JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2002,** *50,* 6521−6526 **6521**

Protective Effect of the Phenolic Fraction from Virgin Olive Oils against Oxidative Stress in Human Cells

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This paper reports the protective effect of the phenolic fraction extracted from extra virgin olive oils (OOPEs) against the cytotoxic effects of reactive oxygen species in human erythrocytes and Caco-2 cells, employed as model systems. Pretreatment of cells with various OOPEs, indeed, provides a remarkable protection against oxidative damages: this effect was strictly dependent on the *o*-diphenolic content of the extracts. Moreover, the protective effects observable in cellular systems were compared with in vitro antioxidant properties, measured by using the FRAP (ferric reducing/antioxidant power) assay; the reducing ability of OOPEs strictly parallels their *o*-phenolic content. The linear relationship demonstrated between biological effects and antioxidant capacity measured by the FRAP assay allows us to propose the use of this rapid colorimetric method in assessing and certifying the antioxidant power of extra virgin olive oil.

KEYWORDS: Mediterranean diet; antioxidant; polyphenol; oxidative stress; hydroxytyrosol; olive oil; functional food

INTRODUCTION

Olive oil represents the typical lipidic source of the Mediterranean diet, a dietary habit which has been associated with a low incidence of several pathologies, including cardiovascular diseases and neurological disorders (*1*−*3*). Moreover, there is clear evidence that olive oil consumption, together with fruit and vegetable consumption, contributes to the significant reduction of breast and colon cancer risk in Mediterranean populations (*4*−*6*).

The beneficial properties of olive oil have been mainly attributed to its high content of monounsaturated oleic acid, which is reported to affect the serum lipidic profile (*7*) and to decrease, both in vivo and ex vivo, LDL susceptibility to oxidation (*8*, *9*). However, in recent years converging evidence indicates that the olive oil nonglyceride fraction, rich in polyphenols, significantly contributes to its benefits on human health (*10*−*12*).

Polyphenols are bioactive molecules, present in all plant species, influencing their morphology, growth, and reproduction as well as their resistance against parasites and environmental stresses (*13*). Polyphenols contribute to the organoleptic properties of olive oil (*14*, *15*); moreover, acting as free radical scavengers, they are chiefly responsible for the intrinsic defense against the autoxidation of unsaturated fatty acids, increasing the shelf life of the oil (*16*). In recent years, the interest of scientists has also been focused on the preventive effect(s) of polyphenols against reactive oxygen species (ROS) mediated degenerative diseases (*12*, *16*, *17*).

Hydroxytyrosol (3,4-dihydroxyphenylethanol; DOPET) is the major *o*-diphenol of virgin olive oil, present in both a free and esterified form (oleuropein aglycon). As reviewed recently (*10*−*12*), DOPET biological properties have been thoroughly explored, including a significant antiproliferative effect in HL-60 cells (*18*). This phenol is able to prevent LDL oxidation (*19*) and to inhibit platelet aggregation (*20*), and it is endowed with potent antimicrobial activity (*21*). We have recently demonstrated that DOPET permeates cell membranes via a passive diffusion mechanism (*22*) and that it rapidly distributes in all organs and tissues, when intravenously injected in rats (*23*).

Literature data on olive oil polyphenols mainly concern purified DOPET, while the antioxidant properties of the total olive oil phenolic extract (OOPE) have been poorly investigated. It should be stressed, in this respect, that the phenolic fraction also contains a variety of antioxidant compounds, including caffeic and protocatechuic acids and lignans (*24*). Therefore, because of the possible interaction among different antioxidants, the measure of the total antioxidant capacity is more representative than the protective effect of a single component. In recent papers, indeed, the influence of OOPE on LDL oxidation in

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10.1021/jf020565+ CCC: $22.00
Published on Web 09/24/2002

vitro (*25*) and on free radical production by the fecal matrix (*26*) has been explored. However, no data are available in the literature concerning the antioxidant activity of total phenols of olive oil in human cell cultures.

The aim of this paper was to investigate the possible protective effect of the phenolic fraction of various extra virgin olive oils against ROS-mediated oxidative injuries in erythrocytes (RBCs) and intestinal (Caco-2) cells. Moreover, the OOPE protective effect was compared with their antioxidant capacity, evaluated by the FRAP assay (ferric reducing/antioxidant power). This test has already been applied to measure the antioxidant properties of several dietary components such as orange juice, wines, and teas (*27*, *28*).

Finally, the potential implications of the reported results on the nutritional value of virgin olive oils will be discussed, also assessing their implications on the market.

MATERIALS AND METHODS

Reagents and Biological Materials. Six extra virgin olive oils, currently on the Italian market, were studied. Dulbecco's modified Eagle's medium (DMEM) with 4.5 mg/mL glucose, Eagle's minimum essential medium (EMEM), fetal calf serum (FCS), nonessential amino acids, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), glutamine, penicillin, streptomycin, PBS tablets, and neutral red were purchased from Gibco Life Science Technologies (S. Giuliano Milanese, Milan, Italy). H_2O_2 (30% aqueous solution), *t*-BHP (70% aqueous solution), Tween 20, Triton X-100, TPTZ (2,4,6-tripyridyl-*s*-triazine), and acetate buffer (CH_3COOH/CH_3COO^-) were from Sigma Chemical Co. (St. Louis, MO).

Extraction of Polyphenols and Their Analysis. The methanolic extract of extra virgin olive oil was obtained according Montedoro et al. (*29*), with some modifications. Methanol/water (20 mL) (80:20 v/v) plus Tween 20 (2% v/w) were added to 20 g of extra virgin olive oil, mixed with an Ultra-Turrax T 25 at 17000*g* for 3 min, and centrifuged at 5000*g* for 10 min. The extraction was repeated twice; to eliminate the oil droplets, the methanolic extract was kept for 24 h at −20 °C. To remove methanol in the biological tests, the methanolic extract was vacuum concentrated to a final volume of 7 mL under a stream of nitrogen at 50−55 °C.

Total phenolic and *o*-diphenolic content was determined colorimetrically using the Folin−Ciocalteau and the Arnow reagent, respectively (*30*); the qualitative analysis of phenolic extracts was performed by HPLC according to Montedoro et al. (*29*).

Preparation of Cell Systems and Induction of Oxidative Stress. Heparinized fresh human blood was obtained from healthy donors after informed consent was obtained. After centrifugation of samples for 10 min at 1000*g*, plasma and buffy coat were removed, and red blood cells (RBCs) were washed three times with isotonic buffered saline (NaCl, 0.9%) and finally suspended in the same buffer to 2% hematocrit. To induce an oxidative stress, *tert*-butyl hydroperoxide (*t*-BHP, 500 μM) was added to the RBC suspension as the oxidizing agent (*31*). Flasks were incubated at 37 °C for 2 h in a shaking water bath; cell-free supernatant was used for the determination of both the extent of hemolysis and the thiobarbituric acid reactive substances (TBARS).

The hemolysis was monitored spectrophotometrically according to Manna et al. (*32*). After oxidative stress, aliquots of the reaction mixture were centrifuged at 1100*g* for 10 min, and the absorption (A) of the supernatant (S1) at 540 nm was measured. Packed ox RBCs were then hemolyzed with 40 volumes of ice-cold distilled water and centrifuged at 1500*g* for 10 min. The supernatant (S2) was then added to S1, and absorption (B) of the combined supernatants (S1 + S2) was measured at 540 nm; percentage hemolysis was calculated from the ratio of the readings (A:B) × 100.

TBARS were determined according to ref *32*. After treatment, ox RBCs were pelleted, and 2 mL of supernatant was mixed with 1 mL of 30% (w/v) trichloroacetic acid and centrifuged at 5000*g* for 15 min. A 2 mL aliquot of acid-soluble supernatant was added to 0.5 mL of 1% (w/v) thiobarbituric acid in 0.05 N NaOH and heated in a boiling water bath for 10 min. The absorbance of the developed pink chromophore was determined at 532 nm.

To assay the capacity of olive oil extracts to protect RBCs from *t*-BHP-induced oxidative injury, the cells were pretreated with 10 μL of the polyphenol extract at 37 °C. After 15 min, the medium was changed to remove the antioxidant before the addition of *t*-BHP. At the end of incubation, the above-mentioned markers were evaluated.

Human colon carcinoma (Caco-2) cells were routinely grown at 37 °C in a humidified incubator with 5% CO_2/95% air atmosphere, in DMEM, supplemented with 10% FCS, glutamine (2 mM), penicillin (50 units/mL), streptomycin (50 units/mL), and 1% nonessential amino acids. For oxidative stress experiments, Caco-2 cells were seeded at a density of 90000 cells/cm^2 in multiwell dishes (*33*), and the medium was changed every 48 h using an iron-free medium (MEM). Twelve to fourteen days after confluence, differentiated monolayers were incubated with 40 mM H_2O_2 for 20 h. At the end of oxidative stress induction, cell viability was evaluated by the test of neutral red uptake according to the procedure of Manna et al. (*34*). The medium was removed and replaced with 0.1 mL of fresh medium containing 1.14 mM neutral red; after 3 h incubation, cells were washed twice with PBS, and the incorporated dye was released from the cells by incubation for 15 min at room temperature in the presence of 1 mL of the cell lysis buffer containing acetic acid (1% v/v) and ethanol (50% v/v). To measure the dye taken up, the cell lysis products were centrifuged and supernatants analyzed at 540 nm.

To assay the OOPEs protective effect on Caco-2 cells from H_2O_2-induced oxidative injury, the cells were pretreated at 37 °C for 4 h in the presence of 100 μL of OOPE, and the medium was changed to remove the extract before the addition of H_2O_2. At the end of incubation, the cell viability was evaluated as above described.

FRAP Assay. The total antioxidant power of OOPE was determined using the FRAP assay, a colorimetric method based on the reduction of a ferric tripyridyltriazine complex to its ferrous form, according to Benzie and Strain (*27*), with minor modifications. OOPE (25 μL) was mixed with 1 mL of working solution, prepared by mixing 25 mL of 300 mM acetate buffer (pH 3.6), 2.5 mL of TPTZ solution (10 mM TPTZ in 40 mM HCl), and 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$. The absorbance at 593 nm was read after 6 min at room temperature, and data were translated into the FRAP value (micromolar) using a methanolic solution of Fe(II) in the range of 100−2000 μM $FeSO_4 \cdot 7H_2O$ for calibration.

Statistical Analysis. Results are reported as means ± SD, $n = 4$; Student's paired *t*-test (Stat-Works software running on Apple Macintosh LC) was routinely utilized.

Table 1. Phenolic Content and FRAP Values in the Methanolic Extracts of Six Virgin Olive Oils

olive oil sample	total phenols (mg/kg)	*o*-diphenols (mg/kg)	FRAP value (μM)
1	34	6	200 ± 10
2	99	12	340 ± 17
3	133	45	600 ± 30
4	133	76	700 ± 35
5	291	143	1200 ± 60
6	358	193	1550 ± 77

RESULTS

It is amply reported that polyphenol concentration of extra virgin olive oils may differ, depending on both cultivars and environmental factors, as well as the degree of ripeness of the olives and the extraction procedures (*35*, *36*). In agreement with the literature data, the concentration of both the total phenolic and the *o*-diphenolic fractions extracted from different oil samples (**Table 1**) indicates a high variability in the phenolic content of the extra virgin olive oils available on the market.

The chemical characterization of three OOPEs, which differ in their phenolic content, was performed by HPLC analysis

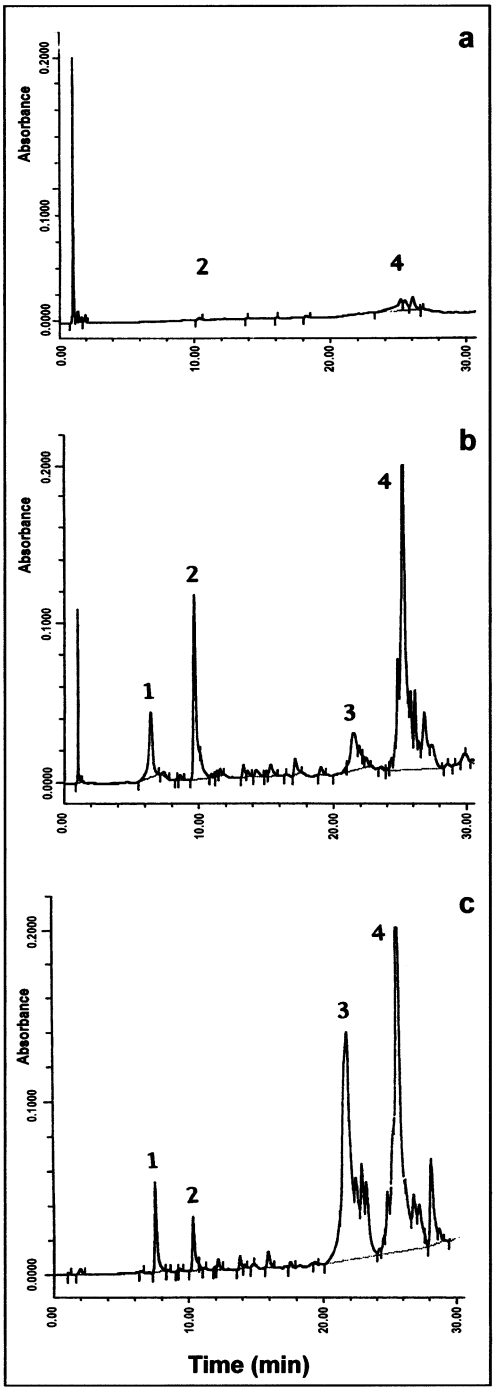

Figure 1. HPLC analysis of different olive oil phenolic extracts. Phenolic extraction and HPLC analysis were performed as described under Materials and Methods. Four major peaks were identified according to their retention times: (1) hydroxytyrosol; (2) tyrosol; (3) oleuropein aglycon; (4) ligstroside aglycon. Panels: (A) olive oil sample 1; (B) olive oil sample 3; (C) olive oil sample 6.

(**Figure 1**); in agreement with previous reports, four major phenolic compounds have been identified on the basis of their retention times, such as DOPET (RT = 6.5 min), tyrosol (4-hydroxyphenylethanol, HPE; RT = 9.5 min), and their secoiridoid precursors oleuropein aglycon (RT = 20.5 min) and ligstroside aglycon (RT = 25.5 min), respectively.

Figure 2 shows the dose dependency of the ferric reducing ability of olive oil phenols DOPET, HPE, and oleuropein compared to that of quercetin, ascorbic acid, and Trolox, a water-soluble analogue of vitamin E (*37*); a linear dose−response

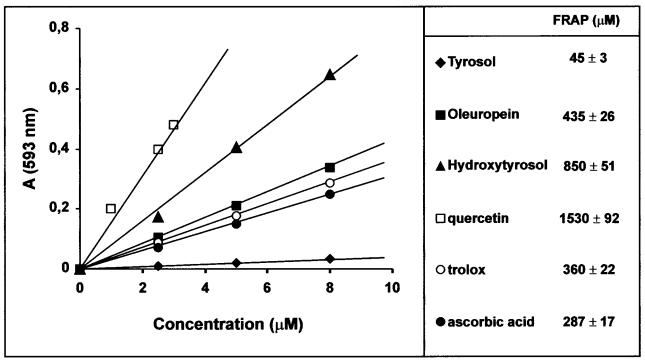

Figure 2. Ferric reducing abilities and FRAP values of standard polyphenols and antioxidant vitamins. Ferric reducing abilities were determined as reported under Materials and Methods; FRAP data (means ± SD, $n = 3$) are expressed as Fe(II) equivalent concentration (μM).

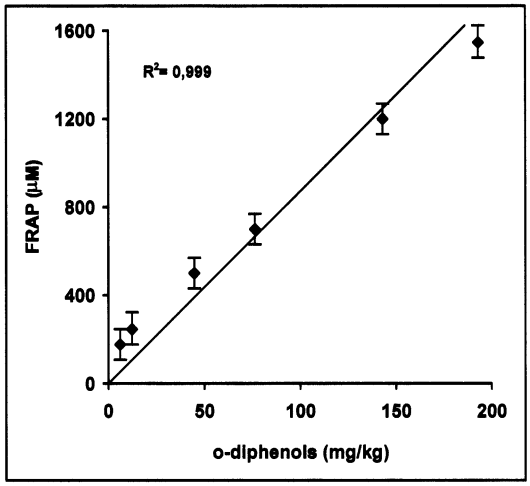

Figure 3. Correlation between FRAP values and *o*-diphenol content for the six olive oil extracts tested. FRAP values were calculated as indicated in Materials and Methods. The correlation coefficient (R^2) was calculated using Pearson's χ^2.

relationship was observable over the range of concentrations selected. To facilitate comparison of the reducing efficiency of selected antioxidants, their FRAP values, calculated using a calibration curve obtained from a methanolic standard solution of Fe(II), are also reported.

DOPET and quercetin FRAP values are significantly higher compared with those of ascorbic acid and TROLOX, confirming the view that reducing activities of flavonoids and simple phenols are higher than those of antioxidant vitamins (*37*). Moreover, the FRAP value of oleuropein is only 50% of that of DOPET, the monophenolic compound being almost inefficient as hydrogen donor.

The FRAP values of OOPEs were then evaluated, as reported in **Table 1**. The reducing ability of OOPEs strictly depends on the degree of hydroxylation of its phenolic components, being proportional to the *o*-diphenolic content. In **Figure 3**, FRAP values of OOPEs versus the respective amounts of *o*-diphenols was plotted; a correlation coefficient of 0.99 has been calculated.

To assess the antioxidant capacity of OOPEs in human cells, RBCs and Caco-2 cells were used as model systems.

Human RBCs were chosen in that these cells are particularly exposed to oxidative hazard, because of their specific role as oxygen carriers. Under physiological conditions ROS are rapidly removed by the endogenous defense systems; however, if ROS are overproduced or if the endogenous defenses are impaired,

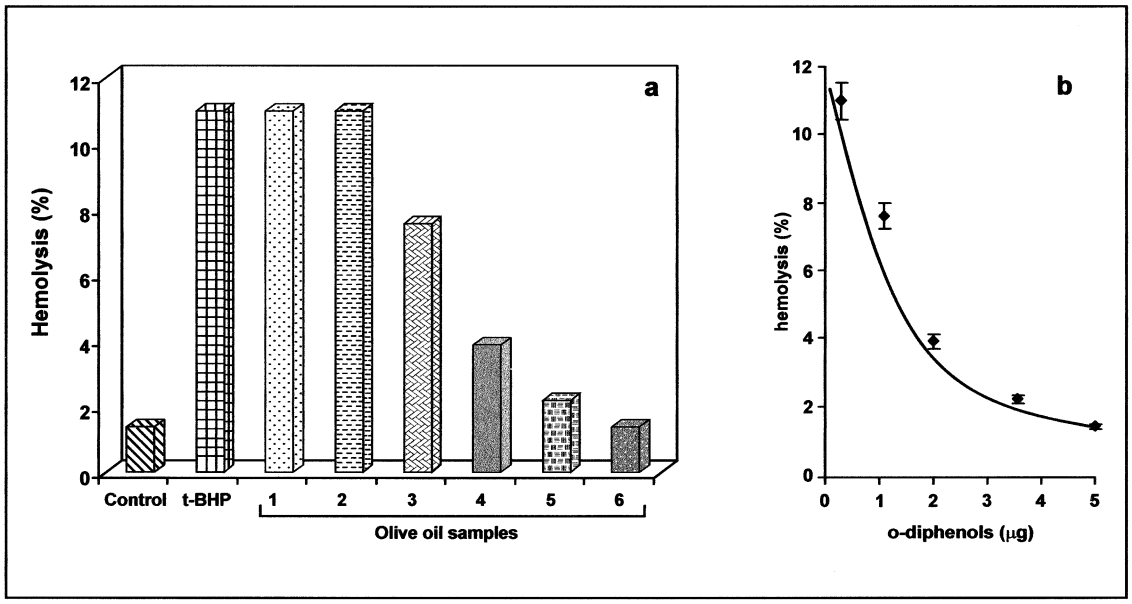

Figure 4. Effect of OOPEs on *t*-BHP-induced hemolysis in RBCs. The cells were pretreated for 15 min at 37 °C with 10 μL of the phenolic fraction extracted from the six olive oil samples tested and then incubated in the presence of 500 μM *t*-BHP. After 2 h, hemolysis was measured as described under Materials and Methods. Values are means ± SD; $n = 4$ (panel a). Panel b reports the *o*-diphenolic concentration dependency of the cytoprotective effect.

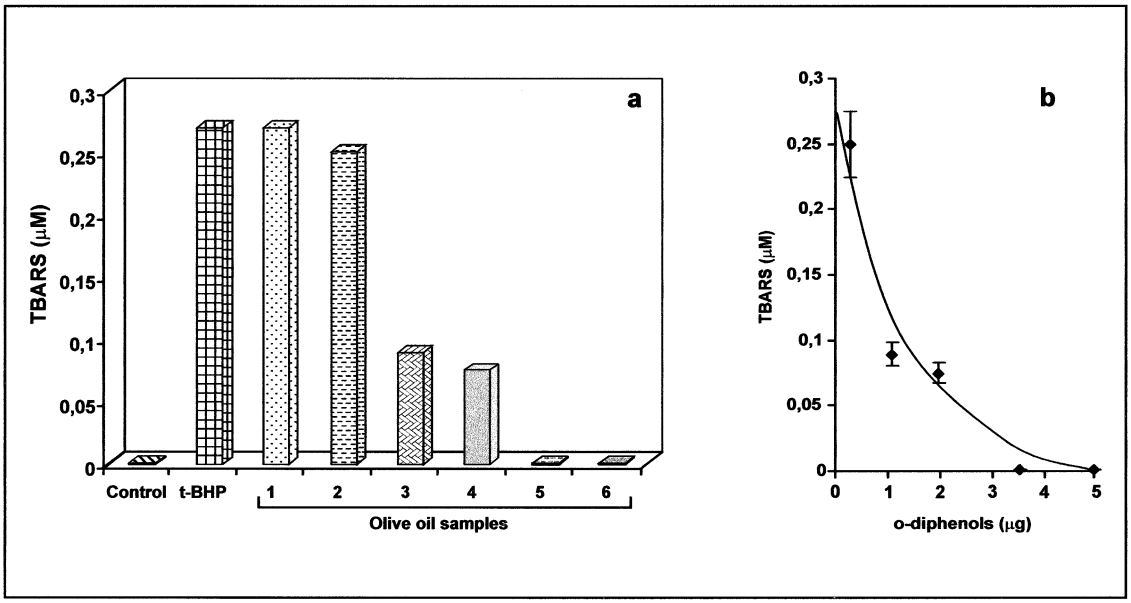

Figure 5. Effect of OOPEs on *t*-BHP-induced TBARS formation in RBCs. The cells were pretreated for 15 min at 37 °C as indicated in **Figure 3.** After 2 h incubation, TBARS were measured as described in Materials and Methods. Values are means ± SD; $n = 4$ (panel a). Panel b reports the *o*-diphenolic concentration dependency of the protective effect.

severe oxidative damages to both plasma membrane and cytosolic components are observable, eventually leading to hemolysis (*32*). To verify the protective effect of OOPEs against oxidative hemolysis, RBCs were treated with 500 μM *t*-BHP for 1 h to induce an oxidative stress. As shown in **Figure 4a**, a significant reduction of hemolysis is observable in cells pretreated with OOPEs from olive oil samples 3−6, containing more than 100 mg/kg total phenol concentration. On the contrary, no protective effect on ROS-mediated cytotoxicity is observable in samples 1 and 2, the antioxidant activity being dependent on the *o*-diphenolic content of OOPEs (**Figure 4b**).

Membrane phospholipids are a major target of oxidative damage in erythrocytes; lipid peroxidation involves the cleavage of polyunsaturated fatty acids at their double bonds, leading to the formation of TBARS. Incubation of RBCs with 500 μM *t*-BHP for 60 min significantly increases TBARS concentration, as a consequence of the oxidative stress. Also, in this case, pretreatment of RBCs with OOPEs provides a remarkable protection only for olive oil samples 3−6, containing a phenolic concentration higher than 100 mg/kg (**Figure 5a**). Even under these experimental conditions, the decrease in TBARS formation was dependent on *o*-diphenolic concentrations of OOPEs (**Figure 5b**).

Colon carcinoma cells (Caco-2 cell line) were selected as a second model system. ROS have been associated with several gastrointestinal injuries (*38*) and may play a major role as mediators of inflammation (*39*). Therefore, this cell line, amply used to examine a variety of intestinal functions (*33*), has also

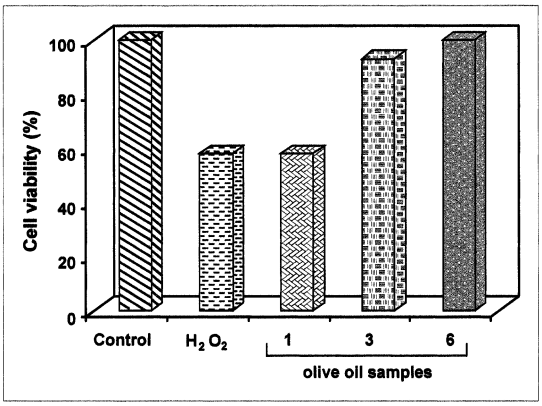

Figure 6. Effect of OOPEs on hydrogen peroxide-induced cytotoxicity of Caco-2 cells. The cells were preincubated for 4 h with 100 μL of the chosen phenolic extract and then treated for 20 h with 40 mM H_2O_2. At the end of incubation, cell viability was measured by the neutral red uptake assay as described in Materials and Methods. Values are means ± SD; $n = 4$.

been employed to investigate the injurious effects of ROS on the gastrointestinal tract (*40*). In this respect, we have previously demonstrated the protection exerted by DOPET on the H_2O_2-induced Caco-2 cell alterations (*34*). To test the antioxidant efficiency of OOPEs in the same model system, Caco-2 cells were preincubated for 4 h with the same volume of olive oil samples 1, 3, and 6 and then exposed to H_2O_2. As shown in **Figure 6**, only extracts from samples 3 and 6 significantly decrease the ROS-induced cell injury, as assessed by checking cell viability with neutral red uptake assay.

DISCUSSION

The hypothesis that polyphenols greatly contribute to the nutritional value of virgin olive oil is supported by literature data describing the biological properties of purified phenolic compounds. The results reported in this paper represent the first evidence that the total olive oil phenolic fraction plays a protective role against ROS-induced oxidative injury in human cells. Moreover, it is possible to conclude that *o*-diphenolic components, mainly DOPET and its esterified form oleuropein aglycon, are the main compounds responsible for this effect. As indicated by the FRAP values, the ferric reducing abilities of DOPET and oleuropein are significantly higher compared to those of ascorbic acid and trolox (*37*). It should be underlined that the FRAP value measured for oleuropein is only 50% compared to that of DOPET, in agreement with data from different in vitro model systems that DOPET antioxidant ability is significantly higher than its precursor oleuropein (*41*, *42*). Finally, also the number of hydroxyl groups on the aromatic ring affects the reducing ability of phenols; the lack of the 3-OH group in HPE, indeed, results in a FRAP value which is dramatically lower compared with that of DOPET, the monophenolic compound being almost inefficient as a hydrogen donor. This result confirms the relevance of the *o*-diphenolic arrangement in determining the antioxidant properties, as previously reported (*34*).

In our experimental system, a complete protection against hemolysis was observed in RBCs pretreated with 5 μg of *o*-diphenols, corresponding to about 300 μL of virgin olive oil containing 100 mg/kg *o*-diphenols. This observation, along with literature data, strongly indicates that olive oil polyphenols could exert their beneficial action also in vivo. Indeed, even though polyphenol concentrations achievable in vivo by the dietary intake of olive oil have not been evaluated, recent papers report that in humans DOPET is dose-dependently absorbed (*43*) and that the intake of high phenol olive oils decreases isoprostane excretion (*44*). Therefore, the daily intake of "functional" extra virgin olive oil, containing high levels of phenolic compounds, could be useful to maximize the antioxidant defenses. Our endogenous defense mechanisms, indeed, are inadequate to completely counteract oxidative damages, and different sources of antioxidants may be important to design dietary strategies for the prevention of those pathologies whose etiology has been related to ROS-mediated injuries.

Finally, the international olive oil markets should be encouraged in the production of extra virgin olive oils rich in polyphenols. To this end, the linear relationship between the antioxidant capacity of OOPEs and their protective effect against oxidative damages in biological systems suggests that FRAP assay can be usefully employed by the oil processing industry, in assessing and certifying the antioxidant power of extra virgin olive oils. This high-quality, functional extra virgin olive oil could be promoted in those countries, for example, Northern Europe and the United States, whose dietary habit is mainly based on animal fats and seed oils.

ABBREVIATIONS USED

Caco-2 cell, human carcinoma colon cells; OOPE, olive oil phenolic extract; RBCs, red blood cells; TBARS, thiobarbituric acid reactive substances; DOPET, 3,4-dihydroxyphenylethanol; HPE, *p*-hydroxyphenylethanol; FRAP, ferric reducing/antioxidant power; DMEM, Dulbecco's modified Eagle's medium; EMEM, Eagle's minimum essential medium; FCS, fetal calf serum; *t*-BHP, *tert*-butyl hydroperoxide.

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Received for review May 15, 2002. Revised manuscript received August 2, 2002. Accepted August 1, 2002. This work was partially supported by a grant from the International Olive Oil Council.

JF020565+