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Influence of Cultivar and Concentration of Selected Phenolic Constituents on the in Vitro Chemiopreventive Potential of Olive Oil Extracts

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ABSTRACT: One of the main olive oil phenolic compounds, hydroxytyrosol (3,4-DHPEA), exerts in vitro chemopreventive activities (antiproliferative and pro-apoptotic) on tumor cells through the accumulation of H_2O_2 in the culture medium. However, the phenol composition of virgin olive oil is complex, and 3,4-DHPEA is present at low concentrations when compared to other secoiridoids. In this study, the in vitro chemopreventive activities of complex virgin olive oil phenolic extracts (VOO-PE, derived from the four Italian cultivars Nocellara del Belice, Coratina, Ogliarola, and Taggiasca) were compared to each other and related to the amount of the single phenolic constituents. A great chemopreventive potential among the different VOO-PE was found following this order: Ogliarola > Coratina > Nocellara > Taggiasca. The antiproliferative and pro-apoptotic activities of VOO-PE were positively correlated to the secoiridoid content and negatively correlated to the concentration of both phenyl alcohols and lignans. All extracts induced H_2O_2 accumulation in the culture medium, but this phenomenon was not responsible for their pro-apoptotic activity. When tested in a complex mixture, the olive oil phenols exerted a more potent chemopreventive effect compared to the isolated compounds, and this effect could be due either to a synergistic action of components or to any other unidentified extract constituent.

KEYWORDS: virgin olive oil, phenols, chemoprevention, proliferation, cell cycle, apoptosis

INTRODUCTION

In the past few years, the positive health effects of virgin olive oil (strengthened by numerous epidemiological studies) have been attributed, at least in part, to its minor components (for reviews, see refs 1 and 2). This small fraction, about 2% of the olive oil weight, includes more than 230 different molecules, the most representative of which are the hydrophilic phenolic compounds.[3] Indeed, the peculiar phenol composition of virgin olive oil is particularly complex and includes different classes of molecules such as phenolic acids, phenolic alcohols, secoiridoids, and lignans.[3] Among the hydrophilic phenols hydroxytyrosol (3,4-dihydroxyphenyethanol, 3,4-DHPEA) has received major attention since the discovery of its strong free radical-scavenging and antioxidant properties,[4] which could be responsible for both the antiatherosclerotic and anticarcinogenic properties of virgin olive oil. Indeed, one mechanism by which 3,4-DHPEA can inhibit the initiation phase of carcinogenesis involves the protection of DNA from oxidative damage, an effect exerted in vitro in a low concentration range (1−10 μM).[5] This DNA preventive activity has also been demonstrated by intervention studies on humans using phenol-rich olive oil.[6,7] In addition, 3,4-DHPEA at higher concentrations is also able to inhibit proliferation and to induce apoptosis on different tumor cell lines such as colon and breast carcinoma cells,[8−12] melanoma,[13] and leukemia HL60 cells.[14,15] Recent studies have surprisingly demonstrated that the antiproliferative and pro-apoptotic properties of 3,4-DHPEA are mediated by a pro-oxidant activity consisting of the generation of hydrogen peroxide (H_2O_2) in the cell culture medium.[16] However, this characteristic is not always shared by other phenolic compounds present in the olive oil, structurally similar to 3,4-DHPEA but with a quite different behavior when compared to 3,4-DHPEA. In particular, tyrosol (*p*-hydroxyphenyethanol: *p*-HPEA) does not induce either apoptosis or H_2O_2 accumulation, whereas the secoiridoid derivative of *p*-HPEA (tyrosol linked to the dialdehydic form of decarboxymethyl elenolic acid, *p*-HPEA-EDA, also called oleocanthal), although unable to generate H_2O_2, induces apoptosis on HL60 cells.[17] Furthermore, the secoiridoid derivative of 3,4-DHPEA (hydroxytyrosol linked to the dialdehydic form of decarboxymethyl elenolic acid, 3,4-DHPEA-EDA) causes an accumulation of H_2O_2, but its pro-apoptotic effect was not inhibited by catalase (enzyme able to remove H_2O_2 from the culture medium).[17]

Since the phenolic composition of olive oil is quite complex and depends upon various agronomic and technological factors

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including the variety of the olive tree (cultivar),[18] the present investigation has the aim to quantify the chemopreventive activity (in terms of antiproliferative and pro-apoptotic potential) of different virgin olive oil phenolic extracts (VOO-PE) derived from 4 Italian cultivars (Nocellara del Belice, Coratina, Ogliarola, Taggiasca), which differed in their phenolic profiles. In addition, the involvement of extracellular production of H_2O_2 on the pro-apoptotic activity of the phenolic extracts has been determined.

MATERIALS AND METHODS

Materials and Phenolic Extracts. RPMI 1640 medium (not containing pyruvate) and heat-inactivated fetal calf serum (FCS) were obtained from Gibco (Gibco BRL, Life Technologies, Paisley, Scotland). (+)-Pinoresinol was obtained from ArboNova (Oy ArboNova Ab, Turku, Finland). All other reagents were purchased from Sigma unless differently specified (Sigma-Aldrich Co. Ltd., Irvine, U.K.). Human promyelocytic leukemia cells (HL60), obtained from the American Type Culture Collection (Rockville, MD), were cultured in complete RPMI 1640 medium supplemented with 10% FCS, 2.0 mM L-glutamine, 100 U/mL penicillin, and 100 U/mL streptomycin. The cells were maintained at 37 °C and 5% CO_2 in a humidified atmosphere and seeded every 4 days at a density of 0.2×10^6 cells/mL. Virgin olive oils were obtained by a mechanical extraction process performed in an industrial plant as follows. Green olives (*Olea europaea* L.) from cultivars Coratina, Ogliarola, Nocellara del Belice, and Taggiasca were crushed using a hammer crusher; malaxation was carried out for 40 min at 25 °C, and the oil was separated by centrifugation (9600*g*; 1 min) using a decanter (Rapanelli model 400 ECO/G) at a low level of water addition. The virgin olive oil phenolic extracts (VOO-PE) (Coratina, Ogliarola, Nocellara del Belice, and Taggiasca) and an olive mill wastewater phenolic extract from Coratina (WW-PE) were obtained according to previously published methods.[19] The HPLC analyses of VOO-PE, performed as previously reported,[19] were conducted using an Agilent Technologies system model 1100 composed of a vacuum degasser, a quaternary pump, an autosampler, a thermostated column compartment, a DAD, and an FLD. The C18 column used was a Spherisorb ODS-1 250 mm × 4.6 mm with a particle size of 5 μm (Phase Separation Ltd., Deeside, U.K.); the injected sample volume was 20 μL. The mobile phase was composed of 0.2% acetic acid (pH 3.1) in water (solvent A)/methanol (solvent B) at a flow rate of 1 mL/min. 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 16 min, and 0% A/100% B for 14 min. This composition was maintained for 10 min and then was returned to the initial conditions and equilibration for 13 min; the total running time was 73 min. The phenolic compounds were detected by DAD with a wavelength of 278 nm.

The VOO-PE were dissolved in ethanol at a concentration of total phenol corresponding to 5 mg/mL and stored at −20 °C in the dark. The extracts were diluted in RPMI 1640 medium just before use to the desired concentrations. All of the solutions were sterilized by filtration on 0.22 μm filters (Celbio S.r.l., Milan, Italy).

Cell Growth and Viability Assays. The proliferation of HL60 cells was followed by counting the viable cells using the trypan blue exclusion method and by applying the MTT (3-[4,5-dimethylthiazol-2-yl)]-3,5-diphenyltetrazolium bromide) assay. HL60 cells were seeded at a density of 0.2×10^6 cells/mL in culture medium in 25 cm^2 flasks (Falcon; Becton Dickinson, Oxnard, CA) and incubated in the presence of different concentrations of phenolic compounds at 37 °C and 5% CO_2. At each established experimental time, 20 μL aliquots were withdrawn from the cell suspensions and diluted 1:10 with a trypan blue solution (2.22 g/L of PBS); after standing at room temperature for 5 min, the viable cells were counted using a Burker chamber. For the MTT assay, HL60 cells were plated in 96-well plates (flat bottom, 100 μL) at 20000 cells per well, and, after 96 h of incubation with the different compounds, the growth rates were quantified by the MTT assay as previously described.[20] This assay measures the production of highly colored formazan as a result of the reduction of MTT by metabolically active cells due to the dehydrogenase enzymes. Ten microliter aliquots of MTT solution (5 mg/mL of MTT in PBS) were added to each well and incubated for 2 h at 37 °C. Lysing buffer (100 μL, 10% SDS dissolved in 0.01 M HCl) was then added, and the plates were incubated for an additional 24 h at 37 °C to dissolve the blue formazan crystals. After incubation, the absorbance of the released formazan was determined at 565 nm.

Apoptosis Assays. The percentage of apoptosis was determined by both fluorescence microscopy and flow cytometry.[21] In the first case, after treatment with the extracts, the cells were recovered by centrifugation and resuspended in complete RPMI medium containing the DNA binding dyes Hoechst 33342 (HO 342, 20 μg/mL in PBS) and propidium iodide (PI, 10 μg/mL in PBS). After 10 min of incubation at room temperature, the cells were examined with a fluorescent microscope (Zeiss, R.G., equipped with a 50-W mercury lamp) with ultraviolet excitation at 340−380 nm. HO 342 is a plasma membrane-permeable compound, which freely enters the cells with intact membranes, as well as cells with damaged membranes, and stains the DNA blue, whereas PI is a highly polar dye that is impermeable to cells with intact plasma membrane and stains the DNA red. Consequently, the viable cells have been identified by their intact nuclei with blue fluorescence (HO 342) and the necrotic cells by their intact nuclei with reddish fluorescence (HO 342 plus PI). Apoptotic cells were detected by their fragmented nuclei, which exhibited either a blue (HO 342) or reddish (HO 342 plus PI) fluorescence depending on the stage of the process. Under each of the experimental condition, three slides were prepared and 100 cells were counted for each slide.

For the flow cytometry detection of apoptosis (sub G_1), the cells were stained with PI as follows. The cell pellet was resuspended in 0.5 mL of hypotonic fluorochrome solution containing 50 μg/mL PI in 0.1% sodium citrate plus 0.1% Triton X-100 in 12 × 75 mm polypropylene tubes (Becton and Dickinson, Lincoln Park, NJ). The tubes were kept at 4 °C for 30 min, and then the PI fluorescence of individual nuclei was measured using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) at a wavelength of 488 nm.

DNA Cell Cycle Analysis. After incubation with VOO-PE HL60 cells were recovered by centrifugation (400*g*, 7 min) and washed twice with cold PBS. The pellet was resuspended in 50 μL of cold PBS plus 450 μL of cold methanol and incubated for 1 h at 4 °C. The cells were then centrifuged, and the pellet was washed twice with cold PBS, resuspended in 500 μL of PBS and 5 μL RNase (20 μg/mL, final concentration), and incubated for 30 min at 25 °C. After incubation, the cells were chilled on ice for 10 min and stained with PI (50 μg/mL final concentration) for 1 h and analyzed by flow cytometry using a FACScan flow cytometer at a wavelength of 488 nm. The percentages of the cells in G_0/G_1, S, and G_2/M phases were calculated using CellFIT Cell-Cycle Analysis version 2.0.2. software.

Measurement of H_2O_2 Concentration in the Culture Medium. The concentration of H_2O_2 in the culture medium was measured by the ferrous ion oxidation−xylenol orange method[22] as follows: medium (20 μL) was mixed with a reaction solution (200 μL) containing 250 μM ammonium iron(II) sulfate, 25 mM H_2SO_4, 100 mM sorbitol, and 125 μM xylenol orange and incubated at room temperature for 30 min. The absorbance was then read at 595 nm, and the concentration of H_2O_2 was derived from a standard curve obtained by adding different concentrations of H_2O_2 in the RPMI medium just before the assay.

Statistical Analyses. All tests were run in triplicate for each experimental condition, and each experiment was repeated at least three

Table 1. Phenolic Composition of Extracts Obtained from Different Virgin Olive Oil (VOO-PE) Cultivars and from an Olive Mill Wastewater (WW-PE) (Milligrams per Gram of Extract)

	VOO-PE (cultivar)				
phenolic compound	Ogliarola	Coratina	Nocellara	Taggiasca	WW-PE
3,4-DHPEA	3.2 ± 0.3 (0.10)[a]	9.9 ± 1.0 (0.32)	25.4 ± 2.3 (0.82)	52.0 ± 4.7 (1.69)	47.4 ± 1.9 (1.49)
p-HPEA	15.3 ± 1.3 (0.55)	4.4 ± 0.4 (0.16)	30.1 ± 2.5 (1.09)	69.8 ± 5.8 (2.53)	4.5 ± 0.2 (0.16)
3,4-DHPEA-EDA	300.3 ± 28.6 (4.70)	485.0 ± 40.5 (7.58)	280.5 ± 26.7 (4.38)	133.9 ± 12.8 (2.09)	548.8 ± 65.9 (8.35)
p-HPEA-EDA (oleocanthal)	111.8 ± 10.3 (1.84)	128.0 ± 12.8 (2.11)	65.6 ± 6.1 (1.08)	39.5 ± 3.7 (0.65)	ND[b]
3,4-DHPEA-EA (oleuropein aglycon)	207.4 ± 19.0 (2.7)	112.6 ± 11.3 (1.49)	128.1 ± 11.7 (1.69)	145.3 ± 11.5 (1.92)	ND
(+)-1-acetoxypinoresinol	21.3 ± 1.8 (0.26)	23.2 ± 2.3 (0.28)	42.9 ± 3.6 (0.51)	64.4 ± 7.6 (0.77)	ND
(+)-pinoresinol	12.0 ± 0.9 (0.17)	17.7 ± 1.8 (0.25)	34.1 ± 2.6 (0.48)	96.7 ± 2.5 (1.35)	ND
verbascoside	ND	ND	ND	ND	17.6 ± 0.15 (0.14)
total phenols	606 ± 7.9 (10.06)	780.9 ± 10.0 (12.18)	671.3 ± 8.9 (10.36)	601.6 ± 6.9 (11.00)	618.2 ± 64 (10.16)

[a] In parentheses are shown the μM concentrations of the different phenolic compounds when the extracts were resuspended in the RPMI medium at a dose of total phenols corresponding to 5 μg/mL. [b] ND, not detected.

times; the results are reported as the mean ± SD. Significant differences among treatments were assessed using both Student's *t* test and one-way ANOVA. When a significant ($P < 0.05$) treatment effect was detected, the mean values were compared using Tukey's post hoc comparisons.

RESULTS

Characteristics of Phenolic Extracts. Among several virgin olive oil phenolic extracts (VOO-PE) available in our laboratory, we selected four extracts derived from different Italian olive oil cultivars (Ogliarola, Coratina, Nocellara, and Taggiasca) on the basis of their phenolic composition and, particularly, for the concentration of secoiridoids and lignans. In addition, an olive mill wastewater phenolic extract (WW-PE) has also been included for comparison. The VOO-PE were characterized by a wide difference in the concentration of their main phenolic components (Table 1). In particular, the main variations were observed for secoiridoid such as *p*-HPEA-EDA, 3,4-DHPEA-EDA, and 3,4-DHPEA-EA and lignans that included pinoresinol and acetoxypinoresinol. As a consequence, the ratio between the sum of secoiridoids and lignans in the four VOO-PE ranged between 1.8 and 18.5 observed in Taggiasca and Coratina VOO-PE, respectively. According to the literature data, VOO-PE did not contain appreciable amounts of verbascoside, whereas in the WW-PE were not present *p*-HPEA-EDA, 3,4-DHPEA-EA, and lignans (pinoresinol and acetoxypinoresinol) (Table 1). The micromolar concentrations of the different compounds, resulting when 5 μg/mL (w/v) of total phenolic was resuspended in the complete cell culture medium (RPMI + 10% FCS), have been also reported (Table 1).

Effect of Phenolic Extracts on Proliferation and Cell Cycle of HL60. To test the antiproliferative activity of the different extracts, HL60 cells were incubated with three concentrations of VOO-PE and followed over time by counting the viable cells every 24 h (Figure 1). At the lowest dose of phenols (1.25 μg/mL) no difference in the growth curves was observed between the control cells and cells incubated with the different VOO-PE (Figure 1A). Instead, at 2.5 μg/mL (Figure 1B) and 5 μg/mL (Figure 1C) there was a time-dependent inhibition of cell growth. Notably, the Taggiasca extract was less active than the others, showing no effect in the first 72 h and a weak effect at 5 μg/mL after 96 h of incubation. Similar antiproliferative

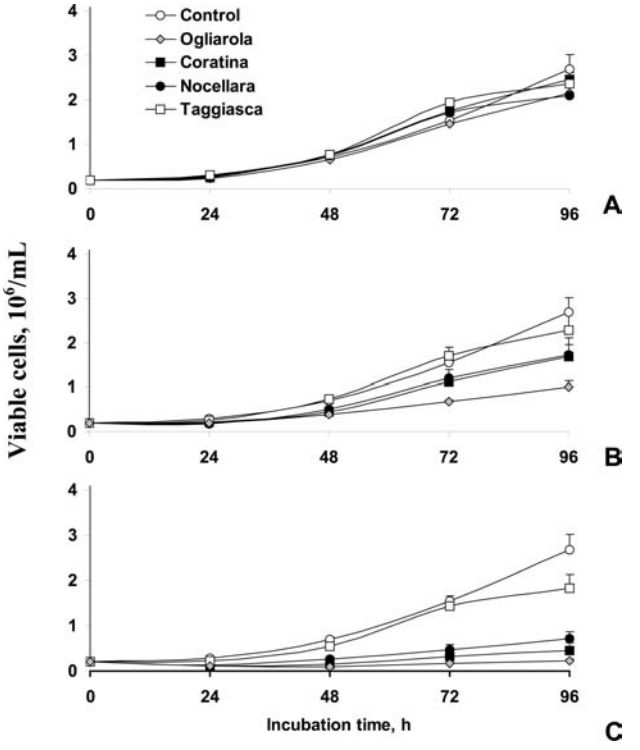

Figure 1. Proliferation of HL60 cells measured by counting the viable cells over time in the presence of solvent (control) and VOO-PE derived from different olive oil cultivars (Ogliarola, Coratina, Nocellara, and Taggiasca) at a concentration of 1.25 (A), 2.5 (B), or 5 (C) μg/mL. Values are the mean ± SD, $n = 5$.

potential was obtained when the cell growth was measured at 96 h of incubation time by the MTT test (Figure 2). The Ogliarola extract was the most effective in inhibiting the HL60 proliferation, whereas the Taggiasca extract reduced significantly the proliferation only at the highest concentration tested (5 μg/mL). Under these experimental conditions (5 μg/mL, 96 h) the WW-PE did not significantly influence the HL60 cell proliferation (data not shown). The $IC_{50\%}$ (the concentration of each VOO-PE necessary to inhibit cell growth by 50% compared to

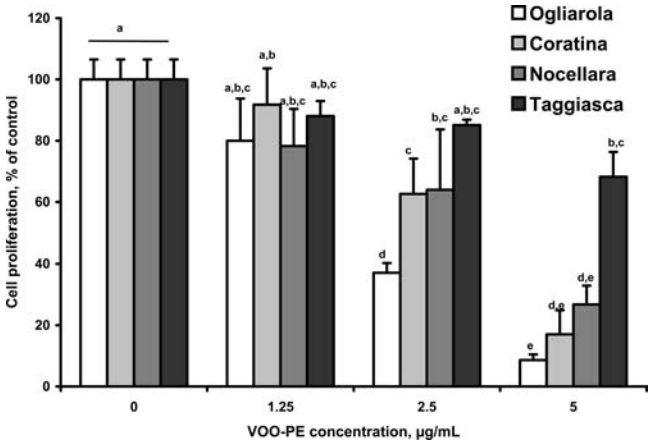

Figure 2. Effect of increasing doses of VOO-PE derived from different olive oil cultivars (Ogliarola, Coratina, Nocellara, and Taggiasca) on proliferation of HL60 cells after 96 h of incubation measured by the MTT assay. Values are the mean ± SD, $n = 5$. Means without a common letter differ, $P < 0.05$.

control) values for the different VOO-PE were as follows: Ogliarola, 2.02 μg/mL; Coratina, 3.18 μg/mL; Nocellara, 3.46 μg/mL; and Taggiasca, >5.00 μg/mL. The effect of VOO-PE on cell cycle distribution was tested after 24 h of treatment by ethidium bromide DNA staining and flow cytometry (Figure 3). The analysis of histogram profiles shows that treatment of HL60 cells with Ogliarola, Coratina, and Nocellara extracts at a concentration of 5.0 μg/mL significantly reduced the percentage of cells in the S phase and, in the case of Coratina and Nocellara, increased the percentage in the G_2 phase (Figure 3). On the other hand, the Taggiasca extract did not influence the cell cycle distribution at the concentrations tested (Figure 3).

Effect of Phenolic Extracts on Apoptosis of HL60 and H_2O_2 Production. Similarly to the registered effects on proliferation, Ogliarola, Coratina, and Nocellara extracts induced a dose-dependent apoptosis on HL60 cells after 24 h of treatment, which was not significant at the lowest concentration (1.25 μg/mL) but was evident at higher doses (Figure 4A). On the other hand, the Taggiasca extract exerted a small pro-apoptotic effect, which was not significant at any of the concentrations tested (Figure 4A). Previous data indicated that some purified olive oil phenolic compounds are able to induce the accumulation of H_2O_2 in the cell culture medium and demonstrated that this was a mechanism by which some of them exerted a pro-apoptotic activity on HL60 cells. We wondered whether the same phenomenon occurs when complex mixtures of VOO-PE were used instead of single compounds. H_2O_2 was measured over time after the addition of the different VOO-PE (5 μg/mL) in the RPMI medium without FCS and HL60 cells (Figure 4B). Indeed, there was a time-dependent accumulation of H_2O_2 in the medium, which did not significantly differ depending on the type of extract used. The addition of catalase to the RPMI medium (CAT, 100 U/mL) completely prevented H_2O_2 accumulation (data not shown). Furthermore, the presence of HL60 cells (0.2×10^6 cells/mL) drastically reduced the H_2O_2 concentration to about l μM after 24 h of incubation (Figure 4B). These results indicate that HL60 cells are able to efficiently metabolize the H_2O_2 produced by the extracts. To further investigate whether the H_2O_2 accumulation was responsible for the pro-apoptotic effects of VOO-PE, the HL60 cells were treated with the different extracts (5 μg/mL) in the presence of CAT for 24 h and then apoptosis was measured (Figure 4C). It was evident that CAT did not reduce the pro-apoptotic effect of the VOO-PE (Figure 4C). For comparison, the pro-apoptotic activity of WW-PE was also measured at the same concentration of VOO-PE (5 μg/mL), but no significant effects were observed (data not shown). To register a significant pro-apoptotic effect, it was necessary to increase the WW-PE concentration 10 times (12.5−50 μg/mL) over that used for the VOO-PE (Figure 4D). Likewise with the VOO-PE, the inclusion of CAT in the medium was not able to inhibit apoptosis induced by WW-PE (Figure 4D).

Correlation between the Phenolic Composition of VOO-PE and Their Chemopreventive Potential. The results reported indicate that together with the wide component variability of the VOO-PE, there was also a different chemopreventive potential, so a correlation study was carried out between the concentrations of phenols in the VOO-PE and their chemopreventive activities. As expected, the antiproliferative and pro-apoptotic abilities of the VOO-PE were highly correlated to each other, but these activities were not correlated to the total amount of phenols present in the VOO-PE (Table 2). Instead, both chemopreventive activities were differently correlated to the concentrations of the different compounds present in the VOO-PE (Table 2). In particular, there was a significant negative correlation with both phenolic alcohols (3,4-DHPEA and *p*-HPEA) and lignans (pinoresinol and acetoxypinoresinol), whereas the correlation with the EDA derivatives of both 3,4-DHPEA and *p*-HPEA (secoiridoids) was positive (Table 2). A much weaker positive correlation was found between the concentration of 3,4-DHPEA-EA and the pro-apoptotic and antiproliferative activities of VOO-PE (Table 2). Because of the surprising negative correlation with lignans, we investigated whether these compounds, in particular pinoresinol, could exert an anti-apoptotic effect. With this aim the Ogliarola extract was enriched with increasing concentrations of pinoresinol and then tested on HL60 cells for its pro-apoptotic activity. The results of this experiment (Figure 5) clearly show that the addition of pinoresinol at concentrations up to 10 μM did not influence the pro-apoptotic activity of the Ogliarola extract.

DISCUSSION

Although numerous studies have investigated the chemopreventive properties of 3,4-DHPEA, little is known about the effects of complex olive oil phenolic extracts on tumor cells. The results herein presented demonstrate that (i) VOO-PE with different phenolic compositions express different in vitro chemopreventive potentials toward HL60 cells; (ii) WW-PE showed a much lower chemopreventive potential compared to VOO-PE; (iii) the secoiridoids 3,4-DHPEA-EDA and, in particular, *p*-HPEA-EDA seem to be primarily responsible for the chemopreventive activity of VOO-PE; and (iv) the antiproliferative and pro-apoptotic activities of both VOO-PE and WW-PE are not due to the phenol-mediated production of H_2O_2 in the cell culture medium. Comparisons between the antiproliferative and pro-apoptotic activities of the different extracts were performed by treating the cells with the same weight doses (μg/mL) of total phenolic compounds, resulting in similar total phenolic molar concentrations (10−12 μM). Therefore, the different chemopreventive potentials shown by the extracts were not a direct consequence of the total amount of phenols but, instead,

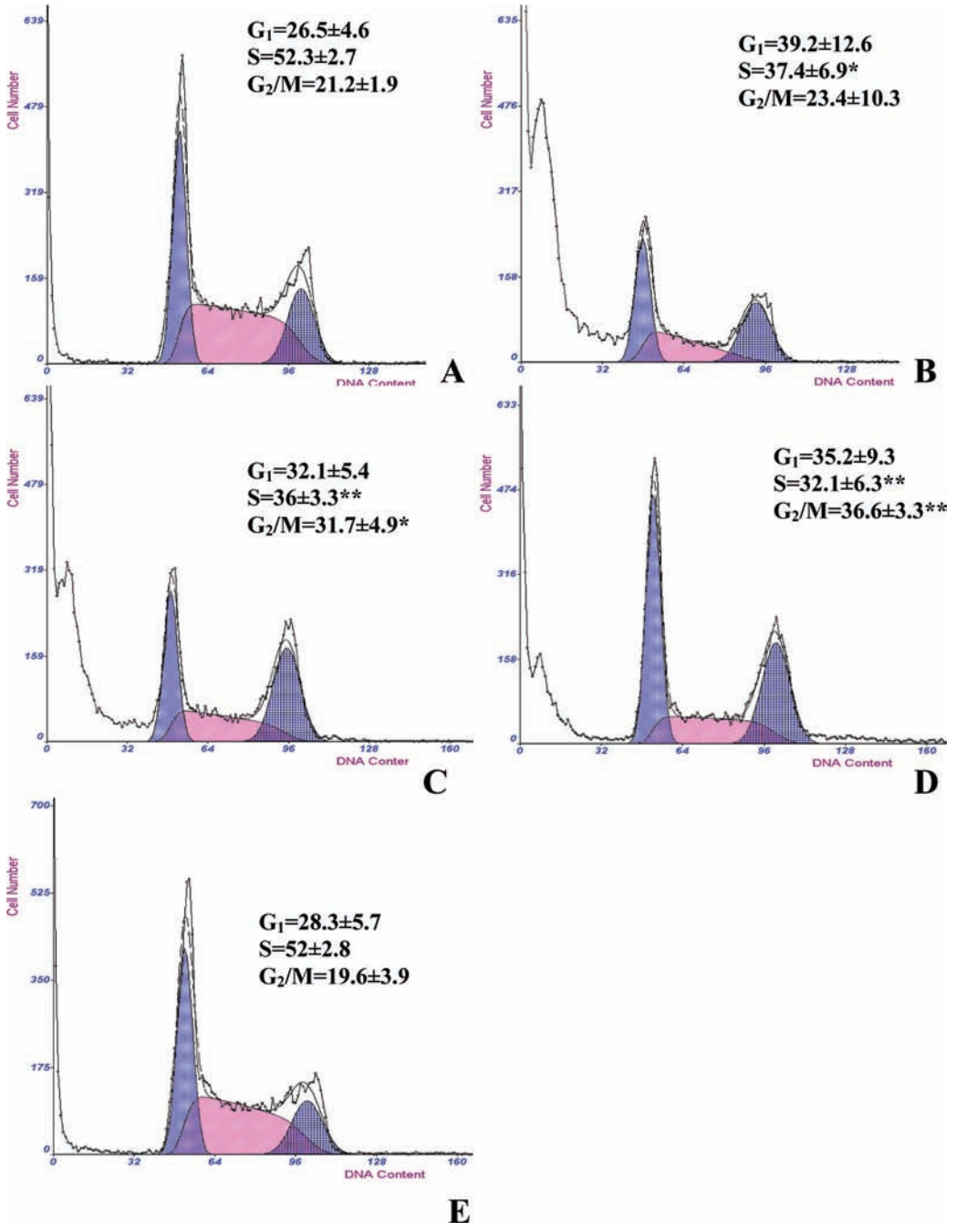

Figure 3. Effect of VOO-PE derived from different olive oil cultivars at a concentration of 5 μg/mL on the cell cycle of HL60 cells. In the figure are shown the flow cytometry histograms of a representative experiment and the percent values of the cells in the different phases of the cell cycle (mean ± SD, $n = 3$) obtained when cells were treated for 24 h with vehicle (A), Ogliarola (B), Coratina (C), Nocellara (D), and Taggiasca (E) extracts. ∗, $P < 0.05$, and ∗∗, $P < 0.01$, compared to the control.

influenced by their composition. Indeed, the antiproliferative and pro-apoptotic effects were positively correlated with the two main components of the extracts, that is, 3,4-DHPEA-EDA and *p*-HPEA-EDA. Previously, these purified compounds have been found to have antiproliferative ($IC_{50\%}$ were 30−35 and 7−8 μM for 3,4-DHPEA-EDA and *p*-HPEA-EDA, respectively) and pro-apoptotic (concentrations that gave 50% of apoptosis were 72 and 12 μM for 3,4-DHPEA-EDA and *p*-HPEA-EDA, respectively) activities toward HL60 cells.[23] However, the concentrations necessary to observe a significant effect were well above of those present in the medium when the VOO-PE were dissolved at a dose of 5 μg/mL. Therefore, synergistic effects with other components may be present. The pivotal role played by *p*-HPEA-EDA in the chemopreventive activity of VOO-PE is suggested by the observation that WW-PE, which is devoid of this compound, showed a much lower effect. In addition, earlier studies showed that *p*-HPEA-EDA is more potent than 3,4-DHPEA-EDA in both suppressing cell growth and inducing apoptosis on HL60 cells,[23,3] further confirming this conclusion. *p*-HPEA-EDA, identified for the first time in olives and olive oil in

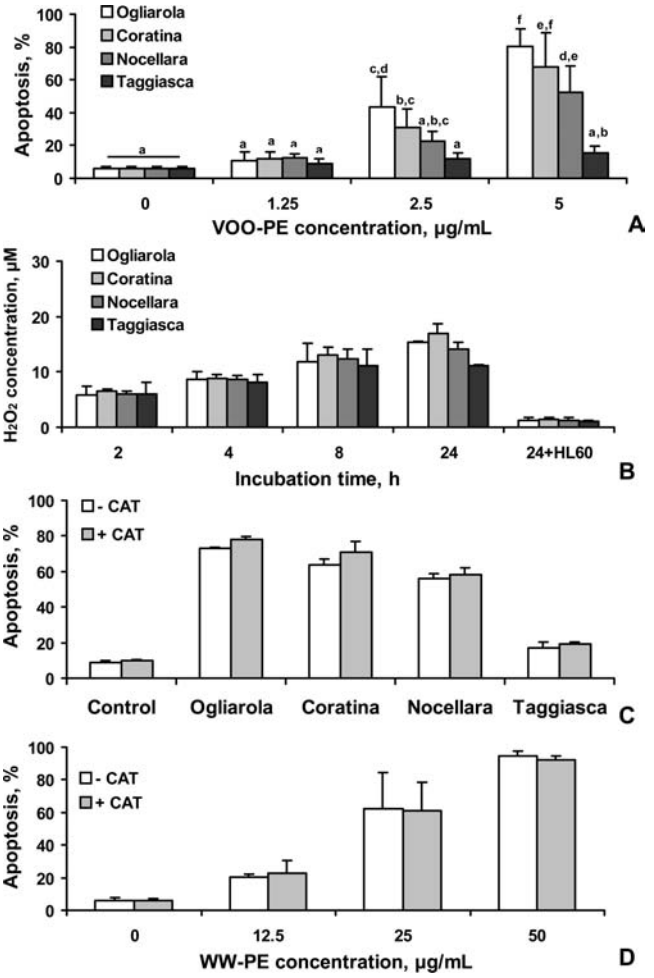

Figure 4. (A) Effect of VOO-PE derived from different olive oil cultivars (Ogliarola, Coratina, Nocellara, and Taggiasca) on apoptosis of HL60 cells after 24 h of incubation. (B) Accumulation of H_2O_2 in the cell culture medium induced by VOO-PE in the absence (2, 4, 8, and 24 h) and in the presence (24 + HL60) of HL60 cells (0.2×10^6/mL). (C) Pro-apoptotic activity of VOO-PE on HL60 cells after 24 h of incubation either in the absence (−CAT) or in the presence (+CAT) of catalase (100 U/mL). (D) Pro-apoptotic activity of WW-PE on HL60 cells after 24 h of incubation either in the absence (−CAT) or in the presence (+CAT) of catalase (100 U/mL). Values are the mean ± SD, $n = 5$. Means without a common letter differ, $P < 0.05$.

Table 2. Pearson Correlation Coefficients (r) between the Concentrations of Different Compounds in the Olive Oil Extracts and Their Pro-apoptosis and Antiproliferative Effects of HL60 Cells*

	apoptosis (%)	proliferation inhibition (%)
apoptosis (%)	1.000	
proliferation inhibition (%)	0.991**	1.000
3,4-DHPEA	−0.998**	−0.986*
p-HPEA	−0.945	−0.957*
3,4-DHPEA-EDA	0.746	0.765
p-HPEA-EDA	0.898	0.869
3,4-DHPEA-EA	0.338	0.254
(+)-1-acetoxypinoresinol	−0.983*	−0.966*
(+)-pinoresinol	−0.983*	−0.998**
total phenols	−0.085	−0.087

* The correlations were calculated using the molar concentrations of compounds in RPMI medium and either their pro-apoptotic effect expressed as percent of apoptotic cells after 24 h of treatment or their antiproliferative effect expressed as percent of inhibition after 72 h of treatment with 5 μg/mL of the olive oil extracts. *, $P < 0.05$; **, $P < 0.01$.

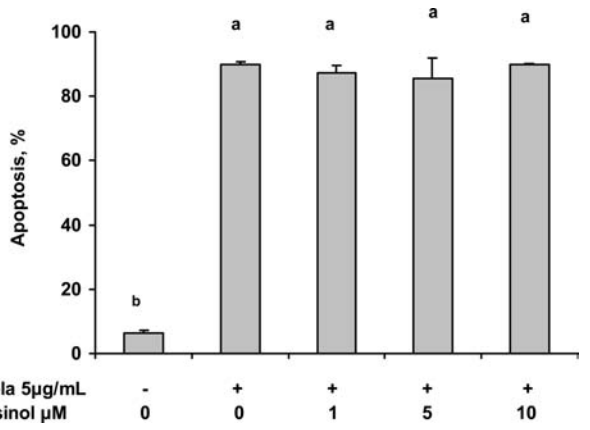

Figure 5. Effect of pinoresinol enrichment of the Ogliarola extract on its pro-apoptotic activity toward HL60 cells after 24 h of incubation. Values are the mean ± SD, $n = 3$. Means without a common letter differ, $P < 0.05$.

1993,[19] was later called oleocanthal and was found to inhibit the activity of cyclooxygenases COX-1 and COX-2, enzymes involved in prostaglandin synthesis.[24] These enzymes could be involved in the regulation of proliferation and apoptosis as suggested by data showing that celecoxib, a selective COX-2 inhibitor, was able to reduce growth and to induce apoptosis in different myeloid leukemia cell lines.[25] In addition, a phenolic extract of olive oil was shown to inhibit proliferation and to reduce the expression of COX-2 in a human colon cancer Caco-2 cell line.[26] Whether COX-2 inhibition is a mechanism by which *p*-HPEA-EDA exerts its antiproliferative and pro-apoptotic effects on HL60 cells remains to be determined. However, two pieces of evidence should be considered: the first is that COX-2 is slightly, if at all, expressed in HL60 cells;[27] the second is that the concentration of *p*-HPEA-EDA (25 μM) necessary to observe an evident COX-2 inhibition (about 50%)[24] is higher than that present in the cell culture medium when the VOO-PE were dissolved at a concentration of 5 μg/mL. Further studies are necessary to clarify this point, considering also that no antiproliferative effect was observed when an oleocanthal-rich olive oil extract was tested on RKO and SW480 colon cancer cell lines.[28]

The finding that both *p*-HPEA and 3,4-DHPEA were negatively correlated with the antiproliferative and pro-apoptotic potentials of the extracts can be explained by the observation that these compounds originate from the hydrolysis of their secoiridoid derivatives (*p*-HPEA-EDA and 3,4-DHPEA-EDA); therefore, they are more abundant in the extracts containing less *p*-HPEA-EDA and 3,4-DHPEA-EDA, and vice versa. In this context, it should also be emphasized that concentrations of *p*-HPEA and 3,4-DHPEA in the culture medium resulting when the VOO-PE were dissolved at 5 μg/mL are much lower than those (>250 μM for *p*-HPEA and >10 μM for 3,4-DHPEA) necessary to observe an effect on HL60 cells.[13]

The observation that the production of H_2O_2 in the cell culture medium is not involved in the pro-apoptotic effects of both VOO-PE and WW-PE agrees with earlier findings showing that *p*-HPEA-EDA and 3,4-DHPEA-EDA induced apoptosis on HL60 cells by a mechanism independent from H_2O_2. In addition, the small amount, always <1 μM, of H_2O_2 produced by the VOO-PE was actively removed by the HL60 cells. We have recently found that HL60 cells are resistant to the treatment with exogenous H_2O_2 (2.5 μM) (data not shown). The involvement of H_2O_2 in the chemopreventive activities of phenolic extracts obtained from other plant foods has been described, sometimes with contrasting results. For instance, Lapidot et al.[29] showed that the ability of an apple phenolic extract to inhibit HepG2 cell proliferation was dependent upon the generation of H_2O_2 in the culture medium, whereas Liu et al.[30] demonstrated that apple extracts were not able to induce H_2O_2 formation in different media.

The role played by lignans in the chemopreventive properties of the VOO-PE seems to be more complex. Because we found a strong negative correlation between their concentration and the pro-apoptotic effect of VOO-PE, it was important to exclude the possibility that these compounds can inhibit apoptosis. In fact, the addition of pinoresinol to the more potent VOO-PE (Ogliarola) did not reduce its pro-apoptotic activity. Instead, pinoresinol has been shown to reduce the viability of SKBR3 breast cancer cells ($IC_{50\%}$ = 72 μM)[31] and to inhibit the proliferation of *p53*-proficient colon cancer cells RKO and HCT116.[28] In this last study it was also found that only a pinoresinol-rich virgin olive oil extract was able to induce apoptosis in *p53*-proficient colon cancer cells. The different results reported in our study could be due to the fact that HL60 is a *p53* null cell line.[32] Preliminary results from our laboratory indicate that pinoresinol itself has a little pro-apoptotic activity but is able to inhibit proliferation on HL60 cells at concentrations (10−100 μM, data not shown) well above those present in the VOO-PE. Finally, during the preparation of this paper work was published in which the phenolic compositions of 12 different extracts of olive oil were compared with their cytotoxic ability against a breast cancer cell line (SKBR3) characterized by an overexpression of *erb*B-2 oncogene. Similar to our results, in that study positive and negative correlations were found between the cytotoxic potential of the extracts and their secoiridoid and lignans content, respectively.[33]

In conclusion, our study demonstrated that when tested in a complex mixture the olive oil phenols exert a more potent chemopreventive effect when compared to the single compounds. This phenomenon could be due to a synergistic interaction among the different compounds present in the extracts. However, it cannot be excluded that the complex extracts contain some other unidentified component(s) having a potent chemopreventive activity.

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