*Free Radical Research*, July 2005; 39(7): 787–795



# DNA protecting and genotoxic effects of olive oil related components in cells exposed to hydrogen peroxide

LAMBROS NOUSIS[1], PASCHALIS-THOMAS DOULIAS[1], NEKTARIOS ALIGIANNIS[2], DIMITRIOS BAZIOS[2], APOSTOLOS AGALIAS[2], DIMITRIOS GALARIS[1], & SOFIA MITAKOU[2]

[1]*Laboratory of Biological Chemistry, University of Ioannina Medical School, 451 10 Ioannina, Greece, and* [2]*Division of Pharmacognosy and Natural Product Chemistry, School of Pharmacy, University of Athens, 15771 Athens, Greece*

Accepted by Professor A. Collins

*(Received 2 April 2004; in revised form 3 August 2004)*



**Abstract**

In search for compounds, able to protect nuclear DNA in cells exposed to oxidative stress, extracts from olive leaves, olive fruits, olive oil and olive mill waste water were tested by using the "single cell gel electrophoresis" methodology (comet assay). Jurkat cells in culture were exposed to continuously generated hydrogen peroxide (11.8 ± 1.5 μM per min) by direct addition into the growth medium of the appropriate amount of the enzyme "glucose oxidase" in the presence or absence of the tested total extracts. The protective effects of the tested extracts or isolated compounds were evaluated from their ability to decrease hydrogen peroxide-induced formation of single strand breaks in the nuclear DNA, while the toxic effects were estimated from the increase of DNA damage when the extracts or isolated compounds were incubated directly with the cells. Significant protection was observed in extracts from olive oil and olive mill waste water. However, above a concentration of 100 μg/ml olive oil extracts exerted DNA damaging effects by themselves in the absence of any $H_2O_2$. Extracts from olive leaves and olive fruits although protective, were also able to induce DNA damage by themselves. Main compounds isolated from the above described total extracts, like oleuropein glucoside, tyrosol, hydroxytyrosol and caffeic acid, were tested in the same experimental system and found to exert cytotoxic (oleuropein glucoside), no effect (tyrosol) or protective effects (hydroxytyrosol and caffeic acid). In conclusion, cytoprotective as well as cytotoxic compounds with potential pharmaceutical properties were detected in extracts from olive oil related sources by using the comet assay methodology.

**Keywords:** *Comet assay, DNA damage, hydrogen peroxide, olive oil, olive mill waste water*

## Introduction

The interest in the Mediterranean diet derives from studies, which demonstrated that the mortality mainly from coronary heart disease but also from other causes was much lower in this area [1,2]. The Mediterranean diet, especially that prevailing some decades ago, was rich in fruits and vegetables, while a central role was played by olive oil. Olive oil, apart from being the source of fatty acids (primarily oleic acid), contains a number of phenolic compounds which are responsible for its stability to oxidative rancidity [3]. In addition, it is plausible to believe that uptake of such compounds through consumption of olive oil may provide resistance toward oxidative stress, which is regarded as major contributor to the development of the above mentioned diseases [1,4,5]. Thus, in last few years a significant increase in the number of publications regarding the determination of the composition and biological profiles of olive oil's phenolic fraction has been observed [6]. Major phenolic compounds present in olive oil, like tyrosol, hydroxytyrosol, oleuropein, caffeic acid and others are regarded as strong antioxidants and radical scavengers [7–9]. On the other hand, fresh olive drupes

Correspondence: D. Galaris, Laboratory of Biological Chemistry, University of Ioannina Medical School, 451 10 Ioannina, Greece. Tel: 30 26510 97562. Fax: 30 26510 97868. E-mail: dgalaris@cc.uoi.gr

ISSN 1071-5762 print/ISSN 1029-2470 online © 2005 Taylor & Francis Group Ltd
DOI: 10.1080/10715760500045806

are brined for several months to remove the bitterness and this process removes a portion of phenolic compounds. It is regarded that only about 1–2% of the available pool of antioxidants in the olive fruits is represented in olive oil, with the rest lost mainly in the waste water which is produced during the process of malaxation [10]. Although, waste water from olive oil mill is characterized by strong polluting activity owing to some harmful compounds included [11], it also possesses phenol compounds which are regarded as strong antioxidants. The results of a relative study have demonstrated that waste water extracts have powerful antioxidant activity and might represent a cheap, as yet unused, source of natural antioxidants [12]. Finally, olive tree leaves are also accumulated as a waste in olive oil mills although they are characterized by high levels of oleuropein glucoside [13], a secoiridoid glycoside that possesses high antioxidant capacity and other beneficial biological properties [14]. In contrast to the antioxidant capacity of the above compounds which has been extensively investigated, the knowledge about the mode of action of olive oil's constituents on intact cells exposed to conditions of oxidative stress, although of high interest, remains limited.

In last few years, we have been interested in the molecular mechanisms of DNA damage induced after exposure of cells to continuous but relatively low rates of generation of oxidants, like $H_2O_2$ and $ONOO^-$, which are known to be generated *in vivo* [15–18]. It has been observed that DNA is more sensitive than other cell constituents and for this reason more suitable as a marker for oxidant-induced effects on cells. Traditional antioxidants, when incubated with the cells before the addition of the $H_2O_2$, offered relatively low degree of protection, while iron and $Ca^{2+}$ chelators were strong protecting agents [15,17]. Moreover, in some cases molecules with no apparent antioxidant capacity were able to protect cells from $H_2O_2$-induced DNA damage by yet unknown mechanism(s) [19]. Based on the above observations, we decided to use the same experimental system in order to search in natural sources for molecules able to protect cellular DNA from damage after exposure of cells to oxidants.

In the present work, the ability of total extracts from olive leaves, olive fruits, olive oil and olive mill waste water as well as purified compounds from these extracts to protect nuclear DNA from $H_2O_2$-induced single strand break formation was investigated. Apart from detecting a variable degree of protection in the above extracts, components able to induce DNA damage by themselves in the absence of $H_2O_2$ were also observed.

## Materials and methods

### *Materials*

RPMI 1640 growth medium supplemented with L-glutamine and gentamycin, glucose oxidase (from *Aspergillus nigher*, 18.000 units/g), catalase (from bovine liver) and Hoechst 33342 were from Sigma Chemical Company (St. Louis, MO, USA). Fetal bovine calf serum, Nunc tissue culture plastics and low melting point agarose were obtained from Gibco BRL (Grant Island, NY, USA). Normal melting point agarose was obtained from Serva GmbH (Heidelberg, Germany). Microscope glass super frosted slides were supplied by Menzel-Glaset. All solvents were purchased from Lab-Scan (Stillorgan Ind. Park, Co. Dublin, Ireland) and were of analytical reagent grade except chromatographic analysis solvents which were of HPLC grade. All HPLC-solvents were filtered through an All-Glass Filter Holder System (47 mm, Waters) prior to use. Methanol-$d_3$ (MeOD), silica gel [Merck, 0.04–0.06 mm (flash) and 0.015–0.04 mm (60H)] and glass pre-coated silica gel 60 $F_{254}$ and RP-18 $F_{254}$ sheets were purchased from Merck (Darmstadt, Germany). All other chemicals used were of analytical grade.

### *Plant materials*

Olives of *Olea europaea* var. *koroneiki* were picked randomly from trees of an approximately 50-year-old olive grove in the island of Crete (Greece) during the crop season 2000–2001. Drupes, all of which had a green skin, were refrigerated at 4°C prior to processing. Olive leaves were collected from the branches of the same trees. Sample of Koroneiki variety virgin olive oil was obtained from oil mill located in the same area, filtered with anhydrous $Na_2SO_4$ and stored at 4°C in darkness. Olive waste waters were collected from the same oil mill during the above mentioned crop season.

### *Preparation of total extracts*

Dried fresh leaves (10 g) were pulverized and the obtained powder was extracted with methanol (3 × 50 ml) for 3 h. After filtration, the solvent was removed under reduced pressure (extract of olive leaves or OL). Freshly collected olives were immediately frozen in liquid nitrogen for 30 min, homogenized in a blender and 100 g of the powder obtained were extracted twice (30 min) with methanol:water (50:50, v/v; 200 ml). The combined extracts were filtered, and concentrated under reduced pressure and washed with petroleum ether (3 × 200 ml) to remove oil, free fatty acids and other lipid contaminants. The remaining aqueous solution was partitioned twice against ethyl acetate in a water to organic phase ratio of 1:1, then the ethyl acetate extract was filtered on sodium sulphate anhydrous and evaporated to dryness at 30°C under vacuum (extract of olive fruits or OF). Samples of olive oil (100 g) were extracted with methanol (5 × 30 ml) to receive constituent phenolics. The methanol layer was washed with cyclohexane

(3 × 150 ml) to remove remaining oil and evaporated (extract of olive oil or OO). Olive mill waste water was filtered and extracted with petroleum ether (2 × 750 ml). The water layer was extracted with ethyl acetate (3 × 500 ml), then the organic layer was collected and the solvent was removed under reduced pressure (extract of olive waste waters or OWW).

### *Isolation and identification of pure compounds*

Pulverized leaves of *O. europaea var. koroneiki* (10 g) were extracted with acetone (3 × 50 ml). After filtration, the solvent was removed under reduced pressure. The residue was washed with dichloromethane:methanol 98:2 (2 × 25 ml) and was subjected to vacuum-liquid chromatography on Silica gel (0.015–0.04 mm). Elution with a $CH_2Cl_2$:MeOH 85:15 yielded 0.37 g oleuropein glucoside. The residue of olive mill waste waters (2.1 g) was submitted to medium pressure liquid chromatography (MPLC) using silica gel 60H as static phase. Elution with $CH_2CI_2$:$CH_3OH$ (100:0 → 50:50) afforded 11 fractions. The third of these fractions (0.03 g), eluted with $CH_2Cl_2$:MeOH 97:3, was re-chromatographed on silica gel flash. Tyrosol (11 mg) was eluted with $CH_2Cl_2$:MeOH 98.5:1.5. The seventh fraction (0.1 g), eluted with $CH_2Cl_2$:MeOH 95:5, was chromatographed over silica gel flash and eluted with $CH_2Cl_2$:$CH_3OH$ (100:0 → 90:10) to give hydroxytyrosol (18 mg). A part of the extract of olive oil (OO) was evaporated and the residue was submitted to high pressure liquid chromatography (HPLC), to give caffeic acid (3 mg).

Total extracts and their fractions were routinely monitoring by thin-layer chromatography (TLC) on glass pre-coated silica gel 60 $F_{254}$ and RP-18 $F_{254}$ sheets, with detection under 254 and 366 nm UV lamps and by spraying with a methanolic solution of vanillin sulfate. Flash column chromatography was carried out using silica gel flash (Merck, 0.040–0.060 mm) with an applied pressure of 300 mbar. MPLC was performed with a Büchi model 688 apparatus.

### *HPLC analysis*

A gradient elution program was used for the isolation of oleuropein glucoside. Solvent A was 0.05 M ammonium acetate buffer (adjusted to pH 5.0 with glacial acetic acid), solvent B was acetonitrile, and the flow-rate was 1 ml/min. The gradient changed as follows: 90% A/10% B–80% A/20% B in 4 min, 40% A/60% B in 4 min, 30% A/70% B in 3 min and 90% A/10% B until the end of the run. At the end of each run, i.e. 15 min, the column was left to equilibrate at the starting mobile phase composition (i.e. 90% A–10% B) for an additional 5 min, giving a total chromatographic analysis time of 20 min. All mobile phases were vacuum filtered through a 0.2-μm Titan membrane filter (Scientific Resources, USA) and degassed in an ultrasonic bath prior to HPLC analysis. The column was maintained at 40°C throughout all experiments with the aid of an electronically controlled oven. UV spectra of all substances were recorded with the aid of the diode array detection system and the maxima of absorbance were determined at 280 nm for oleuropein glucoside. Identification and quantisation of the eluting peaks was performed by comparing their retention time values ($t_R$) and the corresponding UV spectra (obtained from the diode array data) with those of the standards (Table I).

### *Structural analysis of the isolated compounds*

Structural elucidation of the isolated compounds was achieved by means of spectral data (nuclear magnetic resonance (NMR) 1D and 2D, MS). $^1$H NMR spectra were measured on a Bruker DRX400 spectrometer (400 MHz) and $^{13}$C NMR on a Bruker AC200 spectrometer (50 MHz). The 2D (COSY, COSY-LR, HMQC, and HMBC) experiments were performed using standard Bruker microprograms. High resolution electrospray ionization mass spectrum (HR-ES-MS) was obtained in positive mode on a Q-Tof 1 Micromass mass spectrometer equipped with a standard Z-spray source. Optical rotations were measured with a Perkin-Elmer 341 polarimeter. The purity of components was verified by comparison of these values and spectra data with previously reported data.

### *NMR data of the isolated compounds*

*Tyrosol.* $^1$H NMR ($CD_3OD$): 7.05 (2H, d, $\mathcal{J}$ = 8.3 Hz, H-2/H-6), 6.71 (2H, d, $\mathcal{J}$ = 8.3 Hz, H-3/H-5), 3.70 (2H, t, $\mathcal{J}$ = 7.0 Hz, H-8), 2.73 (2H, t, $\mathcal{J}$ = 7.0 Hz, H-7); $^{13}$C NMR ($CD_3OD$): 157.5 (C-4), 131.6 (C-5/C-3), 131.6 (C-1), 65.1 (C-8), 40.3 (C-7).

Table I. Concentrations of phenolic compounds in olive leaves, olive fruits, olive and olive mill waste waters.

| | Oleuropein glucoside | Tyrosol | Hydroxytyrosol | Caffeic acid |
|---|---|---|---|---|
| Olive leaves (mg/g of dry weight) | 2.86 | – | – | – |
| Olive fruits (mg/g of dry weight) | 5.26 | 0.41 | 1.05 | 1.70 |
| Olive oil (mg/kg) | 2.80 | 0.21 | 0.15 | 0.04 |
| OMWW (mg/100ml) | – | 4.10 | 13.1 | 0.40 |

*Hydroxytyrosol*. $^1$H NMR ($CD_3OD$): 6.82 (1H, d, $\mathcal{J}$ = 1.7 Hz, H-2), 6.72 (1H, d, $\mathcal{J}$ = 7.8 Hz, H-5), 6.66 (1H, dd, $\mathcal{J}$ = 1.7, 7.8 Hz, H-6), 3.86 (3H, s, $CH_3O$), 3.72 (2H, t, $\mathcal{J}$ = 7.2 Hz, H-8), 2.75 (2H, t, $\mathcal{J}$ = 7.2 Hz, H-7); $^{13}$C NMR ($CD_3OD$): 147.2 (C-3), 144.0 (C-4), 130.2 (C-1), 120.4 (C-6), 115.9 (C-5), 111.6 (C-2), 62.8 (C-8), 55.6 ($CH_3O$), 40.0 (C-7).

*Caffeic acid*. $^1$H NMR ($CD_3OD$): 7.53 (1H, d, $\mathcal{J}$ = 16.0 Hz, H-7), 7.02 (1H, d, $\mathcal{J}$ = 2.0 Hz, H-2), 6.92 (1H, dd, $\mathcal{J}$ = 8.0, 2.0 Hz, H-6), 6.77 (1H, d, $\mathcal{J}$ = 8.0 Hz, H-5), 6.21 (1H, d, $\mathcal{J}$ = 15.5 Hz, H-8); $^{13}$C NMR ($CD_3OD$): 171.5 (C-9), 149.4 (C-4), 146.9 (C-7), 146.7 (C-3), 128.1 (C-1), 122.8 (C-6), 116.6 (C-5), 116.3 (C-8), 115.2 (C-2).

*Oleuropein glucoside*. $^1$H NMR ($CD_3OD$): 7.50 (s, H-3), 6.79 (d, $\mathcal{J}$ = 8.0 Hz, H-7′), 6.66 (d, $\mathcal{J}$ = 1.9 Hz, H-4′), 6.54 (dd, $\mathcal{J}$ = 8.0, 1.9 Hz, H-8′), 6.07 (br q, $\mathcal{J}$ = 7.2 Hz, H-8), 5.90 (br s, H-1), 4.81 (d, $\mathcal{J}$ = 7.6 Hz, H-1′(Glu)), 4.20 (dt, $\mathcal{J}$ = 10.7, 7.0 Hz, $H_a$-1′), 4.09 (dt, $\mathcal{J}$ = 10.7, 7.0 Hz, $H_b$-1′), 3.96 (dd, $\mathcal{J}$ = 9.2, 4.4 Hz, H-5), 3.70 (3H, s, COOMe), 2.75 (2H, t, $\mathcal{J}$ = 7.0 Hz, H-2′), 2.70 (dd, $\mathcal{J}$ = 14.1, 4.4Hz, $H_a$-6), 2.42 (dd, $\mathcal{J}$ = 14.1, 9.2 Hz, $H_b$-6), 1.65 (3H, dd, $\mathcal{J}$ = 7.2, 1.3 Hz, H-10); $^{13}$C NMR ($CD_3OD$): 173.1 (C-7), 168.6 (COOMe), 155.1 (C-3), 146.1 (C-5′), 144.8 (C-6′), 130.7 (C-3′), 130.5 (C-9), 124.8 (C-8), 121.3 (C-8′), 117.0 (C-7′), 116.5 (C-4′), 109.4 (C-4), 100.9 (C-1′Glu), 95.2 (C-1), 66.8 C-2′), 51.9 (COOMe), 41.2 (C-6), 35.3 (C-1′), 31.7 (C-5), 13.6 (C-10).

### *Cell culture and treatment*

The ability of the above extracts and the isolated pure compounds to protect cellular DNA from $H_2O_2$-induced damage was investigated by using Jurkat cells (a human T-lymphocytic cell line, ATCC, clone E6-1). One hundred microliters RPMI 1640 growth medium (supplemented with 10% fetal calf serum, penicillin 100 IU/ml, streptomycin 100 and 300 μg/ml glutamine) containing $1.5 \times 10^5$ cells were placed into each of 96 wells of ELISA plastic plates and incubated for 1 h at 37°C, 95% air, 5% $CO_2$. Cells were subsequently treated for 10 min with 60 ng of the enzyme glucose oxidase which was able to generate 11.8 ± 1.5 μM $H_2O_2$ per min in the absence of cells. Additions of the extracts or isolated compounds at the indicated concentrations were done 30 min prior to the addition of glucose oxidase. Following the treatment, cells were collected by centrifugation (250*g*, 4°C for 5 min) for further analysis.

### *Single cell gel electrophoresis*

The assay used was essentially the same as previously described [16,17,20,21]. Cells were suspended in 1% low-melting-point agarose in PBS (pH 7.4) and pipetted onto superfrosted glass microscope slides precoated with a layer of 1% of normal-melting-point agarose (warmed at 37°C prior to use). The agarose was allowed to set at 4°C for 10 min and then the slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris at pH 10, 1% Triton X-100 v/v) at 4°C for 1 h in order to remove cellular proteins. Slides were then placed in single rows in a 30-cm wide orizontal electrophoresis tank containing 0.3 m NaOH and 1 mM EDTA, pH > 13 at 4°C for 40 min in order to allow for separation of the two DNA strands (alkaline unwinding). Electrophoresis was performed in the unwinding solution at 30 V (1 V/cm), 300 A for 30 min. The slides were then washed three times for 5 min each with 0.4 M Tris, pH 7.5 at 4°C before staining with Hoechst 33342 (20 μg/ml).

### *Image analysis and scoring*

Stained nucleoids were examined under a UV microscope with an excitation filter of 435 nm and a magnification of 400. The damage was not homogeneous and visual scoring of the cellular DNA on each slide was based on characterization of 100 randomly selected nucleoids. The comet-like DNA formations were categorized into 5 classes (0, 1, 2, 3 and 4) representing an increasing extent of DNA damage seen as a "tail". Each comet was assigned a value according to its class. Accordingly, the overall score for 100 comets ranged from 0 (100% of comets in class 0) to 400 (100% of comets in class 4). In this way the overall DNA damage of the cell population can be expressed, in arbitrary units [22]. Visual scoring expressed in this way correlates near linearly with other parameters such as percent of DNA in tail estimated after computer image analysis using a specific software package [22,23]. Observation and analysis of the results were always carried out by the same experienced person, using a specific pattern when moving along the slide.

### *Statistical analysis*

Student's *t*-test analysis was used in order to examine statistically significant differences. The differences between total minus control (background) values were estimated and tested for statistical significance. Each value represents the mean ± SD of triplicate measurements of two independent experiments.

## Results

Addition of $H_2O_2$ to the culture medium of Jurkat cells induced a rapid and strong induction in the formation of single strand breaks in the nuclear DNA (Figure 1). However, incubation of the cells for 30 min before the addition of $H_2O_2$ with increasing

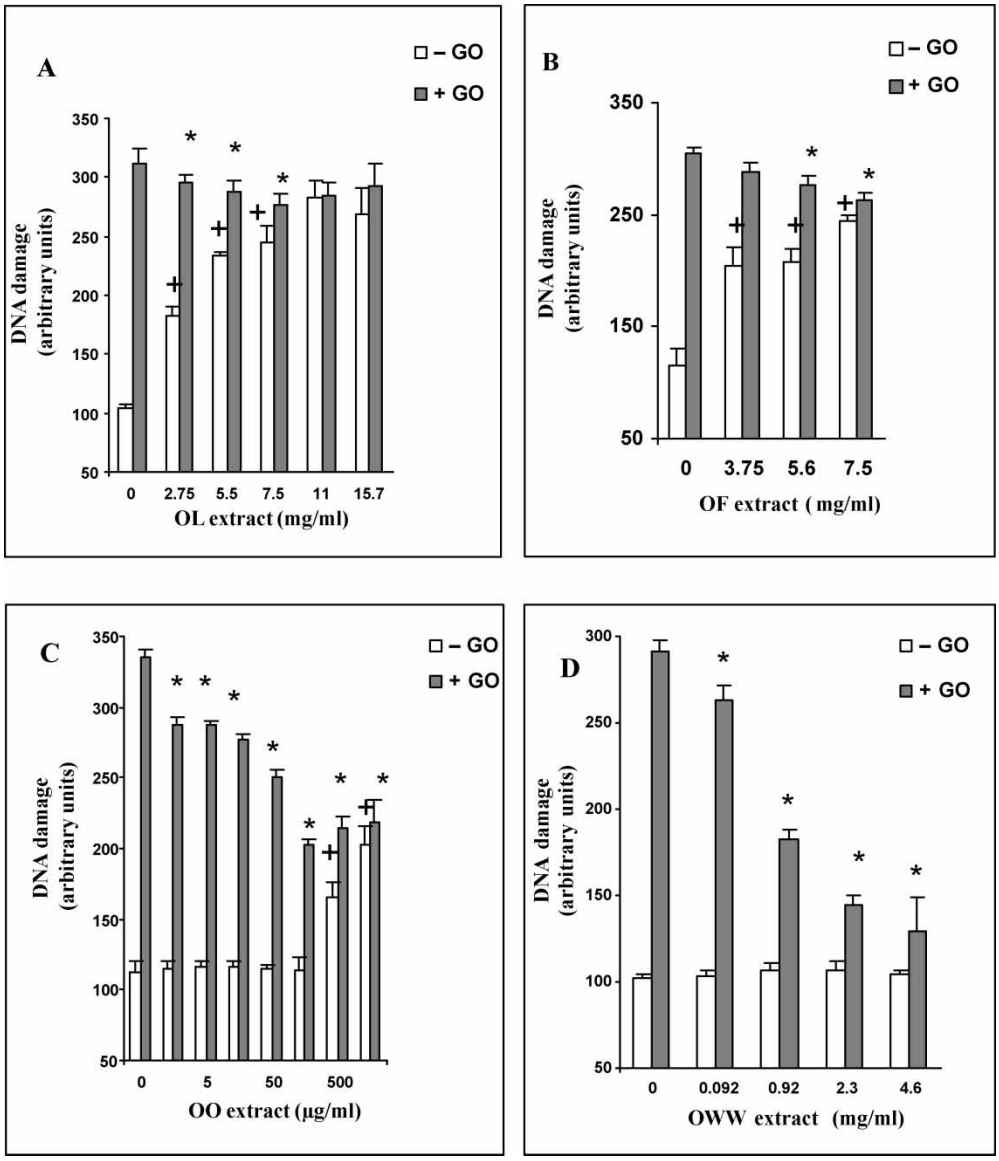
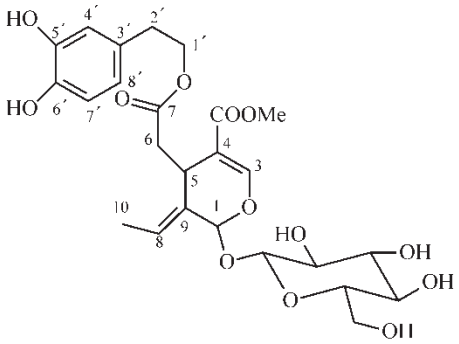


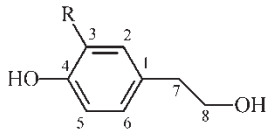

Figure 1. Effects of extract pre-incubation on $H_2O_2$-induced single strand break formation in DNA. One hundred micro-liters of culture medium containing $1.5 \times 10^6$ Jurkat cells/ml were placed into each of 96 wells of ELISA plastic plate and incubated for 30 min with the indicated concentrations of olive leaf (OL) (A) olive fruit (OF) (B), olive oil (OO) (C) and olive mill waste water (OMWW) (D) extracts. Following, glucose oxidase (GO) (60 ng/well, able to generate about 12 μM $H_2O_2$ per min) (filled bars) or the vehicle (open bars) was added. After 10 min, the DNA damage of individual cells was estimated by comet assay and expressed in arbitrary units, as described in "Materials and Methods". Each value represents the mean ± SD of triplicate measurements of two independent experiments (*$p < 0.01$ versus $H_2O_2$-treated samples, $^+p < 0.001$ versus untreated samples).

concentrations of extracts from olive leaves (OL), and olive fruits (OF), isolated as described in "Materials and Methods", significantly decreased the ability of $H_2O_2$ to induce DNA damage (Figure 1A and B, respectively) ($p < 0.01$). The same extracts, however, were able to induce DNA damage by themselves in the absence of $H_2O_2$, as indicated by the increased formation of single strand breaks (open bars in Figure 1A and B) ($p < 0.001$). Incubation of the same cells with extracts from olive oil (OO) offered strong protection (about 60% decrease of DNA damage) at relatively low concentrations (up to 75 μg/ml), while at concentrations of 100 μg/ml or higher they exerted genotoxic effects (Figure 1C). Finally, extracts from olive mill waste waters (OMWW) offered significant protection (58 and 84% reduction at concentrations 0.92 and 4.6 mg/ml, respectively) while genotoxicity was not observed in this case at the concentrations tested (Figure 1D). These results clearly indicate the presence of both protective and genotoxic agents in OL, OF and OO extracts, while only protective compounds were detected in the OWW extract.

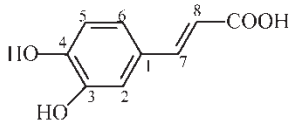
Individual components from the above tested sources, like oleuropein glucoside, tyrosol, hydroxytyrosol and caffeic acid (see chemical formulas in Scheme 1) were purified as described in "Materials




Oleuropein glucoside




R=H: Tyrosol
R=OH: Hydroxytyrosol




Caffeic Acid

Scheme 1. Chemical structures of the tested compounds.

and Methods" (Table I) and tested in the same experimental system. As shown in Figure 2A, the main constituent of olive leave and olive fruit extracts, namely oleuropein glucoside, while not protective against $H_2O_2$-induced DNA damage, was able to create single strand breaks by itself in the absence of $H_2O_2$ ($p < 0.001$ for all concentrations tested). These results are in accordance with previous reports that oleuropein glucoside, in spite of its well known antioxidant and membrane protecting properties was cytotoxic against human cells in culture [24]. On the other hand, hydroxytyrosol, but not tyrosol, which lacks the hydroxyl group at position 3, was able to protect cells from $H_2O_2$-induced DNA damage, indicating the need of an ortho-dihydroxy moiety for protective activity (Figure 2B and C). In addition to hydroxytyrosol, caffeic acid which contains the same ortho-dihydroxy catechol moiety was also protective (Figure 2D) albeit at higher concentrations, compared to hydroxytyrosol ($IC_{50}$s 1.5 and 0.25 mM respectively), indicating a negative influence of the carboxyl group on the activity of caffeic acid.

Furthermore, when 0.1 mM hydroxytyrosol was pre-incubated with the cells for increasing periods of time, a rapid protection was observed (about 50% protection after 5 min), which was gradually decreased as the incubation time was extended up to 2 h (Figure 3A), indicating probably the metabolic inactivation of this compound. When cells were pre-incubated with 1.0 mM caffeic acid for increasing periods of time, a gradual increase in protection was observed up to 30 min of incubation, while at longer incubation periods genotoxic effects were apparent (Figure 3B) indicating that the same compound can act either as protective or genototoxic depending on the duration of incubation with the cells.

Treatment of the cells with hydrogen peroxide or extracts or isolated compounds for the time periods used in this study did not induce any direct cytotoxicity as indicated by trypan blue exclusion tests, thus excluding the possibility that the observed genotocic effects are simply a secondary effect of cytotoxicity. However, caffeic acid could be cytotoxic if incubated for long periods (12–24 h) (results not shown).

## Discussion

The capacity of several extracts or isolated compounds from olive-related products to protect cells against $H_2O_2$-induced DNA damage was investigated in the present study. For this purpose, extracts from olive leaves, olive fruits, virgin oil and waste waters from the olive oil production process were used. The results presented in Figure 1 clearly indicate the presence of cytoprotective as well as cytotoxic compounds in the above extracts. It is well known, indeed, that a great number of mutagenic and carcinogenic compounds are present in several natural sources [25].

The fact that a considerable part of DNA protective capacity was found in the olive mill waste water raises the question of appropriate procedures during olive oil production. The modern technologies used today, in contrast to previous decades when the main epidemiological studies took place, probably achieve higher gains of the final product but may lose substances with important roles in health promoting effects of olive oil. Thus, the quality of olive oil consumed today, compared to the quality of olive oil produced some decades earlier, may be one out of many factors explaining World Health Organization statistics which indicate a steady increase of chronic diseases in Greece [26]. Isolation and reuse of natural antioxidants from waste water of olive oil industry apart from the economical importance may contribute to decrease the environmental effects of this by-product [27].

In an attempt to identify individual compounds with cytoprotective or cytotoxic properties, the above extracts were fractionated and several main compounds were isolated and tested in the same experimental system. Comparing the effects of tyrosol with those of hydroxytyrosol and caffeic acid (Figure 2B, C and D) it is reasonable to conclude that the presence of aromatic rings with ortho-dihydroxy moieties are required in order for the compounds to be effective. It has to be noted that hydroxytyrosol and caffeic acid have been reported previously to exert tective effects in a number of

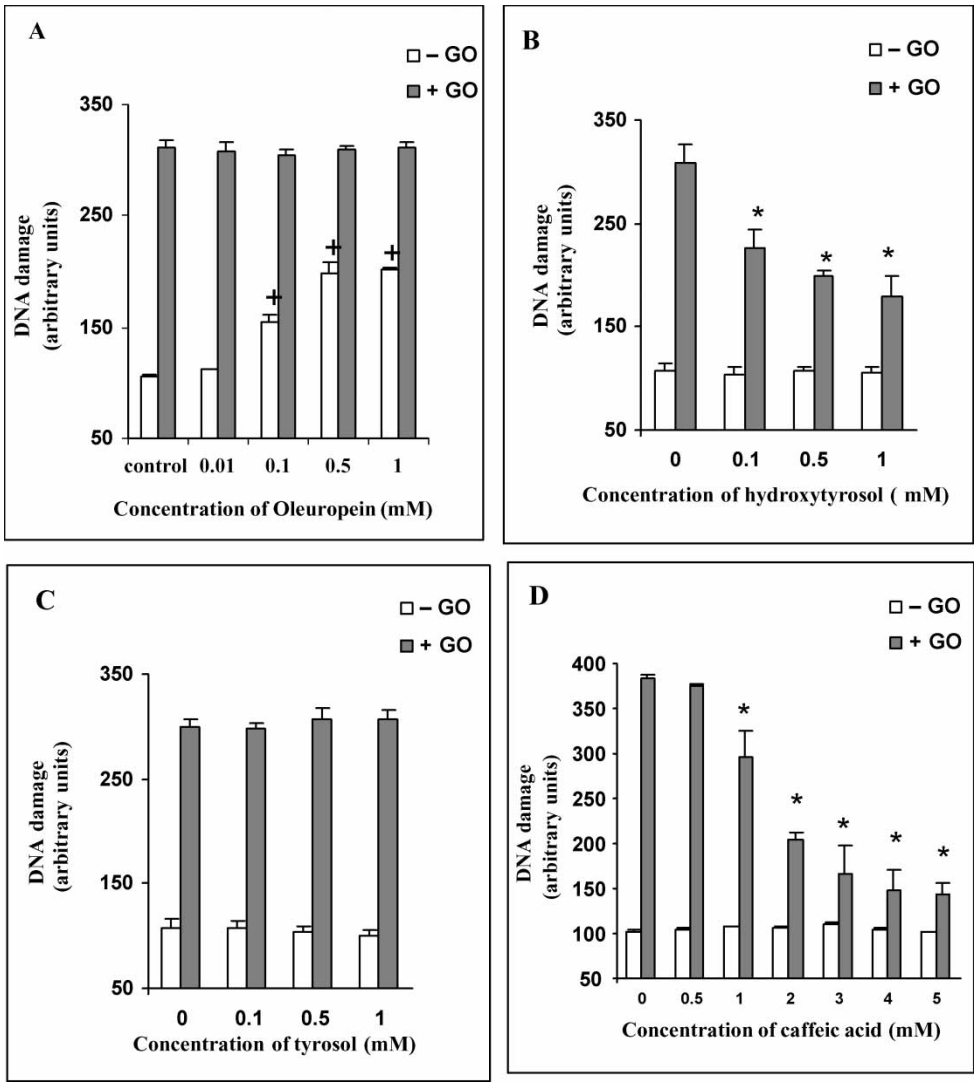



Figure 2. Effects of isolated compounds on $H_2O_2$-induced nuclear DNA damage. Conditions were as in Figure 1, except that pure oleuropein (A), hydroxytyrosol (B), tyrosol (C) and caffeic acid (D) was incubated for 30 min with the cells before the addition of the $H_2O_2$ generating system (60 ng glucose oxidase/well). Each value represents the mean ± SD of triplicate measurements of two independent experiments. ($^{*}p < 0.001$ versus $H_2O_2$-treated samples, $^{+}p < 0.001$ versus untreated samples).

experimental systems [28–30]. The basis of the need for this chemical configuration in order for a compound to be able to protect is not clear at present. It has been proposed that ortho-dihydroxy groups may stabilize the free radical forms of the respective compounds thus increasing their free radical scavenging capacity [31,32]. However, other properties of this moiety, like binding of redox-active iron ions may also contribute to their apparent protective capacity. It is worth noting that previous studies in our laboratory have shown that iron chelating agents with different chemical characteristics exerted strong protection in this particular experimental system [18,33,34]. The fact that hydroxytyrosol was more effective than caffeic acid indicates a negative effect from the carboxyl group in the side chain of the latter (Scheme 1). This may be related to the charge of the carboxyl group that may have a negative influence on its penetration through plasma membrane. This notion was further supported by the observation that the protective action of hydroxytyrosol was more rapid than that of caffeic acid (see Figure 3A and B). It was also shown that the protective potential of hydroxytyrosol was gradually decreased as the incubation time increased, indicating most probably its metabolic inactivation. Indeed, glutathionylation and O-methylation of catecholic compounds have been proposed previously to take place intracellularly [35,36].

On the other hand, oleuropein glucoside in spite of baring an ortho-dihydroxy moiety similar to that of hydroxytyrosol and caffeic acid did not exert any protection against $H_2O_2$-induced DNA damage (Figure 2A). This observation may be due to the bulky and hydrophilic properties of this molecule that may hinder its uptake by the cells. However, the oleuropein glucoside was able to induce DNA damage

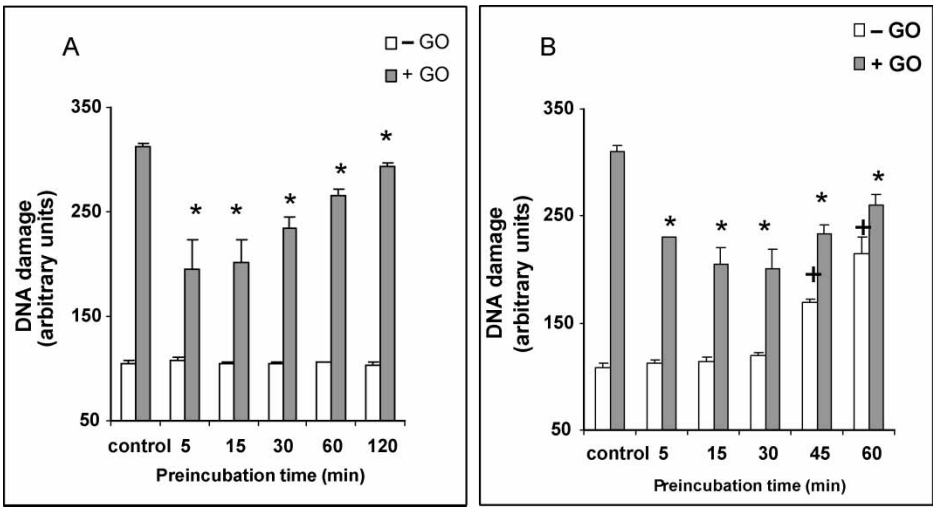



Figure 3. Effects of incubation period on $H_2O_2$-induced nuclear DNA damage. Conditions were as in Figure 1, except that Jurkat cells ($1.5 \times 10^6$ cells/ml) were pre-incubated for the indicated periods of time with 0.1 mM hydroxytyrosol (A) or 2.0 mM caffeic acid (B). Each value represents the mean ± SD of triplicate measurements of two independent experiments. ($^*p < 0.01$ versus $H_2O_2$-treated samples, $^+p < 0.001$ versus untreated samples).

by itself although the molecular mechanism(s) of this effect remains obscure. It has to be stressed that this compound is hydrolyzed *in vivo* when in the acidic environment of the stomach, giving rise to hydroxytyrosol and elenolic acid.

It has to be noted that this *in vitro* study does not necessarily indicate respective *in vivo* effects, especially since the concentrations used are far higher than are achievable *in vivo*. However, it may contribute to a better understanding of the possible biological action(s) of olive-related compounds. Moreover, these observations may also contribute in the epidemiological debate associated with olive oil consuming populations.



## Acknowledgements

This work was supported by grant PENED-2001 01ED450 of General Secretariat of Research and Technology, Athens, Greece.

## References

[1] Keys A, Aravanis C, Van Buchen FSP, Blackburn H, Buzina R, Djordjevic BS. The diet and all-causes death rate in Seven Countries Study. Lancet 1981;2:58–61.
[2] Gerber M. Olive oil and cancer. In: Hill MJ, Giacosa A, Caygill CPG, editors. Epidemiology of diet and cancer. Chichester: Ellis Horwood; 1994. p 263–275.
[3] Boskou D. Olive oil chemistry and technology Illinois: AOCS Press; 1996. p 115–117.
[4] Bartsch H, Nair J, Owen RW. Dietary PUFA's and cancer of the breast and colorectum: Compelling evidence for their role as risk modifiers. Carcinogenesis 1999;20:2209–2218.
[5] Bartsch H, Nair J, Owen RW. Exocyclic DNA adducts as oxidative stress markers in colon carcinogenesis: Potential role of lipid peroxidation, dietary fats and antioxidants. Biol Chem 2002;383:915–921.
[6] Tuck KL, Hayball PJ. Major phenolic compounds in olive oil: Metabolism and health effects. J Nutr Biochem 2002;13:636–644.
[7] De la Puerta R, Dominguez MEM, Ruiz-Gutierrez V, Flavill JA, Hoult JRS. Effects of virgin olive oil phenolics on scavenging of reactive nitrogen species and upon nitrergic neutrotransmission. Life Sci 2001;69:1213–1222.
[8] Visioli F, Bellomo G, Galli C. Free radical-scavenging properties of olive oil polyphenols. Biochem Biophys Res Commun 1998;247:60–64.
[9] Owen RW, Mier W, Giacosa A, Hull WE, Spiegelhalder B, Bartsch H. Phenolic compounds and squalene in olive oils: The concentration and antioxidant potential of total phenols, simple phenols, secoiridoids, lignans and squalene. Food Chem Toxicol 2000;38:647–659.
[10] Owen RW, Haubner R, Mier W, Ciacosa A, Hull WE, Spiegelhalder B, Bartsh H. Isolation, structure elucidation and antioxidant potential of the major phenolic and flavonoid compounds in brined olive drupes. Food Chem Toxicol 2003;41:703–717.
[11] Rodis PS, Karathanos VT, Mantzavinou A. Partitioning of olive antioxidants between oil and water phases. J Agric Food Chem 2002;50:596–601.
[12] Capasso R, Cristinzio G, Evidente A, Scognamiglio F. Isolation, spectroscopy and selective phytotoxic effects of polyphenols from vegetable waste waters. Phytochemistry 1992;31:4125–4128.
[13] Visioli F, Vinceri FF, Galli C. 'Waste waters' from olive oil production are rich in natural antioxidants. Experimentia 1995;51:32–34.
[14] Le Tutour B, Guedon D. Antioxidants activities of *Olea europaea* leaves and related phenolic compounds. Phytochemistry 1992;31:1173–1178.
[15] Panagiotidis M, Tsolas O, Galaris D. Glucose oxidase-produced $H_2O_2$ induces $Ca^{2+}$-depended DNA damage in human peripheral blood lymphocytes. Free Radic Biol Med 1999;26:548–556.
[16] Doulias P-T, Barbouti A, Galaris D, Ischiropoulos H. SIN-1 induced DNA damage in isolated human peripheral blood

lymphocytes as assessed by single cell gel electrophoresis (Comet Assay). Free Radic Biol Med 2001;30:679–685.

[17] Barbouti A, Doulias P-T, Zhu BZ, Frei B, Galaris D. Intracellular iron, but not copper, plays a critical role in hydrogen peroxide-induced DNA damage. Free Radic Biol Med 2001;31:490–498.

[18] Barbouti A, Doulias P-T, Nousis L, Tenopoulou M, Galaris D. DNA damage and apoptosis in hydrogen peroxide-exposed Jurkat cells: Bolus addition versus continuous generation of $H_2O_2$. Free Radic Biol Med 2002;33:691–702.

[19] Tselepis A, Doulias PT, Lourida E, Glantzounis G, Tsimoyiannis E, Galaris D. Trimetazidine protects low-density lipoproteins from oxidation and cultured cells exposed to $H_2O_2$ from DNA damage. Free Radic Biol Med 2001; 30:1357–1364.

[20] Ostling O, Johanson KJ. Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. Biochem Biophys Res Commun 1984; 123:291–296.

[21] Singh 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;184:461–470.

[22] Collins AR, Ma A, Duthie SJ. The kinetics of repair of oxidative DNA damage (strand breaks and oxidized pyrimidines) in human cells. Mutat Res 1995;336:69–77.

[23] Duthie SJ, Headon A. DNA instability (strand breakage, uracil misincorporation, and defective repair) is increased by folic acid depletion in human lymphocytes *in vitro*. FASEB J 1998;12:1491–1497.

[24] Babich H and Visoli F. *In Vitro* Cytotoxity to human cells in culture of some phenolics from olive oil. IL Farmaco 2003; 58:403–407.

[25] Ames BN. Dietary carcinogens and anticarcinogens. Oxygen radicals and degenerative diseases. Science 1983; 221:1256–1264.

[26] World Health Organization. World health statistics annual. Geneva; 1991. p 308–315.

[27] Moure A, Cruz JM, Franco D, Dominguez JM, Sineiro J, Dominguez H, Numez MJ, Parajo JC. Natural antioxidants from residual sources. Food Chem 2001;72:145–171.

[28] Fabiani R, De Bertolomeo A, Rosignoli P, Servili M, Montedoro GF, Morozzi G. Cancer chemoprevention by hydroxytyrosol isolated from virgin olive oil through G1 cell cycle arrest and apoptosis. Eur J Cancer Prev 2002; 11:351–358.

[29] Deiana M, Aruoma OI, De Lourdes Bianchi MP, Spencer JPE, Kaur H, Halliwell B, Aeschbach, R., Banni, S., Assunda Dessi, M. and Corongiu, F.P.. Inhibition of Peroxynitrite dependent. DNA base modification and tyrosine nitration by the extra virgin olive oil-derived hydroxytyrosol. Free Red Biol Med 1999;26:762–769.

[30] Manna C, Galletti P, Cucciolla V, Montedoro G, Zappia V. Olive oil hydroxytyrosol protects human erythrocytes against oxidative damages. J Nutr Biochem 1999;10:159–165.

[31] Rice-Evans C, Miller JN, Paganga G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic Biol Med 1996;20:933–956.

[32] Meyer SA, Heinonen M, Frankel NE. Antioxidant interactions of catechin, cyanidin, caffeic acid, quercetin and ellagic acid on human LDL oxidation. Food Chem 2000;61:71–75.

[33] Doulias P-T, Christoforidis S, Brunk U, Galaris D. Endosomal and lysosomal effects of desferioxamine: Protection of HeLa cells from hydrogen peroxide-induced DNA damage and induction of cell cycle arrest. Free Radic Biol Med 2003; 35:719–728.

[34] Doulias P-T, Nousis L, Zhu B-Z, Frei B, Galaris D. Protection by tropolones against $H_2O_2$-induced DNA damage and apoptosis in cultured Jurkat cells. Free Radic Res, In press.

[35] Spencer PE, Kunhle CGG, Williams JR, Rice-Evans C. Intracellular metabolism and bioactivity of quercetin and its *in vivo* metabolites. Biochem J 2003;372:173–181.

[36] Moridani YM, Scobie H, O'Brien JP. Metabolism of caffeic acid by isolated rat hepatocytes and subcellular fractions. Toxicol Lett 2002;133:141–151.