (α-tocopherol, BHT) had a marked protective effect, lowering the production of MDA.

The capacity of hydroxytyrosol and hydroxytyrosyl acetate, palmitate, and oleate to prevent the lipid peroxidation phenomena in brain homogenate was similar to that of α-tocopherol, whereas that in bulk oil was significantly greater. In oil-in-water emulsions (*23*), the antioxidant effect of hydroxytyrosol and hydroxytyrosyl acetate was smaller than that of α-tocopherol. In brain homogenate, hydroxytyrosyl linoleate showed greater

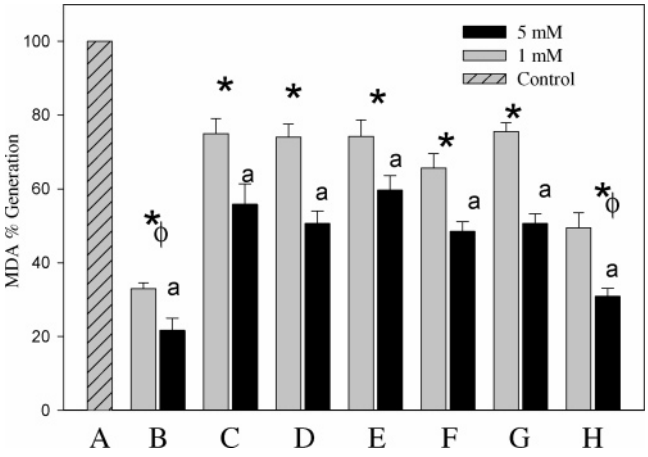

Figure 3. Inhibitory capacity against lipid peroxidation. Lipid peroxidation was performed by induction with CH, and inhibitory capacity was evaluated by malondialdehyde production in the presence and absence of antioxidants. The results are the mean ± SEM of five independent experiments. Key: (*) Samples with BHT (lane B), α-tocopherol (lane C), **1** (lane D), **2** (lane E), **5** (lane F), **7** (lane G), and **8** (lane H) are significantly different in MDA level from that of the antioxidant-free sample (lane A) ($p < 0.001$). (ϕ) Results with BHT (lane B) and **8** (lane H) are significantly different from those of the other antioxidants. (a) Results at concentrations of 5 mM are significantly different from those at 1 mM ($p < 0.001$).

activity than the other esters, whereas in bulk oil the activities of all hydroxytyrosyl esters were similar. In brain homogenate BHT was the most effective antioxidant, whereas in bulk oil it was the least effective. This different action of the antioxidants in biological systems is attributed to a multitude of factors, including the location in different phases, the complex interfacial effects, the type of lipid substrate, and the method to assess oxidation (*49*). The data in **Figure 3** also show that the protection was concentration dependent.

Protein Oxidation. Carbonyl groups are introduced into proteins by oxidative mechanisms occurring in a variety of physiological and pathological processes and are an established marker of protein oxidation (*50*). In brain homogenate, the reaction of CH with proteins resulted in a rapid increase of carbonyl groups, whose formation was quantified using DNPH (*42*).

The measurement of carbonyl content and determination of whether a specific protein becomes oxidized in this process was followed by labeling the carbonyl groups with DNPH and subjecting the proteins to SDS−PAGE and Western blotting. The total protein was detected in parallel blots by Coomassie blue staining. The protein molecular weight profile does not show any differences in amounts or in molecular weight between the samples; in contrast, the protein oxidation profile changes strongly.

After incubation of brain homogenate with CH in the absence of a protective substance, protein oxidation increased dramatically, becoming very manifest and extensive (**Figure 4**, lane A). The presence of hydroxytyrosyl esters (**Figure 4**, lanes E−H) prevented protein oxidation. Hydroxytyrosol produced a weak protection (lane D) similarly to hydroxytyrosyl acetate (lane E), but this behavior changed when a lipophilic chain was present in the molecule, as in hydroxytyrosyl esters **5** (lane F), **7** (lane G), and **8** (lane H), which showed higher capacities to prevent the protein oxidation induced by CH. Thus, the antioxidant function is improved as a result of a better distribution and localization of these fatty esters at the emulsion interface.

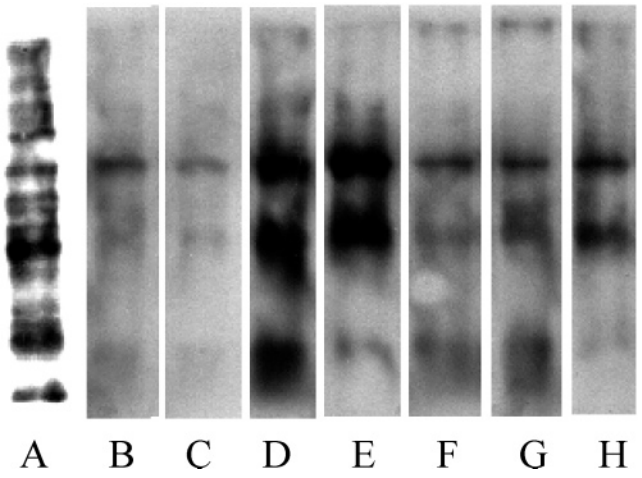

Figure 4. Evaluation of protein oxidation inhibitory capacity. After incubation of a brain homogenate with CH alone or in the presence of antioxidants, aliquots of the reaction mixture were subjected to SDS–PAGE followed by immunoblotting with anti-DNPH. Detection of oxidatively modified proteins of brain homogenate after treatment with CH alone (lane A) or in the presence of BHT (lane B), α-tocopherol (lane C), **1** (lane D), **2** (lane E), **5** (lane F), **7** (lane G), or **8** (lane H).

Table 3. Evaluation of Protein Inhibitory Oxidation Capacity[a]

	optical density (×10^5 pixels)	% protein inhibitory oxidation capacity		optical density (×10^5 pixels)	% protein inhibitory oxidation capacity
A	228.4 ± 12	100.00 ± 5.25	E	51.2 ± 3.2	22.76 ± 1.42
B	5.7 ± 0.2	2.55 ± 0.08	F	8.0 ± 0.5	3.59 ± 0.22
C	4.9 ± 0.2	2.17 ± 0.08	G	27.3 ± 0.1	12.14 ± 0.04
D	72.1 ± 5.1	32.05 ± 2.26	H	19.5 ± 0.1	8.67 ± 0.04

[a] Quantification of carbonyl levels generated in proteins determined by densitometric analysis from immunoblotting as described in the Materials and Methods, after treatment with CH alone (lane A) or in the presence of **1** (lane D) and its derivatives **2** (lane E), **5** (lane F), **7** (lane G), and **8** (lane H) and standard antioxidants BHT (lane B) and α-tocopherol (lane C).

BHT (**Figure 4**, lane B) and α-tocopherol (**Figure 4**, lane C) showed a greater protective activity than hydroxytyrosol and its acetate. However, introduction of a lipophilic chain in the hydroxytyrosol molecule, as in palmitate (**5**), oleate (**7**), and linoleate (**8**) derivatives, increased the protein protection, their efficacy levels approaching those of BHT and α-tocopherol.

The results from these immunoblots were quantified by measuring the optical density (which is related to the level of protein oxidation) and are shown in **Table 3**.

This antioxidant activity is of particular importance, since the loss of protein function caused by oxidative modification may affect the activity of enzymes, receptors, and membrane transporters, among other functions. Under oxidative stress, proteins suffer various structural modifications, which have been extensively studied (*51*, *52*). The physiological consequences depend on whether such alterations occur at the active site of the enzyme, on the number of molecules affected, and on the importance of the protein involved.

In conclusion, the lipophilic hydroxytyrosyl esters investigated showed a high free-radical-scavenging capacity, preventing protein oxidation and lipid peroxidation when cells ex vivo were exposed to active-oxygen substances and/or free radicals. This property makes them potentially useful in the formulation of solid and liquid food for the prevention and treatment of chronic pathological states associated with a high generation of active-oxygen substances and/or free radicals and as additives for preserving foods from oxidation processes.

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