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Antioxidant, Antiproliferative, and Pro-apoptotic Capacities of Pentacyclic Triterpenes Found in the Skin of Olives on MCF-7 Human Breast Cancer Cells and Their Effects on DNA Damage

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This research aimed to investigate erythrodiol, uvaol, oleanolic acid, and maslinic acid scavenging capacities and their effects on cytotoxicity, cell proliferation, cell cycle, apoptosis, reactive oxygen species (ROS) level, and oxidative DNA damage on human MCF-7 breast cancer cell line. The results showed that erythrodiol, uvaol, and oleanolic acid have a significant cytotoxic effect and inhibit proliferation in a dose- and time-dependent manner. At 100 μM, erythrodiol growth inhibition occurred through apoptosis, with the observation of important ROS production and DNA damage, whereas uvaol and oleanolic acid growth inhibition involved cell cycle arrest. Moreover, although all tested triterpenes did not show free radical scavenging activity using ABTS and DPPH assays, they protected against oxidative DNA damage at the concentration 10 μM. Uvaol and oleanolic and maslinic acids, tested at 10 and 100 μM, also reduced intracellular ROS level and prevented H_2O_2-induced oxidative injury. Overall, the results suggest that tested triterpenes may have the potential to provide significant natural defense against human breast cancer.

KEYWORDS: Pentacyclic triterpenes; *Olea europaea*; olive oil; human breast cancer; antiproliferative activity; cell cycle arrest; apoptosis; antioxidant activity; DNA damage

INTRODUCTION

Breast cancer is the leading cause of mortality in women in developing countries (*1*). Of all environmental factors known to influence breast cancer, diet appears to be one of the most significant (*2*). Table olives and olive oil constitute regular dietary components of the traditional Mediterranean diet, which has been associated with a low incidence and prevalence of certain types of cancers, including breast cancer (*3*, *4*). This healthy property is mainly ascribed to oleic acid (*5*), phenolic compounds (*6*), and squalene (*7*,*8*). Nonetheless, other minor components have showed relevant interesting activities. Among them, erythrodiol, uvaol, oleanolic acid, and maslinic acid (**Figure 1**) are the main pentacyclic triterpenes located in the skin of olive fruits (*9*). These constituents are present in virgin olive oil (*10*) with higher concentration in olive pomace oil (*11*, *12*). Among other biological activities, including anti-inflammatory and cardioprotective (*13*−*15*), these triterpenes were reported to possess antioxidant and antitumor properties. Indeed, they were shown to prevent lipid peroxidation (*16*, *17*), protect low-density lipoproteins (LDL) against oxidation (*15*, *18*), and suppress superoxide anion generation (*19*, *20*). Furthermore, these bioactive compounds were found to inhibit the growth of tumor cell lines from various human cancers (*21*−*25*). Despite these numerous papers, to the authors' best knowledge, to date there are no available studies regarding the effects of these triterpenes on breast cancer cells. Therefore, the present study was undertaken to investigate the antioxidant capacity of erythrodiol, uvaol, oleanolic acid, and maslinic acid and its relationship to their antiproliferative capacity and oxidative DNA damage protection, using MCF-7 cells as a model for malignant breast cancer cells. For this purpose, we studied triterpenes' scavenging activity and their effects on cell growth, cell cycle profile, apoptosis, intracellular oxidative stress, and DNA oxidative damage.

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MATERIALS AND METHODS

Chemicals and Reagents. The following reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO): Hepes buffer; sodium pyruvate; nonessential amino acids mixture 100× (NEAA); 2′,7′-dichlorofluorescein diacetate (DCFH-DA); 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide inner salt (XTT sodium salt); 5-methylphenazinium methyl sulfate, *N*-methylphenazonium methyl sulfate (PMS); DL-*all-rac*-α-tocopherol (vitamin E); 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox); 2,2-diphenyl-1-picylhydrazyl (DPPH); 2,2′-azinobis(3-ethylbenzthiazolie-6-sulfonic acid) diammonium salt tablets (ABTS); phosphate buffer saline (PBS); and Hank's buffered salt solution (HBSS). Minimum essential medium with Eagle's salts (MEM), fetal bovine serum (FBS), and phenol-red-free Roswell Park Memorial Institute 1640 medium (RPMI) were obtained from PAA Laboratories GmbH (Pasching, Austria). TrypLE Express and propidium iodide (PI) were

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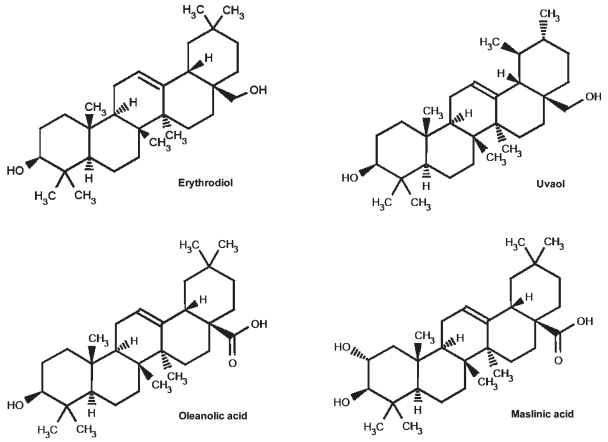

Figure 1. Chemical structures of erythrodiol, uvaol, oleanolic acid, and maslinic acid.

obtained from Invitrogen (Eugene, OR). $K_2S_2O_8$ was obtained from Panreac Quimica S.A.U (Barcelona, Spain). Culture plates were obtained from NUNC (Roskilde, Denmark). The PI/RNase Staining Buffer kit, FITC-conjugated annexin V, and binding buffer were obtained from BD Biosciences Pharmigen (San Diego, CA.). The comet assay kit was purchased from Trevigen, Inc. (Helgerman CT, Gaithersburg, MD).

Erythrodiol, uvaol, and oleanolic acid (purity ≥ 97, 98.5, and 99%, respectively) were purchased from Extrasynthese (Genay, France). Maslinic acid (purity > 80%) was provided by Dr. A. Garcia-Granados, Department of Organic Chemistry, University of Granada, Spain, and was obtained according to the patented method (*26*). Stock solutions of these compounds were prepared in ethanol and frozen at −20 °C until use. For cell experiments, these stock solutions were then diluted in MEM to reach the desired concentration. The final concentration of ethanol in the medium was < 0.6% for the highest concentration tested and had no significant effect on cell viability. Controls also received the same amount of ethanol in all experiments.

ABTS Radical Scavenging Assay. ABTS cation radical scavenging activity was determined using a previously reported procedure (*27*). ABTS radicals ($ABTS^{\bullet+}$) were obtained by ABTS/H_2O 0.5 mM reaction with $K_2S_2O_8$ for 16 h in the dark at room temperature. $ABTS^{\bullet+}$ was diluted in ultrapure water until the absorbance at 734 nm was 0.7 (±0.1). Stock solutions of tested triterpenes and Trolox (as antioxidant reference) in ethanol (10 mM) were diluted with ultrapure water to reach the assayed concentrations. Triterpene concentrations ranged from 12.5 to 800 μM, whereas Trolox concentrations were from 50 to 800 μM. Twenty microliters of each concentration of triterpene, standard (Trolox), blank (ultrapure water), or ethanol control (8%) was added to a 96-well plate. The reaction was initiated by the addition of 280 μL of $ABTS^{\bullet+}$. Absorbance readings were taken every 5 min at 30 °C during 120 min in a microplate reader (TECAN, GENios Plus). All determinations were carried out in triplicate. The inhibition of $ABTS^{\bullet+}$ was calculated according to the following percentage of free radical scavenging activity (% RSA) formula:

$$\%\ \mathrm{RSA} = [(A_{C(0)} - A_{A(t)})/A_{C(0)} \times 100]$$

Where $A_{C(0)}$ is the absorbance of the control (blank) at $t = 0$ and $A_{A(t)}$ is the absorbance in the presence of the triterpene or standard sample at $t = 60$ min.

DPPH Free Radical Scavenging Assay. The antioxidant activity of the tested triterpenes against the stable radical DPPH was measured as previously reported by Brand-Williams et al. (*28*) with some modifications. Briefly, a 100 μM ethanolic solution of DPPH was mixed with different ethanolic solutions of triterpenes in 96-well plates at 0.13, 0.25, 0.5, 1, 2.5, 5, and 10 mol of antioxidant/mol of DPPH. α-Tocopherol was used as a standard antioxidant control in the range of concentration 0.13−1.00 mol ratio. A sample without antioxidant was also measured as a blank control. The decrease in absorbance at 520 nm was determined immediately and every 5 min during 120 min in a microplate reader (TECAN GENios plus). Measurements were performed in triplicate. The inhibition of DPPH radical was calculated according to the percentage of free radical scavenging activity (% RSA) described above (at $t = 60$ min).

Cell Lines and Culture. The MCF-7 (primary human breast cancer) cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and was maintained at 37 °C in a humidified atmosphere under 5% CO_2 in MEM supplemented with 10% FBS, 1% Hepes buffer, 1% sodium pyruvate, and 1% NEAA. Cells in the exponential growth phase were used for all experiments.

MDA-MB-231 (metastatic human breast cancer) and U937 (human histiocytic lymphoma) cell lines were used to compare the effect of triterpenes on apoptosis induction. These cell lines were cultivated using the same MCF-7 culture conditions.

Cytotoxicity Assay. The effect of the tested triterpenes on cell viability was determined by the XTT assay, which is based on the ability of live cells to cleave the tetrazolium ring, thus producing formazan, which absorbs at 570 nm (*29*). A total of 5×10^3/well MCF-7 cells were grown onto 96-well plates for 24 h prior to treatment with increasing concentrations of triterpenes, from 12.5 to 100 μM, for another 24 h. Thereafter, plates were incubated with XTT in RPMI without phenol red for 3 h, and absorbance was measured at 450 nm wavelength (620 nm as reference) in a plate reader (TECAN GENios Plus). All measurements were performed in triplicate, and each experiment was repeated at least three times.

Cell Proliferation Assay. In the proliferation assay, MCF-7 cells were seeded at a density of 2×10^3/well onto 96-well culture plates and allowed to adhere for 24 h. Thereafter, medium was replaced with fresh medium containing increasing concentrations of tested triterpenes, from 12.5 to 100 μM, and plates were incubated for 24, 48, or 120 h. After each time point, medium was substituted by a fresh culture medium and cells were allowed a proliferation period of 120, 96, or 24 h, respectively. Then, plates were incubated with XTT in RPMI without phenol red for 3 h, and absorbance was measured at 450 nm wavelength (620 nm as reference) in a plate reader (TECAN GENios Plus) (*8*). All measurements were done in triplicate, and each assay was made twice.

Cell Cycle Assay. MCF-7 cells were seeded in 12-well culture plates at a density of 1×10^5/well. After 24 h, the cells were treated with or without (control) 10 and 100 μM triterpenes under study and incubated for an additional 24 h. They were then fixed with cold 70% ethanol and stored at −20 °C for at least 24 h. Cells were subjected to flow cytometry analysis on an EPICS XL-MCL cytofluorometer (Beckman Coulter, Spain) after propidium iodide labeling (PI/RNase staining buffer). The percentage of cells in sub G1, G0/G1, S, and G2/M phases was calculated using the FlowJo program (v5.7.2). The experiment was repeated at least three independent times.

Apoptosis Assay. The percentage of apoptotic cells was determined using a double staining assay with FITC-conjugated annexin V and propidium iodide (PI). MCF-7 cells (1×10^5/well) in 12-well culture plates were treated after 24 h with or without 10 and 100 μM triterpenes for another 24 h. Subsequently, cells were harvested, washed twice in cold PBS, and resuspended in 100 μL of annexin binding buffer. Cells were stained with 5 μL of annexin V−FITC and 1 μL of PI solution, gently vortexed, and incubated for 15 min at room temperature in the dark before flow cytometric analysis. The experiment was repeated at least three independent times.

Detection of Intracellular Reactive Oxygen Species. The generation of intracellular reactive oxygen species (ROS) was evaluated on the basis of the intracellular peroxide-dependent oxidation of DCFH-DA to form a fluorescent compound, DCF, as described by Warleta et al. (*8*). Briefly, MCF-7 cells were seeded at a density of 1×10^4/well on 96-well plates and grown for 24 h to allow adhesion. Subsequently, the medium was substituted with a fresh one containing 1, 10, or 100 μM tested triterpenes, and cells were incubated for another 24 h. Thereafter, cells were washed two times with HBSS and incubated with fresh DCFH-DA (100 μM) for 30 min at 37 °C in 5% CO_2. Cells were then washed twice and read in a plate reader for 30 min (Ex λ_{485}/Em λ_{535}, gain 60). The intracellular ROS level percentage was calculated as

$$F = [(F_{t=30} - F_{t=0})/F_{t=0} \times 100]$$

where $F_{t=0}$ is the fluorescence at $t = 0$ min and $F_{t=30}$ the fluorescence at $t = 30$ min.

Hydrogen peroxide (H_2O_2), a widely used model of in vitro oxidative stress, can directly damage DNA, lipids, and other macromolecules, causing oxidative injury to the cell (*30*). To evaluate whether erythrodiol, uvaol, oleanolic acid, or maslinic acid is able to reduce induced oxidative

Table 1. Percentage of Free Radical Scavenging Activity of Erythrodiol, Uvaol, Oleanolic Acid and Maslinic Acid Measured by Decolorization of $ABTS^{\bullet+}$ (**A**) and Reduction of the DPPH Radical (**B**)[a]

(**A**)

	Trolox	erythrodiol	uvaol	oleanolic acid	maslinic acid
12.5 μM	ne	3.49 ± 0.34	4.08 ± 0.55	3.69 ± 0.43	4.39 ± 0.36
25 μM	ne	2.61 ± 0.39	3.59 ± 0.36	3.19 ± 0.39	3.91 ± 0.24
50 μM	17.61 ± 0.38	2.74 ± 0.26	3.57 ± 0.63	3.19 ± 0.43	4.83 ± 0.49
100 μM	32.77 ± 0.77	2.30 ± 0.41	5.02 ± 0.86	2.66 ± 0.66	4.34 ± 0.37
200 μM	58.70 ± 1.32	2.83 ± 0.56	5.25 ± 1.04	3.62 ± 1.09	5.86 ± 0.46
400 μM	94.99 ± 0.77	2.08 ± 0.64	4.37 ± 1.05	5.92 ± 1.76	10.03 ± 0.68
800 μM	99.06 ± 0.24	2.28 ± 0.16	4.60 ± 1.36	5.60 ± 1.67	18.32 ± 0.86

(**B**)

mol AH/mol/DPPH	α-tocopherol	erythrodiol	uvaol	oleanolic acid	maslinic acid
0.13	44.33 ± 1.71	—	2.86 ± 0.63	—	0.20 ± 0.07
0.25	71.64 ± 2.10	—	1.69 ± 0.65	—	0.33 ± 0.11
0.50	81.50 ± 1.25	—	1.46 ± 0.61	—	0.75 ± 0.22
1.00	81.24 ± 1.19	—	2.26 ± 0.57	0.60 ± 0.35	2.08 ± 0.11
2.50	ne	3.47 ± 1.14	4.40 ± 0.64	3.42 ± 0.29	24.45 ± 0.23
5.00	ne	3.35 ± 0.69	3.42 ± 0.69	2.61 ± 0.61	42.37 ± 0.44
10.00	ne	8.24 ± 0.63	12.51 ± 1.19	10.48 ± 1.08	71.93 ± 0.38

[a] Trolox and α-tocopherol were used as standard antioxidants control. Values represent the mean ± SEM of three independent experiments at t = 60 min. ne, not estimated. —, free radical scavenging capacity was not detected.

stress, cells were challenged with 500 μM H_2O_2 30 min before fluorescence quantification.

All measurements were performed in triplicate, and each experiment was repeated at least three times.

Alkaline Single-Cell Gel Electrophoresis (Comet Assay). MCF-7 cells were seeded at the density of 1×10^5/well into 12-well plate cultures. After 24 h, they were treated with or without 10 and 100 μM triterpenes for an additional 24 h. They were then scraped, washed twice (300*g*, 10 min, 4 °C) with cold 1× PBS (Ca^{2+}/Mg^{2+} free), and resuspended in 1 mL of cold 1× PBS. After that, the comet assay was performed according to the method of Warleta et al. (*8*).

To assess whether triterpenes are able to protect against oxidative DNA damage, cell suspensions were exposed for 10 min to 50 μM H_2O_2 at 4 °C. Cells washed twice with cold 1× PBS and resuspended in 1 mL of cold 1× PBS were then subjected to the comet assay procedure.

Slide Scoring and Analysis. DNA strand breaks were examined in a fluorescence microscope (Zeiss Axiovert 200) equipped with a Luca EMCCD camera (Andor Technology, Belfast, U.K.) under 494 nm excitation and 521 nm emission wavelength using the Komet 5.5 software package (Kinetic Imaging Ltd., Liverpool, U.K.). Fifty cell images were randomly characterized per sample using 20× magnification. Relative fluorescence between head and tail through the Olive tail moment (Olive_TM) was used to determine DNA damage.

Statistical Analysis. The results of free radical scavenging activity of tested triterpenes (% RSA) are presented as the mean of three independent experiments ± the standard error of the mean (SEM).

For cell assays, data are displayed as the mean of at least three independent experiments ± SEM, and results are expressed as a percentage relative to the untreated control cells, which was defined as 100%. A general variance analysis (ANOVA) was carried out on all data followed by Fisher's LSD test. A p value of < 0.05 was considered to be statistically significant. These statistical analyses were performed using Statgraphics Plus 5.1 statistical software (Statpoint Technologies, Inc., Warranton, VA).

RESULTS

Antioxidant Activity. The free radical scavenging capacity of triterpenes under study was evaluated by means of ABTS and DPPH assays. As shown in **Table 1**, erythrodiol, uvaol, and oleanolic acid have shown a weak free radical scavenging activity in both tests. Maslinic acid also exhibited a weak antiradical activity up to 800 μM and 2.50 mol ratio; however, from 5.00 mol ratio, a high DPPH scavenging activity was observed (RSA > 70% at 10.00 mol ratio).

Effects of Triterpenes on Cell Survival. MCF-7 cells were exposed to increasing concentrations, from 12.5 to 100 μM, of erythrodiol, uvaol, oleanolic acid, and maslinic acid for 24 h, and then cell survival, compared with untreated controls, was evaluated using the XTT assay. As shown in **Figure 2**, the percentage of living cells decreased in a dose-dependent manner, respectively, from 25 and 50 μM erythrodiol and uvaol. It is worth noting that at the same concentration of 100 μM, the cytotoxic effect induced by erythrodiol was significantly stronger than that induced by uvaol (respectively, 12 and 64% of the cells still viable). Related to triterpenic acids, cell survival dropped slightly by 50 or 100 μM oleanolic acid (89% of the cells still viable), whereas it was unaffected by the presence of maslinic acid.

Effects of Triterpenes on Cell Proliferation. The effects of tested triterpenes on MCF-7 cell proliferation was evaluated with concentrations ranging from 12.5 to 100 μM after 24, 48, and 120 h of exposure (**Figure 3**). A dose- and time-dependent decrease in cell proliferation rate was achieved by erythrodiol and oleanolic acid. Cell proliferation was almost completely inhibited 24 h after MCF-7 exposure to 50 μM erythrodiol and 100 μM oleanolic acid (**Figure 3A**,**C**). Uvaol, however, showed a biphasic behavior; an increase in proliferation rate was observed at the concentration of 12.5 μM, followed by a dose- and time-dependent proliferation inhibition from 25 μM (**Figure 3B**). Finally, maslinic acid increased the MCF-7 cell proliferation rate (**Figure 3D**).

Effects of Triterpenes on Cell Cycle and Apoptosis. Figure 4 shows the effects of triterpenes on the different phases of the cell cycle. MCF-7 treatment with 10 μM erythrodiol induced a significant increase in the population in G0/G1 phase (8%), whereas a 100 μM concentration of this compound caused a marked decrease in the percentage of cells in G0/G1 (33%) and an increase in the percentage of cells in sub G1 phase (10-fold higher than untreated control cells) (**Figure 4A**). This sub G1 is normally associated with apoptosis. When cells were treated with a 10 μM

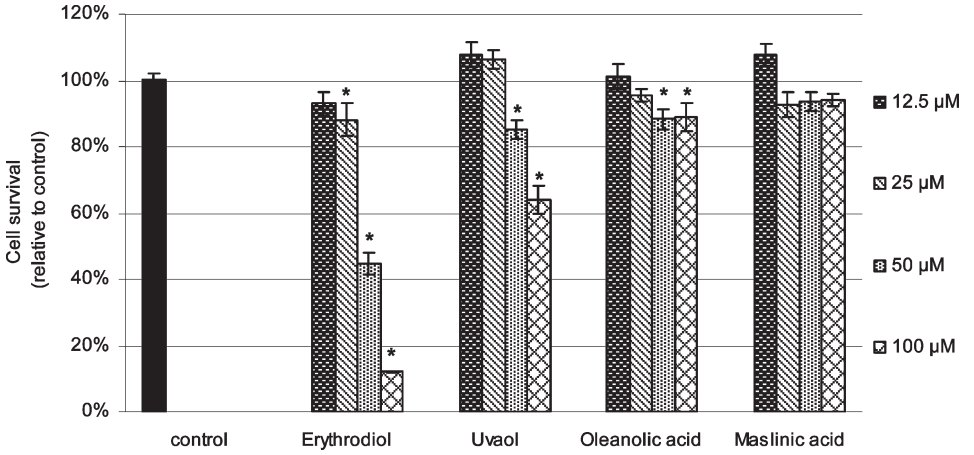

Figure 2. Effects of triterpenes on MCF-7 cell survival. Cells were treated with different concentrations of erythrodiol, uvaol, oleanolic acid, and maslinic acid for 24 h, and then cell survival was determined by the XTT assay. The results represent the mean ± SEM of three independent experiments. * denotes statistically significant difference compared with control, which was considered to be 100% ($p < 0.05$).

concentration of either uvaol or oleanolic acid, no changes on cell cycle profiles were detected with respect to untreated controls. At the concentration of 100 μM, uvaol induced a significant increase in G0/G1 phase (8%) and a decrease in S phase (41%) (**Figure 4B**). Similarly, 100 μM oleanolic acid caused an increase in the population in G0/G1 (18%), with a concomitant decrease in the percentage of cells in the S phase (50%) and G2/M phase (34%) (**Figure 4C**). At both concentrations tested, maslinic acid did not induce significant change in the cell cycle profile (**Figure 4D**).

Assessment of apoptosis was performed by flow cytometry using annexin V−FITC and PI labeling, and the percentage of apoptotic cells was calculated from the ratio of the sum of early plus late apoptotic cells. Consistent with cell cycle analysis, 100 μM erythrodiol strongly induced apoptosis (the apoptotic rate increased from 12% in control untreated cells to 64%), whereas no effect on apoptosis induction was observed by the rest of the triterpenes (**Figure 5A**).

In the same way, 100 μM erythrodiol strongly induced apoptosis in MDA-MB-231 and U937 cells (the apoptotic rate increased from 7 to 83% and from 1 to 80%, respectively). The rest of the triterpenes did not induce apoptosis in MDA-MB-231, but they did in U937 cells (**Figure 5B**).

Effects of Triterpenes on Intracellular ROS Level. Measurement of intracellular ROS level using the DCFH-DA probe showed a significant decrease in ROS level in MCF-7 cells after 24 h of treatment with uvaol, oleanolic acid, and maslinic acid. However, in the case of erythrodiol, a marked increase in intracellular ROS level was observed at the concentration of 100 μM, whereas no significant effect was observed at a 1 or 10 μM concentration of this triterpene (**Figure 6A**).

To induce intracellular oxidative stress, H_2O_2 was added before fluorescence measurement. As shown in **Figure 6B**, H_2O_2 induced oxidative injury on MCF-7 cells. Thereafter, to investigate the in vitro preventive effect of triterpenes against H_2O_2 oxidative injury, the intracellular ROS level was measured in MCF-7 cells previously treated with 1, 10, and 100 μM triterpenes. Our results revealed that uvaol, oleanolic acid, and maslinic acid protected against oxidative injury as compared to H_2O_2 control cells in the range of concentrations tested, whereas, as expected, 100 μM erythrodiol did not show any protective effect, showing again an important increase in intracellular ROS level.

Effects of Triterpenes on DNA Integrity and H_2O_2-Induced DNA Damage. The effects of triterpenes under study on DNA integrity and H_2O_2-induced DNA damage in MCF-7 cells was evaluated using alkaline single-cell gel electrophoresis (comet assay), and results were expressed as Olive_TM. Olive_TM incorporates a measure of both the smallest detectable size of migrating DNA (reflected in the comet tail length) and the number of relaxed/broken pieces (represented by the intensity of DNA in the tail) (*31*). Results showed that neither uvaol, oleanolic acid, or maslinic acid, at both doses tested, nor erythrodiol, at the lowest dose, affected DNA integrity. However, significant DNA damage was produced by 100 μM erythrodiol as compared with control (**Figure 7A**).

MCF-7 treatment with 50 μM H_2O_2 alone for 10 min (control H_2O_2) significantly increased Olive_TM when compared with control (**Figure 7B**). Preincubation of MCF-7 cells with a 10 μM concentration of either erythrodiol, uvaol, or oleanolic acid exerted a preventive effect against H_2O_2-induced DNA damage (respectively, 63, 43, and 26% reduction), whereas at 100 μM no longer was a preventive effect observed. Otherwise, MCF-7 cells treated with maslinic acid significantly decreased the formation of single-strand breaks (up to 76% reduction at 100 μM) (**Figure 7B**).

DISCUSSION

In the present study, we have focused on erythrodiol, uvaol, oleanolic acid, and maslinic acid, four pentacyclic triterpenes found in *Olea europaea* (*9*−*12*). Previous papers have described the antiproliferative properties of these triterpenes against various cancer cells (*21*−*25*). However, their antitumor capacity against breast cancer has not yet been studied. Here, we report on the antiproliferative and antioxidant capacities of these triterpenes in the MCF-7 human breast cancer cell line, the first such study to date. Our results suggest that tested triterpenes may have the potential to provide significant natural defense against human breast cancer. This conclusion is based on the following findings: antiproliferative and cytotoxic effects, cell cycle arrest and/or induction of apoptosis, reduction of intracellular ROS level, and prevention against oxidative DNA damage, all depending on dose and chemical structure.

Cytotoxicity, a common preliminary method, is helpful to determine whether tested compounds have potential antineoplastic properties (*32*). Furthermore, increased proliferation and decreased cell death (apoptosis) are two major processes that contribute to the progression of tumor cell growth. The cytotoxic effect of oleanolic and maslinic acids has been previously reported on several tumor cell lines such as A549 (non-small-cell lung cells), SK-OV-3 (ovary), SK-MEL-2 (melanoma), XF-498 (central nervous system), HCT-15 (colon), HSC-2 (oral squamous cell carcinoma), and

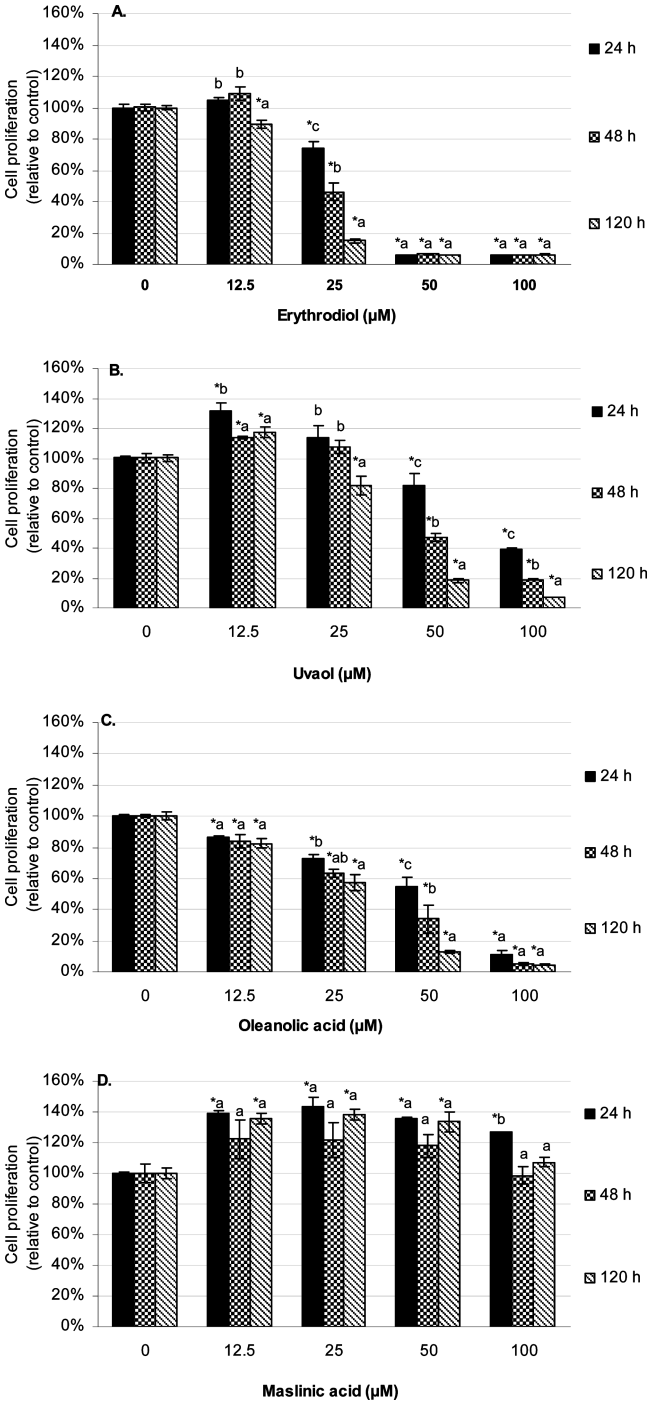

Figure 3. Effects of triterpenes on MCF-7 cell proliferation. Cells were treated with different concentrations of erythrodiol (**A**), uvaol (**B**), oleanolic acid (**C**), and maslinic acid (**D**) for 24, 48, and 120 h, and then cell proliferation, using the XTT assay, was determined after a proliferation period of up to 6 days. The results represent the mean ± SEM of two independent experiments and are expressed as percentage of the control, which was defined as 100%. ∗ denotes statistically significant difference between concentrations for each time ($p < 0.05$). Different letters denote statistically significant difference between times for each concentration ($p < 0.05$).

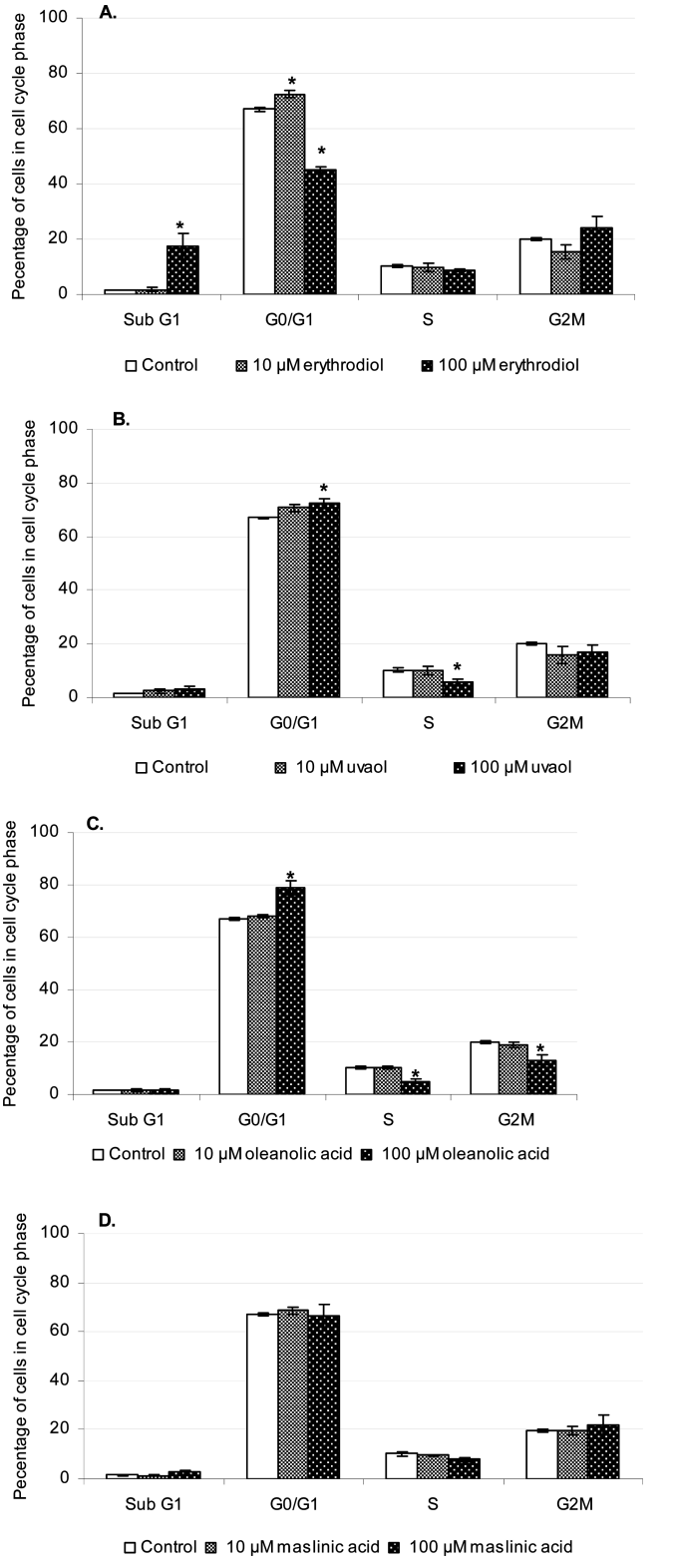

Figure 4. Effects of triterpenes on MCF-7 cell cycle distribution. Cells treated with 10 and 100 μM erythrodiol (**A**), uvaol (**B**), oleanolic acid (**C**), and maslinic acid (**D**) for 24 h were collected and stained with PI after fixation by 70% ethanol. Following flow cytometry, cell cycle distribution was analyzed using the FlowJo program. The results represent the mean ± SEM of four independent experiments. ∗ denotes statistically significant difference compared with control ($p < 0.05$).

HSG (salivary gland tumor) (*33*, *34*). In addition, the antiproliferative capacity of erythrodiol, uvaol, oleanolic, and maslinic acids was described in Caco-2 and HT-29 colon cancer cell lines (*21*−*23*) and in 1321N1 astrocytoma cell line (*24*, *25*). In this study, our results showed that erythrodiol, uvaol, and oleanolic acid significantly inhibited cell growth and proliferation in a dose- and time-dependent manner, the effect of erythrodiol being more pronounced. Hence, these data suggest that these triterpenes effectively reduced the malignancy and suppressed the generation

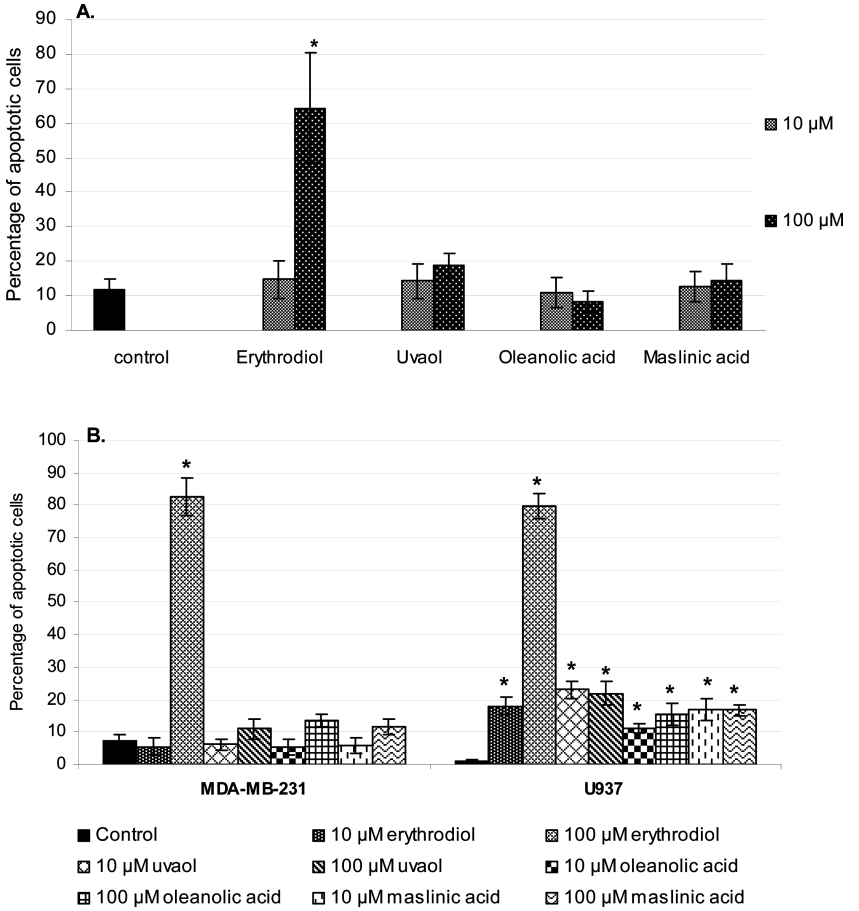

Figure 5. Effects of triterpenes on apoptosis induction on MCF-7 (**A**) and MDA-MB-231 and U937 cells (**B**). Cells treated with 10 and 100 μM erythrodiol, uvaol, oleanolic acid, and maslinic acid for 24 h were collected and stained with FITC-conjugated annexin V and PI. Following flow cytometry, the percentage of apoptotic cells was calculated from the ratio of the sum of early plus late apoptotic cells using the FlowJo program. The results represent the mean $\pm$ SEM of three independent experiments. $*$ denotes statistically significant difference compared with control ($p < 0.05$).

potential of cancer cells. Uvaol showed a significant proliferative effect at the lowest dose of 12.5 μM. It has been reported that at low doses, certain antioxidants can stimulate the growth of some types of cancer. For instance, vitamin C was found to stimulate the growth of both human parotid carcinoma cells and human leukemic cells in vitro (*35*), and vitamin E was found to enhance the growth of various prostate cancer cell lines (*36*). Future work is needed to clarify the mechanism of stimulation of cancer cell growth by some antioxidants.

It has been reported that some anticancer agents cause growth inhibition through interfering with the processes of cell cycle (*37*) and some others cause cell death by apoptosis (*38*). On the one hand, cell cycle is under strict regulation in the cell with numerous control points that allow correct progression of the different phases. In this sense, a delay in progression of the G0/G1, S, or G2/M cell cycle phases would constitute a cellular defense mechanism to allow action of the DNA repair systems (*39*). On the other hand, apoptosis is considered to be a physiologically important process that functions to eliminate undesired cells during development and homeostasis of multicelular organisms. Therefore, to determine whether cell cycle arrest or apoptosis is involved in growth inhibition, we examined cell cycle phase distribution and induction of apoptosis of the triterpene-treated cells by flow cytometry. Our results indicated that at the concentration of 100 μM, uvaol and oleanolic acid inhibition of proliferation appeared to result from inhibition of cell cycle progression, because these compounds were found to be involved in the action of the G0/G1 checkpoint and inhibition of DNA replication. The literature lacks studies on the potential antiproliferative capacity of uvaol. Nonetheless, recently Martín et al. (*25*) reported that uvaol growth-inhibiting activity is associated with the induction of apoptosis in 1321N1 astrocytoma cells. These authors observed that exposure of 1321N1 cells to 50 and 100 μM uvaol for 18 h induces apoptosis around 40−60%. Instead, and in agreement with our results, Li et al. (*40*) and Cipak et al. (*41*) reported that oleanolic acid, at the concentration of 60 μM, inhibition of proliferation occurred through G0/G1 phase arrest, respectively, after 72 and 24 h of exposure in HCT-15 colon adenocarcinoma cell line and HL-60 leukemic cell line. By contrast, in another study it was found that oleanolic acid inhibition of proliferation is mediated via apoptosis in 1321N1 astrocytoma cells treated for 18 h with a 25 μM concentration of this triterpenic acid (*24*). Differences in uvaol and oleanolic acid responses are therefore most likely due to the specific cell type and concentration and treatment time used. Moreover, in our study, it was found that uvaol and oleanolic acid did not induce apoptosis in MDA-MB-231 human breast adenocarcinoma cell line but did in U937 human leukemic cell line, and it was not dose-dependent. Otherwise, our results suggest that 100 μM erythrodiol inhibition of MCF-7 cell proliferation appeared to result from the occurrence of apoptosis. Erythrodiol was also found to strongly induce apoptosis in MDA-MB-231 and U937 cells. Our findings are in agreement with others previously reported (*23*, *25*) describing that erythrodiol antiproliferative activity is associated with the induction of apoptosis and that erythrodiol apoptotic potential appears to be a generalized event.

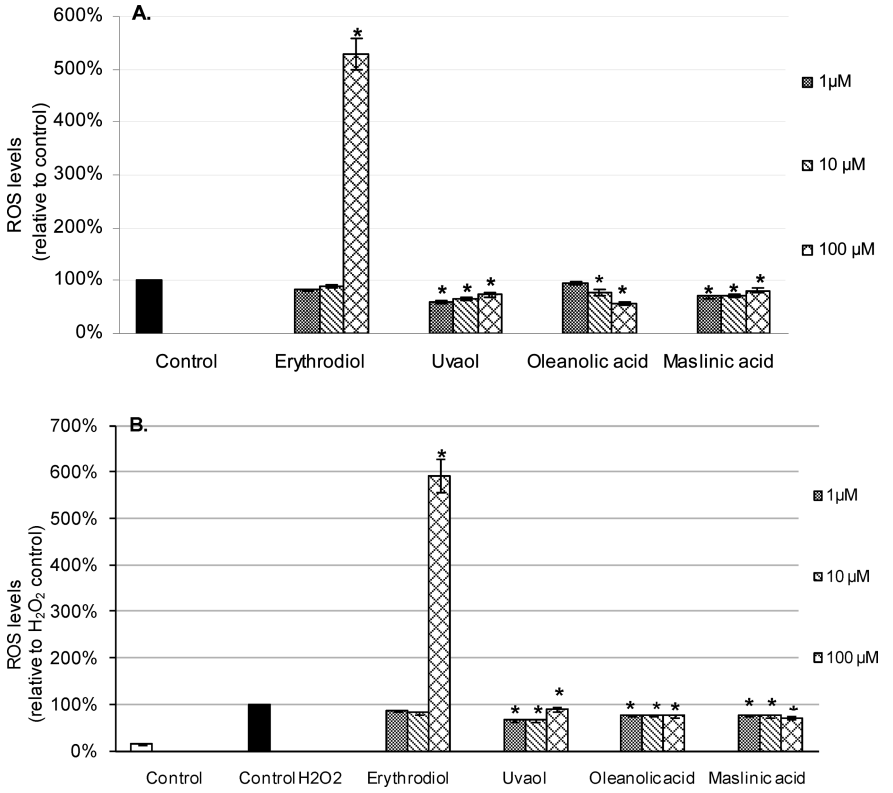

Figure 6. (**A**) Intracellular ROS level in MCF-7 cells after triterpene treatment. Cells were treated with different concentrations of erythrodiol, uvaol, oleanolic acid, and maslinic acid for 24 h, and then intracellular ROS level was measured by DCFH-DA probe. The results represent the mean ± SEM of three independent experiments. ∗ denotes statistically significant difference compared with control, which was considered to be 100% ($p < 0.05$). (**B**) Capacity of triterpenes to reduce MCF-7 oxidative stress induced by H_2O_2 addition measured by DCFH-DA probe. The results represent the mean ± SEM of three independent experiments. ∗ denotes statistically significant difference compared with positive control (control H_2O_2), which was considered to be 100% ($p < 0.05$).

Under our experimental conditions, incubation of MCF-7 cells for 24 h with maslinic acid did not exert any signs of cytotoxicity or alter cell cycle parameters, nor did it induce apoptosis. However, it increased proliferation rate. Our results are in contrast with others reported previously describing the antiproliferative and apoptosis-inducing effect of maslinic acid on colon and brain cancers (*21*, *22*, *24*, *42*, *43*). On the other hand, it was observed that maslinic acid induces apoptosis in U937 leukemic cells. Differences in susceptibility to maslinic acid may be therefore due to differences in the nature of the cell line. Further studies should be done to understand the mechanism by which maslinic acid stimulates MCF-7 cells proliferation.

It has been reported that many anticancer compounds exert antitumor activity including antioxidant mechanism (*44*). The triterpenes under study have been previously reported to possess antioxidant properties in a variety of experimental systems (*15*−*20*). However, to the authors' best knowledge, very little is known about the triterpenes' free radical scavenging activity. Using ABTS and DPPH chemical assays, our results indicated that the tested triterpenes lack free radical scavenging capacity. Accordingly, Yang et al. (*45*) reported that oleanolic and maslinic acids did not possess DPPH scavenging capacity up to the concentration of 200 μM. Nonetheless, in our study it was found that at very high mole ratio (up to 5.00 mol), maslinic acid exhibited a high DPPH scavenging capacity. Furthermore, we recently reported that maslinic acid, unlike the rest of the triterpenes, acts as an efficient peroxyl radical scavenger as assessed by the ORAC assay (*15*).

On the other hand, at present, overwhelming evidence indicates that ROS are involved in both the initiation and progression of cancer (*46*). In this sense, the cancer chemopreventive properties of antioxidants are generally believed to be due to their ability to scavenge endogenous ROS (*47*). Furthermore, oxidative stress may result in an increase in oxidative damage and can be caused either by an overproduction of free radicals and ROS or by an impairment of the endogenous antioxidant system. In this paper, DCFH-DA assay results showed that uvaol and oleanolic and maslinic acids decreased significantly the steady-state generation of ROS by MCF-7 in culture. In addition, it was found that increased levels of ROS generated during the oxidative stress period (30 min of H_2O_2 exposure) were quenched in cells pretreated with these triterpenes. Overall, our data indicate that in the cellular system, uvaol and oleanolic and maslinic acids behaved as antioxidants, reducing both endogenous and exogenous ROS levels.

On the other hand, ROS levels have been shown to play an important role in the initiation and execution of apoptosis of many anticancer compounds (*48*). In this sense, erythrodiol, uvaol, oleanolic acid, and maslinic acid induction of apoptosis was found to be preceded by ROS generation in astrocytome cells (*24*, *25*). The same effect was observed by maslinic acid in HT-29 cells (*42*). On basis of these observations, in our study the increase in intracellular ROS level in MCF-7 cells treated with 100 μM erythrodiol may be related to apoptosis induction. Further experiments should be performed to study the detailed mechanism of apoptosis.

The comet assay provides a simple and effective method for evaluating DNA damage at the single-cell level. As expected, MCF-7 cell treatment with uvaol, oleanolic acid, or maslinic acid showed no genotoxicity at both doses tested. Similarly, 10 μM erythrodiol did not induce DNA damage. By contrast, and consistent with apoptosis and ROS results, 100 μM erythrodiol was found to produce high DNA damage.

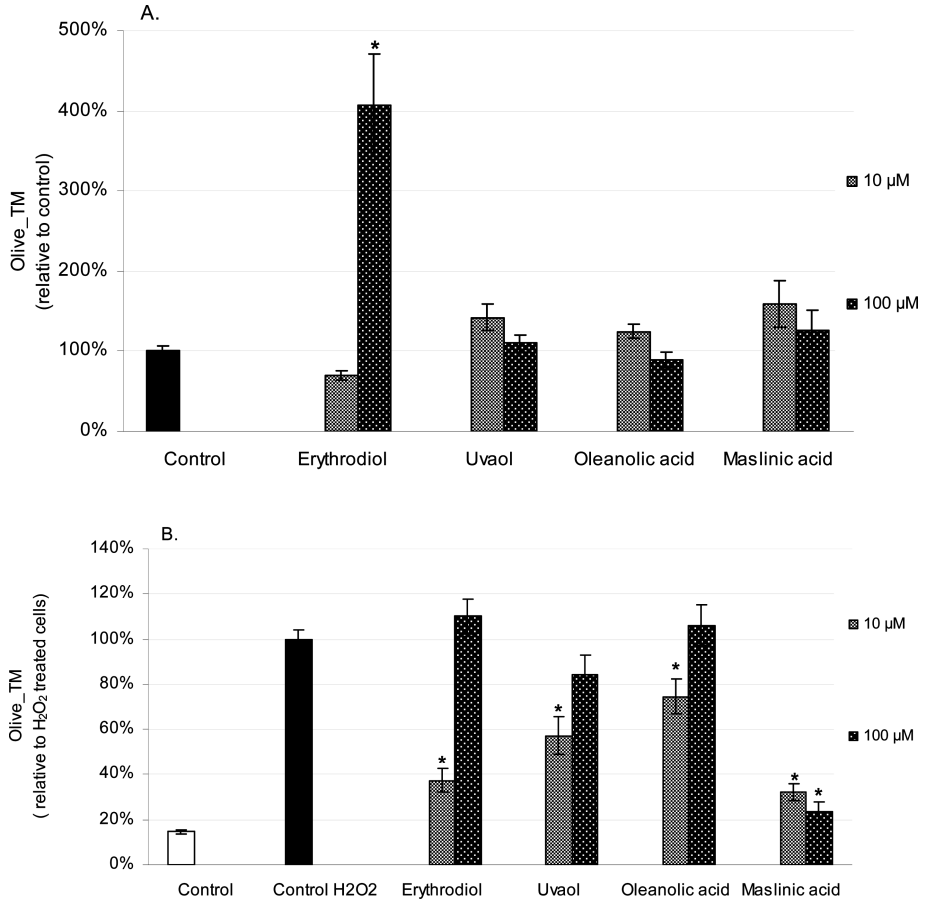

Figure 7. (**A**) Effects of triterpenes on DNA integrity assessed by the comet assay. MCF-7 cells treated with 10 and 100 μM erythrodiol, uvaol, oleanolic acid, and maslinic acid for 24 h were subjected to comet assay. Olive_TM values represent the mean ± SEM. * denotes statistically significant difference compared with control, which was considered to be 100% ($p < 0.05$). (**B**) Effects of triterpenes on DNA oxidative damage induced by H_2O_2 addition evaluated by the comet assay. Olive_TM values represent the mean ± SEM. * denotes statistically significant difference compared with positive control (control H_2O_2), which was considered to be 100% ($p < 0.05$).

H_2O_2 is suggested to cause DNA strand breakage by the generation of hydroxyl radicals ($OH^{\bullet}$) through the Fenton or Fenton-like reactions (*49*). In fact, in our study it was found that preincubation of MCF-7 cells with H_2O_2 for 10 min caused marked DNA damage. Very little is known regarding the effect of the tested triterpenes against DNA-induced oxidative injury, and only Ovesná et al. (*50*) have previously reported the effect of oleanolic acid against H_2O_2-induced DNA damage. These authors concluded that this triterpene significantly reduced DNA-induced oxidative injury in leukemic L1210, K562, and HL-60 cells in the concentration range from 2.5 to 10 μM. In agreement, in our experiment it was found that at 10 μM, oleanolic acid, as well as erythrodiol and uvaol, exhibited a protective effect against DNA damage caused by H_2O_2. However, no longer was a protective capacity observed when tested at 100 μM. By contrast, maslinic acid displayed a protective capacity at both doses tested.

According to Iliakis et al. (*51*), the DNA damage response is a hierarchical process; to allow time for DNA repair, the cells activate checkpoint pathways that delay the normal progression of the cell cycle. Checkpoints, together with repair and apoptosis, are integrated in a circuitry that determines the ultimate response of a cell to DNA damage. Therefore, our results, taken together, suggest that at low dose (10 μM), erythrodiol, uvaol, oleanolic acid, and maslinic acid in a dose-independent manner act as antioxidants scavenging superoxide and hydroxyls radicals produced by H_2O_2, thus preventing the impairment of mitochondrial function and consequently avoiding MCF-7 cell death. However, the absence of protective activity against DNA-induced damage in MCF-7 cells pretreated with high doses (100 μM) of erythrodiol, uvaol, and oleanolic acid may be linked to their capacity to activate other signaling pathways, resulting in cell cycle arrest and/or apoptosis and, thus, proliferation inhibition. Additional experiments to get a better understanding of the triterpenes' mechanisms of action are in progress.

In relation to the chemical structures, these pentacyclic triterpenes can be classified as alcoholic or acid (**Figure 1**). Although the structure and activity relationships of these pentacyclic triterpenes are far from clear, it seems that the $-CH_2OH$ group at C-28 enhances the cytotoxic capacity of the triterpenic diols as compared to triterpenic acids ($-COOH$ at C-28). In addition, the pro-apoptotic capacity of erythrodiol may be related to the presence of two methyl groups at C-20. Maslinic acid differs from the other triterpenes by the presence of an additional $-OH$ group at C-2, which appears to suppress the cytotoxic effect and confer it antioxidant activity.

To the authors' best knowledge, to date, there are no studies reporting the bioavailability of these triterpenes. Therefore, it is difficult to predict the presence of these compounds or their derived metabolites in the blood after consumption of a triterpene-rich diet. In any case, the concentrations used in the present study are similar to those previously reported (*21*−*25*).

In conclusion, our results provide new insight into the anticarcinogenic action of erythrodiol, uvaol, oleanolic acid, and maslinic acid in human breast cancer. Moreover, our findings

support the hypothesis that these triterpenes, isolated in appreciable higher amounts from the non-glyceride fraction of pomace olive oil, may be considered as valuable molecules for use as cancer chemotherapeutic or chemopreventive agents.

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