

# Oxidative DNA Damage Is Prevented by Extracts of Olive Oil, Hydroxytyrosol, and Other Olive Phenolic Compounds in Human Blood Mononuclear Cells and HL60 Cells<sup>1,2</sup>

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## Abstract

Our aim in this study was to provide further support to the hypothesis that phenolic compounds may play an important role in the anticarcinogenic properties of olive oil. We measured the effect of olive oil phenols on hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)-induced DNA damage in human peripheral blood mononuclear cells (PBMC) and promyelocytic leukemia cells (HL60) using single-cell gel electrophoresis (comet assay). Hydroxytyrosol [3,4-dihydroxyphenyl-ethanol (3,4-DHPEA)] and a complex mixture of phenols extracted from both virgin olive oil (OO-PE) and olive mill wastewater (WW-PE) reduced the DNA damage at concentrations as low as 1  $\mu$ mol/L when coincubated in the medium with H<sub>2</sub>O<sub>2</sub> (40  $\mu$ mol/L). At 10  $\mu$ mol/L 3,4-DHPEA, the protection was 93% in HL60 and 89% in PBMC. A similar protective activity was also shown by the dialdehydic form of elenoic acid linked to hydroxytyrosol (3,4-DHPEA-EDA) on both kinds of cells. Other purified compounds such as isomer of oleuropein aglycon (3,4-DHPEA-EA), oleuropein, tyrosol, [p-hydroxyphenyl-ethanol (p-HPEA)] the dialdehydic form of elenoic acid linked to tyrosol, caffeic acid, and verbascoside also protected the cells against H<sub>2</sub>O<sub>2</sub>-induced DNA damage although with a lower efficacy (range of protection, 25–75%). On the other hand, when tested in a model system in which the oxidative stress was induced by phorbol 12-myristate 13-acetate-activated monocytes, p-HPEA was more effective than 3,4-DHPEA in preventing the oxidative DNA damage. Overall, these results suggest that OO-PE and WW-PE may efficiently prevent the initiation step of carcinogenesis in vivo, because the concentrations effective against the oxidative DNA damage could be easily reached with normal intake of olive oil. J. Nutr. 138: 1411–1416, 2008.

## Introduction

Epidemiological evidence and numerous animal studies indicate that olive oil may possess anticarcinogenic properties. Case control studies have shown an inverse correlation between olive oil consumption and cancer in different sites (1–8) and animal studies demonstrated a protective activity against chemically induced carcinogenesis, such as Dimethylbenz[a]anthracene-induced mammary tumors (9) and Azoxymethane-induced colon carcinoma (10). Furthermore, olive oil may protect from UV-induced skin cancer (11) and it reduces the incidence of spontaneous appearance of liver tumors in mice (12).

Mutations in somatic cells play a central role both in cancer initiation and in other stages of the carcinogenic process (13). Such genetic alterations are caused by exposure to genotoxic substances

of environmental origin and/or are endogenously produced. Among the endogenously produced genotoxic substances, the reactive oxygen species seem to be of particular importance, because they are continuously produced in all aerobic organisms both as by-products of normal oxygen metabolism and as bactericidal agents by activated phagocytic cells (14). Therefore, the oxidative stress has been strongly correlated to the onset of various degenerative diseases, particularly cancer (15). For these reasons, the chemoprevention ability of olive oil has been ascribed to minor phenolic compounds that possess a potent antioxidant activity (16,17). The phenolic composition of olive oil is rather complex and includes the phenolic alcohols hydroxytyrosol [3,4-dihydroxyphenyl-ethanol (3,4-DHPEA)]<sup>5</sup> and tyrosol

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<sup>5</sup> Abbreviations used: 3,4-DHPEA, 3,4-dihydroxyphenyl-ethanol or hydroxytyrosol; 3,4-DHPEA-EA, isomer of oleuropein aglycon; 3,4-DHPEA-EDA, dialdehydic form of elenoic acid linked to hydroxytyrosol; OO-PE, olive oil phenolic extract; p-HPEA, p-hydroxyphenyl-ethanol or tyrosol; p-HPEA-EDA, dialdehydic form of elenoic acid linked to tyrosol; PMA, phorbol 12-myristate 13-acetate; WW-PE, wastewater phenolic extract.

[*p*-hydroxyphenylethanol (p-HPEA)] and their secoiridoid precursors such as the dialdehydic form of elenoic acid linked either to hydroxytyrosol (3,4-DHPEA-EDA) or to tyrosol (p-HPEA-EDA), and the isomer of oleuropein aglycon (3,4-DHPEA-EA) (18). Because of their hydrophilic properties, a consistent amount of phenols is lost during olive oil production in the olive mill wastewater (19), which could therefore be a relevant source of such compounds (20–22).

The antioxidant activity of olive oil phenols has been demonstrated in several *in vitro* systems; for example, it was shown that hydroxytyrosol: 1) prevents the tert-butylhydroperoxide-induced death of HepG2 cells (23); 2) counteracts the low-density lipoprotein oxidation both chemically produced (24) and cell mediated (25); and 3) protects different cell types such as CaCo-2 (26), erythrocytes (27), and PC12 (28) from hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)-induced cytotoxicity as evidenced by several methods like the leakage of lactate dehydrogenase and the 3-[4,5-dimethyl(thiazol-2-yl)]-3,5-diphenyltetrazolium bromide assay. More recently, some intervention studies have investigated the DNA protective potential of olive oil phenols with conflicting results. Weinbrenner et al. (29) found a decreased amount of 8-oxo-7,8-dihydro-2'-deoxyguanosine in mitochondrial DNA of mononuclear cells and in urine after short-term consumption of olive oil with a linear trend significantly correlated to the content of phenols. Similarly, Salvini et al. (30) showed a 30% reduction of oxidative DNA damage in peripheral blood lymphocytes during intervention on postmenopausal women with virgin olive oil containing high amounts of phenols. On the other hand, no significant effect was detected on urinary excretion of etheno-DNA adducts after consumption of phenol-rich olive oil (31). Furthermore, it was shown that the urinary excretion of oxidation products of guanine was not modified after assumption of olive oil with low, medium, and high phenolic content (32).

In this study, we have investigated the potential protective activity of different olive oil phenols on oxidative DNA damage induced either by H<sub>2</sub>O<sub>2</sub> or by phorbol-myristate-acetate (PMA) activated monocytic cells. This last *ex vivo* model, recently developed in our laboratory (33), has the advantage that, instead of treating the cells with extreme nonphysiological levels of a single oxidant, it exposes the cells to several oxidative species, which are continuously produced at low concentrations and represent the natural oxidative stress in more realistic conditions. The oxidative DNA damage was detected by the highly sensitive comet assay (34).

## Materials and Methods

**Materials and cell line.** Human promyelocytic leukemia cells (HL60), obtained from the American Type Culture Collection, were cultured in RPMI 1640 medium as previously described (33). 3,4-DHPEA was obtained from Cayman Chemicals, oleuropein glucoside was purchased from Extrasynthèse, p-HPEA from Janssen Chemical, and caffeic acid from Fluka.

**Preparation of phenol extracts from olive oil and olive mill wastewater.** The mechanical oil extraction process was performed at industrial plant as follows. Green olives (*Olea europaea* L.) from cultivar Coratina at the ripening stage of 0.90, evaluated as the pigmentation index, were crushed using a hammer crusher; the malaxation was carried out for 40 min at 25°C and the oil was extracted by centrifugation (9600 × g; 1 min) using a decanter (Rapanelli Mod. 400 ECO/G) at a low level of water addition. A phenol methanolic extract was obtained from the virgin olive oil (OO-PE), which contained 650 mg/kg of total phenols, as reported by Montedoro et al. (31). The mill wastewater phenolic extract (WW-PE) was obtained by liquid/liquid (methanol/water) extraction from a concentrate deriving from wastewater treatment by membrane filtration

(patent pending); the wastewater used was obtained from olives (Moraiole) processed using the extraction system described above. The liquid/liquid extraction was performed as follows: 100 mL of wastewater concentrate (11.7 g/L of total phenols) was homogenized for 1 min with ethyl acetate (50 mL), then the organic phase was recovered and the aqueous residual subjected to another extraction. The collected organic phase, after saturation with sodium sulfate to remove water, was filtered and the solvent totally evaporated. The obtained residual was solubilized in 5 mL of ethanol and then evaporated until dry under nitrogen flow.

The HPLC analyses of phenolic extracts were conducted according to Montedoro et al. (35). The OO-PE and WW-PE were analyzed by HPLC with an Agilent Technologies system model 1100 composed of a vacuum degasser, a quaternary pump, an autosampler, a thermostatted column compartment, a diode array detector, and a fluorescent detector. The C18 column used was a Spherisorb ODS-1 250 × 4.6 mm with a particle size of 5 μm (Phase Separation); the injected sample volume was 2 μL. The mobile phase was 0.2% acetic acid (pH 3.1) in water (A)/methanol (B) at a flow rate of 1.5 mL/min. The total running time was 55 min and the gradient changed as follows: 95% A/5% B for 2 min, 75% A/25% B for 8 min, 60% A/40% B for 10 min, 50% A/50% B for 10 min, and 0% A/100% B for 10 min, maintained for 5 min, return to initial conditions in 10 min. For the detection of all the phenolic compounds, a DAD was employed; the wavelength used was 278 nm (36).

**Purification of phenolic compounds.** The separation of the secoiridoids' derivatives was performed from the OO-PE by semipreparative HPLC as previously reported (37), whereas verbascoside was extracted from olive fruit according to the procedure reported in a previous article (38). Briefly, the phenols were extracted from 5g of freeze-dried olive pulps (Moraiole cultivar at 2.5 of ripening stage) using 50 mL of a mixture of methanol:water 80:20 (v:v) at low temperature; the extraction procedure was performed 3 times. The purity of the 3,4-DHPEA-EDA, 3,4-DHPEA-EA, p-HPEA-EDA, and verbascoside preparations was evaluated by HPLC (36) and the chemical structure was confirmed by NMR (37).

**Isolation of peripheral blood mononuclear cells, monocytes, and lymphocytes.** Peripheral blood mononuclear cells (PBMC) were isolated from leukocyte-enriched human peripheral blood by a density gradient (39). Monocytes and lymphocytes were isolated by plating the PBMC suspension (200 μL/well) on 96-well flat-bottom microtiter plates (Falcon; Becton Dickinson) and incubated for 2 h at 37°C and 5% CO<sub>2</sub>. After incubation, nonadherent lymphocytes were removed and washed by centrifugation (400 × g; 7 min) with RPMI + 5% fetal calf serum, while monocytes that adhered to the plastic were washed in the wells with warm RPMI + 5% FCS and used for the subsequent experiments. This cell preparation contained over 90% of monocytes as judged by indirect immunofluorescence analysis with a monoclonal antibody to the CD11b antigen (Boehringer Mannheim).

**Treatment of cells with phenols and exposure to H<sub>2</sub>O<sub>2</sub> and activated monocytes.** The phenolic extracts were dissolved in a solution of ethanol/water (1/3, v:v) at 9.532 g/L for OO-PE and 5.266 g/L for WW-PE to obtain a total concentration of 3,4-DHPEA + 3,4-DHPEA-containing compounds (3,4-DHPEA-EDA, 3,4-DHPEA-EA, and verbascoside) corresponding to 10 mmol/L. Similarly, the purified phenolic compounds were dissolved in the same solution at the concentration of 10 mmol/L. All samples were then divided into aliquots and stored at –20°C in the dark. The compounds were thawed just before use and diluted in RPMI 1640 medium (Bio-Whittaker, Boehringer Ingelheim) to the desired concentrations. All the solutions were sterilized by filtration on 0.22-μm filters (Celbio).

We investigated the antioxidant potential of olive oil phenols in 2 different conditions as follows: 1) the cell suspensions (HL60 or PBMC) were enriched with different compounds in RPMI 1640 complete medium and then immediately treated with 40 μmol/L H<sub>2</sub>O<sub>2</sub> for 30 min at 37°C; 2) the cells (lymphocytes) were exposed to the reactive oxygen species produced by activated monocytes. For this purpose, cell suspensions were enriched with different compounds in RPMI 1640 complete medium and then cocultured for 1 h at 37°C and 5% CO<sub>2</sub>

with the adherent monocytes (prepared as reported above) either activated or not with PMA (2  $\mu\text{mol/L}$ ). In both cases, after incubation, the cells were recovered and we evaluated the viability and the DNA damage using the trypan blue exclusion technique and the comet assay, respectively (33).

**Single cell gel electrophoresis (comet assay).** The single cell gel electrophoresis assay was performed essentially as described by Singh et al. (40). Briefly, cells were included in the low-melting agarose (0.7% in PBS) and placed in a lysis solution (2.5 mol/L NaCl, 100 mmol/L Na<sub>2</sub>EDTA, 10 mmol/L Tris-HCl, pH 10, containing freshly added 1% Triton  $\times$ 100 and 10% dimethylsulfoxide) for 1 h at 4°C. Electrophoresis was carried out in freshly made buffer (1 mmol/L Na<sub>2</sub>EDTA, 300 mmol/L NaOH, pH 13.0) for 20 min at a fixed voltage of 25V (300 mA). After electrophoresis, the slides were neutralized (0.4 mol/L Tris-HCl, pH 7.5) and stained with ethidium bromide (20 mg/L).

**Comet detection and statistical analysis.** The comets ( $n = 100$ ) were analyzed 24 h after staining at 400 $\times$  magnification using a fluorescence microscope (Zeiss, R.G.) equipped with a 50-W mercury lamp. The damage for each slide was expressed in arbitrary units, which is a parameter derived from the “tail moment” as previously described (41).

The results of each experiment, repeated 5 times ( $n = 5$ ) using PBMC preparations obtained from different donors, were compared using a 1-way ANOVA. When a significant ( $P < 0.05$ ) treatment effect was detected, the means were compared using Tukey's post hoc comparison test.

## Results

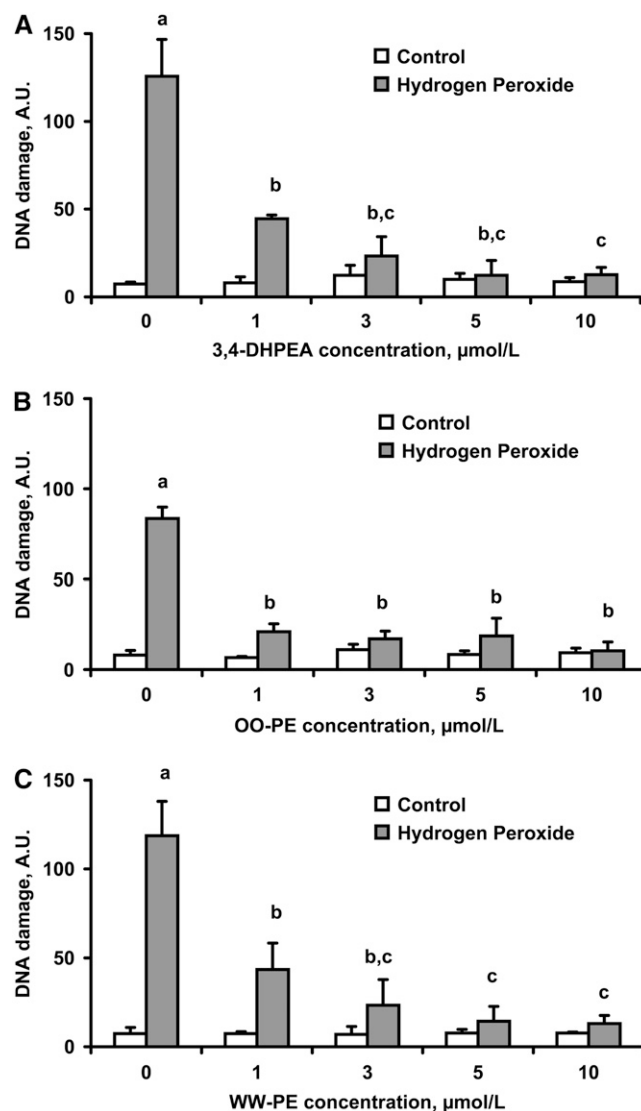
HPLC analysis of the phenolic composition of OO-PE and WW-PE showed more 3,4-DHPEA and 3,4-DHPEA-EDA in WW-PE than in OO-PE (Table 1). In addition, verbascoside was not found in the OO-PE and 3,4-DHPEA-EA, p-HPEA-EDA, and (+)-1-acetoxypinoresinol were not present in the WW-PE (Table 1). The extracts were dissolved at concentrations of 9.532 g/L (OO-PE) and 5.266 g/L (WW-PE) to have the same molar concentration of 3,4-DHPEA + 3,4-DHPEA-containing compounds in the culture medium used for cell exposure (Table 1). 3,4-DHPEA was used as reference compound, because several studies have demonstrated its potent antioxidant activity.

We performed preliminary experiments to determine both the concentration of H<sub>2</sub>O<sub>2</sub> and the time of exposure of the cells, which gave an appreciable genotoxic effect without cytotoxicity. We found that the optimal conditions for treating cells were 40  $\mu\text{mol/L}$  H<sub>2</sub>O<sub>2</sub> and 30 min of incubation at 37°C. Enrichment of the exposure medium during the H<sub>2</sub>O<sub>2</sub> treatment with purified 3,4-DHPEA, OO-PE, and WW-PE reduced the DNA damage to

**TABLE 1** Concentrations of different compounds present in OO-PE and from WW-PE

|                                      | Extract |                   | Culture medium    |       |
|--------------------------------------|---------|-------------------|-------------------|-------|
|                                      | OO-PE   | WW-PE             | OO-PE             | WW-PE |
|                                      | mg/g    |                   | $\mu\text{mol/L}$ |       |
| 3,4-DHPEA                            | 8.0     | 48.7              | 0.50              | 1.65  |
| 3,4-DHPEA-EDA                        | 205.7   | 502.1             | 6.13              | 8.20  |
| 3,4-DHPEA-EA                         | 133.6   | n.d. <sup>1</sup> | 3.37              | n.d.  |
| Verbascoiside                        | n.d.    | 17.7              | n.d.              | 0.15  |
| Total 3,4-DHPEA-containing compounds | 347.3   | 568.5             | 10                | 10    |
| p-HPEA                               | 7.1     | 4.3               | 0.49              | 0.16  |
| p-HPEA-EDA                           | 238.3   | n.d.              | 7.47              | n.d.  |
| (+)-1-Acetoxypinoresinol             | 86.5    | n.d.              | 1.98              | n.d.  |
| Total phenols                        | 679.2   | 572.8             | 19.94             | 10.16 |

<sup>1</sup> n.d., Not detected.

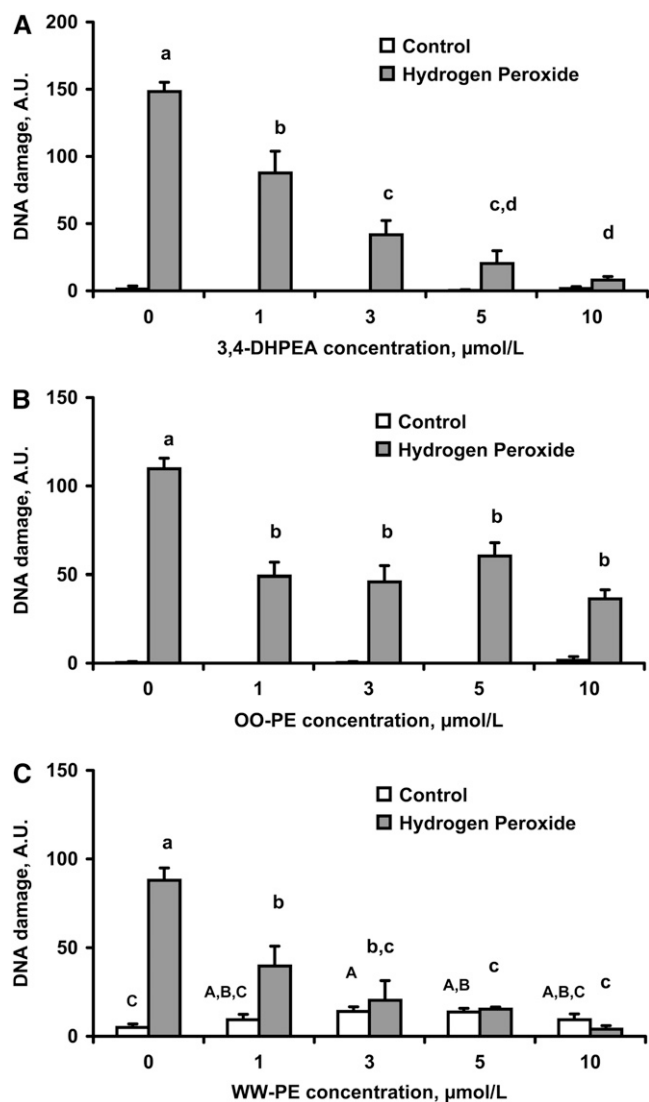


**FIGURE 1** Effect of increasing concentrations of 3,4-DHPEA (A), OO-PE (B), and WW-PE (C) on DNA damage of HL60 cells that were untreated or treated with H<sub>2</sub>O<sub>2</sub> 40  $\mu\text{mol/L}$  for 30 min. Values are means  $\pm$  SD,  $n = 5$ . Means without a common letter differ,  $P < 0.05$ .

both PBMC (Fig. 1) and HL60 cells (Fig. 2). Both 3,4-DHPEA and phenol mixtures significantly decreased the H<sub>2</sub>O<sub>2</sub> genotoxicity at concentrations as low as 1  $\mu\text{mol/L}$ . In addition, WW-PE slightly but significantly affected DNA damage in the absence of H<sub>2</sub>O<sub>2</sub> (Fig. 2C).

Different phenolic compounds, structurally related to 3,4-DHPEA and present in OO-PE and WW-PE, reduced the DNA damage caused by H<sub>2</sub>O<sub>2</sub> (Table 2). In both cell types, the protective activity of 3,4-DHPEA-EDA was similar to that of 3,4-DHPEA (considered the reference compound), whereas the protective activities of 3,4-DHPEA-EA, oleuropein, p-HPEA, and p-HPEA-EDA were significantly lower compared with that of 3,4-DHPEA (Table 2). In addition, caffeic acid and verbascoside had less effect than 3,4-DHPEA in HL60 cells (Table 2).

The ability of the phenols to prevent DNA damage also was investigated in a model system in which the oxidative stress was induced by PMA-activated monocytes. Freshly isolated lymphocytes were coincubated with monocytes (attached to the bottom of the 96-well plate) either stimulated or not with 2  $\mu\text{mol/L}$  of PMA, a protein kinase C activator. After 1 h of incubation at



**FIGURE 2** Effect of increasing concentrations of 3,4-DHPEA (A), OO-PE (B), and WW-PE (C) on DNA damage of PBMC either untreated or treated with  $\text{H}_2\text{O}_2$  40  $\mu\text{mol/L}$  for 30 min. Values are means  $\pm$  SD,  $n = 5$ . Means without a common letter differ,  $P < 0.05$ .

37°C, lymphocytes were removed and assayed for DNA damage. The presence of PMA increased DNA damage in lymphocytes (Fig. 3). When lymphocytes were exposed to PMA-activated monocytes in the presence of the different phenolic compounds, the DNA damage was significantly reduced (Fig. 3). Surprisingly, in this experimental system, p-HPEA was more effective than 3,4-DHPEA in preventing oxidative DNA damage. 3,4-DHPEA reduced DNA damage 45% and p-HPEA, 69% (Fig. 3).

## Discussion

Although different in vitro systems have shown that olive oil phenols possess a potent antioxidant activity (16,17) and prevent the reactive oxygen species-mediated cell injury (26–30), there is limited and contradictory evidence for such a protective role on DNA damage. For instance, 3,4-DHPEA promotes both the bleomycin-Fe(III)-dependent DNA damage (42) and the copper-dependent chemical modification of DNA bases (24). In addition, olive oil extracts and purified oleuropein were found to exert a genotoxic effect on Jurkat cells when

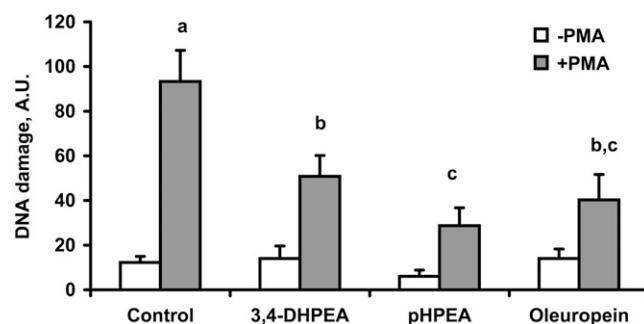
**TABLE 2** Preventive effect of different compounds (10  $\mu\text{mol/L}$ ) on DNA damage induced by treatment with  $\text{H}_2\text{O}_2$  (40  $\mu\text{mol/L}$ ) on HL60 cells and PBMC<sup>1</sup>

| Compounds, 10 $\mu\text{mol/L}$ | DNA damage reduction       |                              |
|---------------------------------|----------------------------|------------------------------|
|                                 | PBMC                       | HL60                         |
|                                 | %                          |                              |
| 3,4-DHPEA                       | 93 $\pm$ 4 <sup>a</sup>    | 89 $\pm$ 12 <sup>a</sup>     |
| 3,4-DHPEA-EDA                   | 93 $\pm$ 4 <sup>a</sup>    | 83 $\pm$ 11 <sup>a</sup>     |
| 3,4-DHPEA-EA                    | 62 $\pm$ 7 <sup>b</sup>    | 67 $\pm$ 15 <sup>a,b</sup>   |
| Oleuropein                      | 60 $\pm$ 4 <sup>b</sup>    | 25 $\pm$ 2 <sup>c,d</sup>    |
| p-HPEA                          | 67 $\pm$ 15 <sup>b</sup>   | 23 $\pm$ 16 <sup>d</sup>     |
| p-HPEA-EDA                      | 65 $\pm$ 14 <sup>b</sup>   | 30 $\pm$ 9 <sup>c,d</sup>    |
| Caffeic acid                    | 75 $\pm$ 8 <sup>a,b</sup>  | 50 $\pm$ 16 <sup>b,c</sup>   |
| Verbascoside                    | 78 $\pm$ 12 <sup>a,b</sup> | 48 $\pm$ 14 <sup>b,c,d</sup> |

<sup>1</sup> Values are means  $\pm$  SD,  $n = 5$ . Means in a column without a common letter differ,  $P < 0.05$ .

tested at concentrations of 100  $\mu\text{g/L}$  and 100  $\mu\text{mol/L}$ , respectively (43). Nevertheless, oleuropein and p-HPEA, in concentrations ranging from 10 to 1000  $\mu\text{mol/L}$ , did not prevent the DNA damage induced by exposure of the Jurkat cells to  $\text{H}_2\text{O}_2$  continuously produced by the enzyme glucose oxidase (43). Furthermore, p-HPEA demonstrated a genotoxic effect on whole blood cells at 50  $\mu\text{mol/L}$  when assayed by the atypical comet assay (44). Recently, oleuropein and olive mill wastewater were demonstrated to be genotoxic by the micronucleus test (45). On the other hand, 3,4-DHPEA showed a protective effect toward the oxidative DNA damage induced by peroxynitrite ( $\text{ONOO}^-$ ) on neuronal hybridoma cells at high concentrations (0.25–1.0 mmol/L) (46). Instead, we demonstrated that phenols, when used both as purified compounds and in a complex crude extract and regardless of the source (i.e. olive oil or olive mill wastewater) may prevent the  $\text{H}_2\text{O}_2$ -induced DNA damage in a very low concentration range (1–10  $\mu\text{mol/L}$ ). These concentrations could be easily reached in the tissues with an ordinary intake of 50 g/d of olive oil, because phenols are effectively absorbed in humans (47). Indeed, a recent intervention study has shown that an intake of 40 mL of olive oil containing a considerable amount of phenols (366 mg/kg) can result in a plasma concentration of 3,4-DHPEA and p-HPEA above 10  $\mu\text{mol/L}$  during the first 4 h after ingestion (48).

Our results agree partially with those published by Quiles et al. (49), who found a slight protective effect (25%) on human prostate cancer cells by 10  $\mu\text{mol/L}$  of 3,4-DHPEA. However, in



**FIGURE 3** Effect of 3,4-DHPEA, p-HPEA, and oleuropein (10  $\mu\text{mol/L}$ ) on DNA damage of lymphocytes coincubated with monocytes either untreated or stimulated with PMA (2  $\mu\text{mol/L}$ ) for 1 h. Values are means  $\pm$  SD,  $n = 5$ . Means without a common letter differ,  $P < 0.05$ .

this study, the experimental conditions were different from our conditions, because the cells were preincubated with the phenols for 24 h and then stressed by  $\text{H}_2\text{O}_2$  60  $\mu\text{mol/L}$  for 5 min in incomplete medium. In addition, we obtained further evidence supporting the hypothesis that the phenolic compounds may efficiently prevent the oxidative DNA damage at low concentrations by means of the ex vivo system of PMA-activated monocytes. This model simulates more closely the oxidative stress in vivo, because the treatment of monocytes with PMA activates both the NADPH oxidase, which catalyses the 1-electron reduction of oxygen to form  $\text{O}_2^{\cdot-}$  and the NO synthase, which produces NO. These highly reactive compounds may be the precursors of a wide spectra of reactive species that are able to induce DNA damage on lymphocytes (33). It is important to emphasize that in the PMA-activated monocyte system, p-HPEA was more efficient than 3,4-DHPEA in preventing DNA damage. These results were unexpected, because the *ortho*-diphenols, i.e. 3,4-DHPEA, are more potent radical scavengers and effective antioxidants than simple phenols, i.e. p-HPEA (50). However, the reactive species produced by activated monocytes are different and more complex than the simple  $\text{H}_2\text{O}_2$ . In addition, a definitive answer to the ability of 3,4-DHPEA and p-HPEA to scavenge different reactive oxygen species is not available. For instance, some studies have shown a potent scavenger activity of 3,4-DHPEA against superoxide anions (51,52), whereas others have found a scavenger activity against  $\text{H}_2\text{O}_2$  but not against superoxide anions (53). The latter result is supported by the finding that 3,4-DHPEA protected the cells from  $\text{H}_2\text{O}_2$ -induced damage but did not affect superoxide anion-induced cell death (28). Data on the effects exerted by p-HPEA on the different reactive oxygen species are not available; therefore, the possibility remains that this compound could efficiently scavenge  $\text{O}_2^{\cdot-}$ , NO, and ONOO. On the other hand, it may be possible that p-HPEA acts on other cell functions, such as the endogenous antioxidant systems and DNA damage repair activity, which are independent from the antioxidant characteristic. It was recently found, for instance, that p-HPEA restored intracellular antioxidant defense in J774 A.1 cells (25). Furthermore, an inhibitory effect of p-HPEA on NADPH oxidase of the monocyte with the consequent reduction of superoxide production cannot be excluded, whereas 3,4-DHPEA does not affect this enzyme activity (53).

When the damage was produced by  $\text{H}_2\text{O}_2$ , the preventive activities of different phenols, in particular 3,4-DHPEA, caffeic acid, and p-HPEA, reflected their antioxidant potential as determined by the 2,2-diphenyl-1-picrylhydrazyl radical scavenging test (54,55). Indeed,  $\text{H}_2\text{O}_2$  causes DNA strand breaks by generation of the hydroxyl radical ( $\text{OH}^{\cdot}$ ) via the Fenton reaction in the presence of a  $\text{Fe}^{2+}$ . Therefore, olive oil phenols may act, in addition to their interference as free radical scavengers, as metal ion chelators (56). This latter property depends, at least in part, upon the presence in the phenol molecule of catechol moieties; in fact, catechol was reported to form a complex with  $\text{Fe}^{2+}$  at pH 7.4 hindering the reaction with  $\text{H}_2\text{O}_2$  to generate  $\text{OH}^{\cdot}$  (28).

Regarding the other secoiridoid compounds, the addition of the dialdehydic form of elenoic acid to both 3,4-DHPEA and p-HPEA does not modify the DNA damage preventive activity (a similar protective activity was found for 3,4-DHPEA and 3,4-DHPEA-EDA and for p-HPEA and p-HPEA-EDA), whereas the addition of the elenoic acid to 3,4-DHPEA reduced the effect (3,4-DHPEA-EA was less active than 3,4-DHPEA). These observations are in agreement with those of Visioli et al. (57), who showed a similar inhibitory potential of these compounds toward oxidation of LDL.

In conclusion, this study showed a potent DNA damage preventive activity of olive oil phenols, providing new evidence to support a possible role of these compounds in the prevention of cancer. In addition, the results obtained with WW-PE suggest that this industrial by-product could be an abundant and inexpensive material to obtain bioactive phenolic compounds.

## Literature Cited

- Garcia-Segovia P, Sanchez-Villegas A, Doreste J, Santana F, Serra-Majem L. Olive oil consumption and risk of breast cancer in the Canary Islands: a population-based case-control study. *Public Health Nutr*. 2006;9:163–7.
- Trichopoulou A, Katsouyanni K, Stuver S, Tzala L, Gnardellis C, Rimm E, Trichopoulos D. Consumption of olive oil and specific food groups in relation to breast cancer risk in Greece. *J Natl Cancer Inst*. 1995;87:110–6.
- Martin-Moreno JM, Willett WC, Gorgojo L, Banegas JR, Rodriguez-Artalejo F, Fernandez-Rodriguez JC, Maisonneuve P, Boyle P. Dietary fat, olive oil intake and breast cancer risk. *Int J Cancer*. 1994;58:774–80.
- La Vecchia C, Negri E, Franceschi S, Decarli A, Giacosa A, Lipworth L. Olive oil, other dietary fats, and the risk of breast cancer (Italy). *Cancer Causes Control*. 1995;6:545–50.
- Norrish AE, Jackson RT, Sharpe SJ, Skeaff CM. Men who consume vegetable oils rich in monounsaturated fat: their dietary patterns and risk of prostate cancer (New Zealand). *Cancer Causes Control*. 2000;11:609–15.
- Hodge AM, English DR, McCredie MR, Severi G, Boyle P, Hopper JL, Giles GG. Foods, nutrients and prostate cancer. *Cancer Causes Control*. 2004;15:11–20.
- Franceschi S, Favero A, Conti E, Talamini R, Volpe R, Negri E, Barzan L, La Vecchia C. Food groups, oils and butter, and cancer of the oral cavity and pharynx. *Br J Cancer*. 1999;80:614–20.
- Braga C, La Vecchia C, Franceschi S, Negri E, Parpinel M, Recarli A, Giacosa A, Trichopoulos D. Olive oil, other seasoning fats, and the risk of colorectal carcinoma. *Cancer*. 1998;82:448–53.
- Lasekan JB, Clayton MK, Gendron-Fitzpatrick A, Ney DM. Dietary olive and safflower oils in promotion of DMBA-induced mammary tumorigenesis in rats. *Nutr Cancer*. 1990;13:153–63.
- Bartoli R, Fernández-Bañares F, Navarro E, Castellà E, Mañé J, Alvarez M, Pastor C, Cabré E, Gassull MA. Effect of olive oil on early and late events of colon carcinogenesis in rats: modulation of arachidonic acid metabolism and local prostaglandin E(2) synthesis. *Gut*. 2000;46:191–9.
- Ichihashi M, Ahmed NU, Budiyo A, Wu A, Bito T, Ueda M, Osawa T. Preventive effect of antioxidant on ultraviolet-induced skin cancer in mice. *J Dermatol Sci*. 2000;23:S45–50.
- Thuy NT, He P, Takeuchi H. Comparative effect of dietary olive, safflower, and linseed oils on spontaneous liver tumorigenesis in C3H/He mice. *J Nutr Sci Vitaminol (Tokyo)*. 2001;47:363–6.
- De Flora S, Izzotti A, D'agostini F, Balansky RM, Noonan D, Albini A. Multiple points of intervention of cancer and other mutation-related diseases. *Mutat Res*. 2001;480/481:9–22.
- Boonstra J, Post JA. Molecular events associated with reactive oxygen species and cell cycle progression in mammalian cells. *Gene*. 2004;337:1–13.
- Halliwell B. Oxidative stress and cancer: have we moved forward? *Biochem J*. 2007;401:1–11.
- Vissers MN, Zock PL, Katan MB. Bioavailability and antioxidant effects of olive oil phenols in humans: a review. *Eur J Clin Nutr*. 2004;58:955–65.
- Baldioli M, Servili M, Perretti G, Montedoro GF. Antioxidant activity of tocopherols and phenolic compounds of virgin olive oil. *Journal of the American Oil Chemists' Society* 1996;73:1589–93.
- Servili M, Montedoro GF. Contribution of phenolic compounds to virgin olive oil quality. *Eur J Lipid Sci Technol*. 2002;104:602–13.
- Niaounakis M, Halvadakis CP. Olive-mill waste management. Athens: Typhito, George Dardanos; 2004.
- Servili M, Baldioli M, Selvaggini R, Miniati E, Macchioni A, Montedoro GF. HPLC evaluation of phenols in olive fruit, virgin olive

- oil, vegetation waters and pomace and 1D and 2D-NMR characterization. *Journal of the American Oil Chemists' Society*. 1999;76:873–82.
21. Servili M, Selvaggini R, Esposto S, Taticchi A, Montedoro GF, Morozzi G. Health and sensory properties of virgin olive oil hydrophilic phenols: agronomic and technological aspects of production that affect their occurrence in the oil. *J Chromatogr A*. 2004;1054:113–27.
22. Obied HK, Allen MS, Bedgood DR, Prenzler PD, Robards K. Bioactivity and analysis of biophenols recovered from olive mill waste. *J Agric Food Chem*. 2005;53:823–37.
23. Goya L, Mateos R, Bravo L. Effect of the olive oil phenol hydroxytyrosol on human hepatoma HepG2 cells: protection against oxidative stress induced by tert-butylhydroperoxide. *Eur J Nutr*. 2007;46:70–8.
24. Aruoma OI, Deiana M, Jenner A, Halliwell B, Kaur H, Banni S, Corongiu FP, Dessì MA, Aeschbach R. Effect of hydroxytyrosol found in extra virgin olive oil on oxidative DNA damage and on low-density lipoprotein oxidation. *J Agric Food Chem*. 1998;46:5181–7.
25. Di Benedetto R, Varl R, Scaccocchio B, Filesi C, Santangelo C, Giovannini C, Matarrese P, D'Archivio M, Masella R. Tyrosol, the major extra virgin olive oil compound, restored intracellular antioxidant defences in spite of its weak antioxidative effectiveness. *Nutr Metab Cardiovasc Dis*. 2007;17:535–45.
26. Manna C, Galletti P, Cucciola V, Moltedo O, Leone A, Zappia V. The protective effect of the olive oil polyphenol (3,4-dihydroxyphenyl)-ethanol counteracts reactive oxygen metabolite-induced cytotoxicity in Caco-2 cells. *J Nutr*. 1997;127:286–92.
27. Manna C, Galletti P, Cucciola V, Montedoro GF, Zappia V. Olive oil hydroxytyrosol protects human erythrocytes against oxidative damages. *J Nutr Biochem*. 1999;10:159–65.
28. Hashimoto T, Ibi M, Matsuno K, Tanigawa T, Yoshikawa T, Yabe-Nishimura C. An endogenous metabolite of dopamine, 3,4-dihydroxyphenylethanol, acts as a unique cytoprotective agent against oxidative stress-induced injury. *Free Radic Biol Med*. 2004;36:555–64.
29. Weinbrenner T, Fito M, de la Torre R, Saez GT, Rijken P, Tormos C, Coolen S, Albaladejo MF, Abanades S, et al. Olive oils high in phenolic compounds modulate oxidative/antioxidative status in men. *J Nutr*. 2004;134:2314–21.
30. Salvini S, Sera F, Caruso D, Giovannelli L, Visioli F, Sapeva C, Masala G, Ceroti M, Giovacchini V, et al. Daily consumption of a high-phenol extra-virgin olive oil reduces oxidative DNA damage in postmenopausal women. *Br J Nutr*. 2006;95:742–51.
31. Hillestrøm PR, Covas MI, Poulsen HE. Effect of dietary virgin olive oil on urinary excretion of etheno-DNA adducts. *Free Radic Biol Med*. 2006;41:1133–8.
32. Machowetz A, Poulsen HE, Gruendel S, Weimann A, Fito M, Marrugat J, de la Torre R, Salonen JT, Nyyssonen K. Effect of olive oils on biomarkers of oxidative DNA stress in Northern and Southern Europeans. *FASEB J*. 2007;21:45–52.
33. Fabiani R, De Bartolomeo A, Rosignoli P, Morozzi G. Antioxidants prevent the lymphocyte DNA damage induced by PMA-stimulated monocytes. *Nutr Cancer*. 2001;39:284–91.
34. Collins AR. The comet assay for DNA damage and repair. *Mol Biotechnol*. 2004;26:249–61.
35. Montedoro GF, Servili M, Baldioli M, Miniati E. Simple and hydrolyzable compounds in virgin olive oil. 1. Their extraction, separation and quantitative and semiquantitative evaluation by HPLC. *J Agric Food Chem*. 1992;40:1571–6.
36. Selvaggini R, Servili M, Urbani S, Esposto S, Taticchi A, Montedoro GF. Evaluation of phenolic compounds in virgin olive oil by direct injection in high-performance liquid chromatography with fluorometric detection. *J Agric Food Chem*. 2006;54:2832–8.
37. Montedoro GF, Servili M, Baldioli M, Selvaggini R, Miniati E, Macchioni A. Simple and hydrolyzable compounds in virgin olive oil. Note 3. Spectroscopic characterization of the secoiridoids derivatives. *J Agric Food Chem*. 1993;41:2228–34.
38. Servili M, Baldioli M, Selvaggini R, Macchioni A, Montedoro GF. Phenolic compounds of olive fruit: one- and two-dimensional nuclear magnetic resonance characterization of nüzhenide and its distribution in the constitutive parts of fruit. *J Agric Food Chem*. 1999;47:12–8.
39. Boyum A. Isolation of mononuclear cells and granulocytes from human blood. *Scand J Clin Lab Invest*. 1968;21:77–89.
40. Sing NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res*. 1988;175:184–91.
41. Collins AR, Ai-guo M, Duthie SJ. The kinetics of repair of oxidative DNA damage (strand breaks and oxidised pyrimidines) in human cells. *Mutat Res*. 1995;336:69–77.
42. Aeschbach R, Loliger J, Scott BC, Murcia A, Butler J, Halliwell B, Aruoma OI. Antioxidant actions of thymol, carvacrol, 6-geringol, zingerone and hydroxytyrosol. *Food Chem Toxicol*. 1994;32:31–6.
43. Nousis L, Doulas PT, Aliagannis N, Bazios D, Agalias A, Galaris D, Mitakou S. DNA protecting and genotoxic effects of olive oil related components in cells exposed to hydrogen peroxide. *Free Radic Res*. 2005;39:787–95.
44. Grasso S, Siracusa L, Spatafora C, Renis M, Tringali C. Hydroxytyrosol lipophilic analogues: enzymatic synthesis, radical scavenging activity and DNA oxidative damage protection. *Bioorg Chem*. 2007;35:137–52.
45. El Hajjoui H, Pinelli E, Guisresse M, Merlina G, Rvel JC, Hafidi M. Assessment of the genotoxicity of olive mill waste water (OMWW) with the *Vicia faba* micronucleus test. *Mutat Res*. 2007;634:25–31.
46. Deiana M, Aruoma OI, Bianchi MP, Spencer JPE, Kaur H, Halliwell B, Aeschbach R, Banni S, Dessi MA, et al. Inhibition of peroxynitrite dependent DNA base modification and tyrosine nitration by the extra virgin olive oil derived antioxidant hydroxytyrosol. *Free Radic Biol Med*. 1999;26:762–9.
47. Miró-Casas E, Covas MI, Fitó M, Farré-Albadalejo M, Marrugat J, de la Torre R. Tyrosol and hydroxytyrosol are absorbed from moderate and sustained doses of virgin olive oil in humans. *Eur J Clin Nutr*. 2003;57:186–90.
48. Covas MI, de la Torre K, Farré-Albadalejo M, Kaikkonen J, Fitó M, López-Sabater C, Pujadas-Bastardes MA, Joglar J, Weinbrenner T, et al. Postprandial LDL phenolic content and LDL oxidation are modulated by olive oil phenolic compounds in human. *Free Radic Biol Med*. 2006;40:608–16.
49. Quiles JL, Farquharson AJ, Simpson DK, Grant I, Wahle KWJ. Olive oil phenolics: effects on DNA oxidation and redox enzyme mRNA in prostate cells. *Br J Nutr*. 2002;88:225–34.
50. Rice-Evans CA, Miller JN, Paganga G. Structure-antioxidant activity relationship of flavonoids and phenolic acids. *Free Radic Biol Med*. 1996;20:933–56.
51. Visioli F, Bellomo G, Galli C. Free radical-scavenging properties of olive oil polyphenols. *Biochem Biophys Res Commun*. 1998;247:64–64.
52. Rietjens SJ, Bast A, Haenen GRMM. New insights into controversies on the antioxidant potential of the olive oil antioxidant hydroxytyrosol. 2007;55:7609–14.
53. O'Dowd Y, Driss F, My-Chan Dang P, Albim C, Gougerot-Pocidalo MA, Pasquier C, El-Benna J. Antioxidant effect of hydroxytyrosol, a polyphenol from olive oil: scavenging of hydrogen peroxide but not superoxide anion produced by human neutrophils. 2004;68:2003–8.
54. Stupans I, Kirlik A, Tuck KL, Hyball PJ. Comparison of radical scavenging effect, inhibition of microsomal oxygen free radical generation, and serum lipoprotein oxidation of several natural antioxidants. *J Agric Food Chem*. 2002;50:2464–9.
55. Carrasco-Pancorbo A, Cerretani L, Bendini A, Segura-Carretero A, Del Carlo M, Gallina-Toschi T, Lercker G, Compagnone D, Fernandez-Gutierrez A. Evaluation of the antioxidant capacity of individual phenolic compounds in virgin olive oil. *J Agric Food Chem*. 2005;53:8918–25.
56. Briante R, Febbraio F, Nucci R. Antioxidant properties of low molecular weight phenols present in the Mediterranean diet. *J Agric Food Chem*. 2003;51:6975–81.
57. Visioli F, Bellomo G, Montedoro GF, Galli C. Low density lipoprotein oxidation is inhibited in vitro by olive oil constituents. *Atherosclerosis*. 1995;117:25–32.