

## Antiproliferative and apoptosis-inducing effects of maslinic and oleanolic acids, two pentacyclic triterpenes from olives, on HT-29 colon cancer cells

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We have previously reported the anticarcinogenic effects of an olive fruit extract composed of pentacyclic triterpenes, the main components of which are maslinic acid (73.25%) and oleanolic acid (25.75%). Here we examined the effects of the individual components on proliferation, necrosis and apoptosis rates by fluorescence-based techniques in human HT-29 colon cancer cells. Oleanolic acid showed moderate antiproliferative activity, with an  $EC_{50}$  of 160.6 (SE 10.6)  $\mu\text{mol/l}$ , and moderate cytotoxicity at high concentrations ( $\geq 250 \mu\text{mol/l}$ ). On the other hand, maslinic acid inhibited cell growth with an  $EC_{50}$  of 101.2 (SE 7.8)  $\mu\text{mol/l}$ , without necrotic effects. Oleanolic acid, which lacks a hydroxyl group at the carbon 2 position, failed to activate caspase-3 as a prime apoptosis protease. In contrast, maslinic acid increased caspase-3-like activity at 10, 25 and 50  $\mu\text{mol/l}$  by 3-, 3.5- and 5-fold over control cells, respectively. The detection of ROS in the mitochondria, which serve as pro-apoptotic signal, evidenced the different bioactivity of the two triterpenes. Confocal microscopy analysis revealed that maslinic acid generated superoxide anions while oleanolic acid-treated cells did not differ from the control. Completion of apoptosis by maslinic acid was confirmed microscopically by the increase in plasma membrane permeability, and detection of DNA fragmentation. In conclusion, the anticancer activity observed for olive fruit extracts seems to originate from maslinic acid but not from oleanolic acid. Maslinic acid therefore is a promising new compound for the chemoprevention of colon cancers.

### Maslinic acid: Oleanolic acid: Apoptosis: HT-29 cells

Olives and olive oil are a major part of the traditional Mediterranean diet, which has been associated with a low incidence and prevalence of cancer<sup>(1,2)</sup>. In support of a hypothesis that constituents of olives may contribute to the cancer protective activity of this diet we recently reported that an extract from the skin of olive fruits composed of maslinic acid (73.25%) and oleanolic acid (25.75%) inhibited proliferation and effectively induced apoptosis in HT-29 human colon cancer cells<sup>(3)</sup>. Maslinic and oleanolic acids (Fig. 1) are pentacyclic triterpenes present in the skin of olive fruits<sup>(4,5)</sup>. These non-nutritive dietary constituents can be detected in olive oils<sup>(4)</sup> with higher concentrations in olive pomace oils, also known as 'orujo oil', than in virgin olive oils<sup>(6)</sup>. In olive fruits maslinic and oleanolic acids are present at concentrations of 681 (SE 63) mg/kg and 420 (SE 20) mg/kg, respectively<sup>(6)</sup>. The hydrolytic processes that take place in the fruit during extraction facilitate the release of these triterpenes from the olive skin. Consequently, they are found in virgin olive oil at concentrations of 194 (SE 14) mg/kg for maslinic acid and 244 (SE 28) mg/kg for oleanolic acid<sup>(6)</sup>.

Pentacyclic triterpenoids are abundant in the plant kingdom, and have been widely used for medicinal purposes in many Asian countries<sup>(7)</sup>. Maslinic acid has received relatively

scant attention, which can mainly be attributed to the lack of a commercial standard. Most studies have therefore used extracts from various plants used in traditional Asian medicine that contained not only maslinic acid. However, maslinic acid has been described as an anti-HIV agent<sup>(8)</sup>, as an antioxidant<sup>(9)</sup>, anti-inflammatory<sup>(10)</sup> and anti-tumoral agents<sup>(11,12)</sup>. Oleanolic acid, whose chemical structure differs from maslinic acid by the lack of the hydroxyl group at the 2-carbon position, is a well-known member of the oleanane triterpene family. The myriad of biological and pharmacological properties described for oleanolic acid include, among others, anti-inflammatory, anti-tumoral, hepatoprotective, anti-diabetogenic and anti-HIV activities<sup>(7,13)</sup>.

Colon cancer development is often characterized in an early stage by a hyperproliferation of the epithelium leading to the formation of adenomas. In this multi-step process an early intervention<sup>(14)</sup> should target the inhibition of enhanced cell proliferation in transformed cells and, probably more important, the induction of the apoptotic pathway to delete cells carrying mutations. Both processes were shown to be modulated by an olive fruit extract composed of 73.25% maslinic and 25.75% oleanolic acid<sup>(3)</sup>. We here present data on the effects of the individual constituents in a human colorectal cancer

**Abbreviations:** DMSO, dimethyl sulphoxide; ROS, reactive oxygen species.

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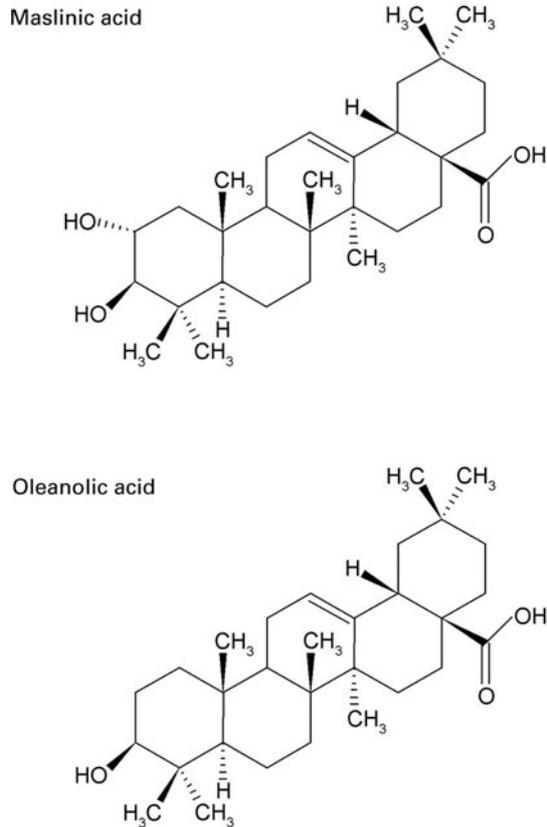


Fig. 1. The chemical structures of maslinic acid and oleanolic acid.

cell model. HT-29 human colon adenocarcinoma cells were treated with maslinic acid or oleanolic acid and cell proliferation and apoptosis rates were determined.

## Experimental methods

### Chemicals and reagents

Maslinic acid was obtained from olive pomace according to the patented method from García-Granados<sup>(15)</sup> and was kindly provided by V. R.-G. from the Instituto de la Grasa, Sevilla. Oleanolic acid was purchased from Extrasynthese (Geney, France). Media and supplements for cell culture were from Invitrogen (Karslsruhe, Germany). Cell culture plates were from Renner (Dannstadt, Germany) and Quadriperm<sup>®</sup> wells were obtained from Merck (Darmstadt, Germany). The fluorophores SYTOX-Green, MitoTracker Red CMXRos and proxyl fluoescamine were from Invitrogen. Hoechst dyes were purchased from Sigma (Deisenhofen, Germany) and the fluorogenic caspase-3 substrate acetyl-aspartyl-glutamyl-valyl-aspartyl-amino-4-methyl-coumarine was obtained from Calbiochem (Bad Soden, Germany).

### Cell culture

HT-29 cells (passage 106) were provided by American Type Culture Collections and were used between passages 150 and 200. Cells were cultured and passaged in RPMI 1640 supplemented with 100 ml/l fetal calf serum and 2 mmol/l glutamine. Antibiotics added to the media were 100 000 U/l penicillin and 100 mg/l streptomycin. The cultures were maintained in a

humidified atmosphere of 95 % air and 5 % CO<sub>2</sub> at 37°C. Fresh medium was given every 2 d and on the day before the experiments were performed. Cells were passaged at pre-confluent densities by the use of a solution containing 0.05 % trypsin and 0.5 mm-EDTA. Maslinic acid and oleanolic acid were applied to the cell cultures dissolved in dimethyl sulphoxide (DMSO) to a final concentration of 2 % (v/v) in all the experiments. Controls were always treated with the same amount of DMSO.

### Necrosis

The potential non-specific toxicity of maslinic and oleanolic acid in HT-29 cells was assessed prior to the study. Therefore, HT-29 cells were seeded at a density of  $5 \times 10^4$  cells/well on to twenty-four-well cell culture plates and allowed to adhere for 4 h. Subsequently, the medium was replaced by a fresh one and the cells were exposed for 3 h to increasing concentrations of each compound. Necrotic cell death was evaluated with SYTOX-Green, which becomes fluorescent after DNA binding. The percentage of dead cells was determined by SYTOX-fluorescence prior to cell lysis in relation to the fluorescence measured after the solubilization of cells with 1 % (v/v) Triton X-100 in isotonic NaCl. Cell numbers were determined based on a calibration curve measured using cell numbers between  $1 \times 10^3$  and  $1.5 \times 10^5$  cells, which had been adjusted after counting the cells in a Neubauer chamber. Fluorescence was measured at 538 nm after excitation at 485 nm using a fluorescence multiwell-plate reader (Fluoroskan Ascent; Thermo Electron, Dreieich, Germany).

### Cell proliferation

In the proliferation assay HT-29 cells were seeded at a density of  $5 \times 10^3$  cells/well on to twenty-four-well cell culture plates and allowed to adhere for 24 h. Thereafter, medium was replaced by a fresh culture medium containing increasing concentrations of maslinic or oleanolic acid. Cells were allowed to grow for another 72 h and total cell counts were determined thereafter. Cells were lysed with 1 % (v/v) Triton X-100 in isotonic NaCl and DNA was stained with SYTOX-Green. Cell numbers were counted using the fluorescence multiwell-plate reader.

### Caspase-3-like activity

Caspase-3-like activity was measured following the method of Nicholson *et al.*<sup>(16)</sup> and used as an early apoptosis marker. Briefly, cells were seeded at a density of  $5 \times 10^5$  per well on to six-well plates and allowed to adhere for 24 h. HT-29 cells were exposed to 150 and 250  $\mu\text{mol/l}$  maslinic and oleanolic acids at different time-points: 3, 8, 12, 24, 36 and 48 h. Cells were trypsinized once the incubation was finished. Cell numbers were determined, and cells were centrifuged at 2500 g for 10 min. Cytosolic extracts were prepared by adding 750  $\mu\text{l}$  of a buffer containing 2 mmol/l EDTA, 1.63  $\mu\text{mol/l}$  3-((cholamidopropyl)-dimethyl-ammonium)-1-propane-sulphonate, 5 mmol/l dithiothreitol, 1 mmol/l phenyl-methyl-sulphonyl-fluoride, 10 mg/l pepstatin A, 20 mg/l leupeptin, 10 mg/l aprotinin and 10 mmol/l HEPES/KOH (pH 7.4) to each pellet and homogenized with ten strokes. The homogenate was centrifuged at 100 000 g at 4°C for 30 min, and the cytosolic supernatant was incubated with the fluorogenic caspase-3 tetrapeptide-substrate acetyl-aspartyl-glutamyl-valyl-aspartyl-amino-4-methyl-coumarine

at a final concentration of 20  $\mu\text{mol/l}$ . Cleavage of the caspase-3 substrate was followed by determination of emission at 460 nm after excitation at 390 nm using the fluorescence plate reader. The study of the time-dependent activation of caspase-3 indicated that 24 h was the optimal incubation time. Consequently, the dose-dependent activation of this caspase was evaluated after HT-29 cells had been incubated for 24 h with maslinic and oleanolic acids.

#### Detection of superoxide radicals

Production of superoxide radicals in mitochondria of HT-29 cells was visualized using a confocal laser scanning microscope (Leica TCS SP2, Bensheim, Germany). Therefore, cells were seeded at a density of  $3 \times 10^4$  per well on glass slides placed in Quadriperm<sup>®</sup> wells. Cells were grown for 24 h to allow adhesion to the slides. Subsequently, medium was replaced by a fresh one containing 150  $\mu\text{mol/l}$  maslinic acid or 150  $\mu\text{mol/l}$  oleanolic acid and cells were incubated for 4 h. Proxyl fluorescamine (50  $\mu\text{mol/l}$ ) was loaded to the cells for the last 2 h of incubation with the aim of determining the production of superoxide anions in the mitochondria. Mitochondria were stained with 500 nmol/l MitoTracker Red CMXRos, which was loaded to the cells for the last 30 min of incubation. Superoxide radicals were detected after excitation with the UV laser at emissions of 440–480 nm and mitochondria were visualized after excitation at 543 nm at emissions of 590–650 nm, respectively.

#### Membrane permeability

HT-29 cells were seeded at a density of  $3 \times 10^4$  cells/well on glass slides placed in Quadriperm<sup>®</sup> wells and allowed to adhere for 24 h. Afterward, culture medium was replaced with a fresh one containing 150  $\mu\text{mol/l}$  maslinic acid and cells were incubated for 8, 16, 20 and 24 h. At the end of the incubation, cells were stained with 1 mg/l Hoechst 33342 and the rate of accumulation of the dye in early apoptotic cells<sup>(17)</sup> was detected using an inverted fluorescence microscope (Leica DMIRBE, Bensheim, Germany) equipped with a band-pass excitation filter of 340–380 nm and a long-pass emission filter of 425 nm. Photographs were taken of at least three independent cell batches, and images were evaluated on a blindly coded basis. Apoptotic cells were determined by the number of cells showing elevated fluorescence versus the total cell counts.

#### Nuclear fragmentation

Nuclear fragmentation as a late marker of apoptosis was determined by the staining of DNA with Hoechst 33258. HT-29 cells ( $3 \times 10^4$  cells/well) were seeded on glass slides, allowed to adhere for 24 h, and were incubated with 150  $\mu\text{mol/l}$  maslinic acid during 8, 16, 20 and 24 h. After each time-point, cells were washed with PBS, allowed to air-dry for 30 min and then were fixed with 2% paraformaldehyde before staining with 1 mg/l Hoechst 33258. Cells were visualized under the inverted fluorescence microscope at emission of 425 nm after excitation of 340–280 nm. Images were evaluated on a blindly coded basis. Apoptotic cells were determined by the number of cells displaying chromatin condensation and nuclear fragmentation versus total cell counts.

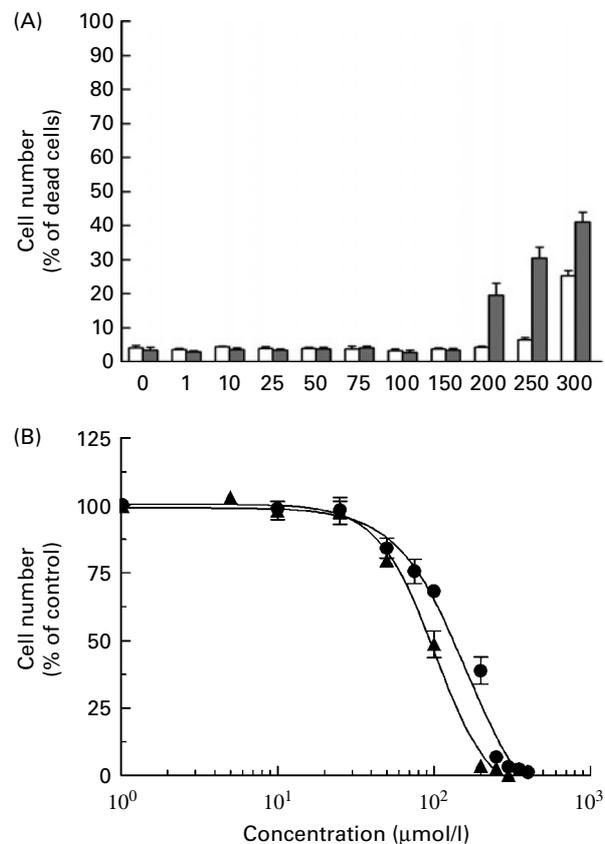
#### Statistics

Results are expressed as means with their standard errors. To derive the  $\text{EC}_{50}$  values for growth inhibition, a non-linear approximation model by the least square method based on a competition curve using one component was applied. Results were tested for normal distribution and for homogeneity of variance by standardized residual plot. When necessary, logarithmic transformations were performed. Normally distributed results were analysed by one-way ANOVA. If the result was found to be significant ( $P < 0.05$ ) the Tukey's multiple comparison test was used to determine specific differences between results. Statistical differences between time and dose were tested by two-way ANOVA and Bonferroni's post-test. The effects with  $P < 0.05$  were considered statistically significant. All statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

## Results

#### Necrosis

Maslinic acid did not exert non-specific cytotoxicity at the concentration range from 1 to 250  $\mu\text{mol/l}$  since 95.3 (SE 0.2)



**Fig. 2.** Effects of maslinic and oleanolic acids on proliferation and necrosis in HT-29 cells. (A), Proliferation was measured over 72 h in the absence (control) or presence of compounds at different concentrations ( $\square$ , maslinic acid;  $\blacksquare$ , oleanolic acid). (B), Necrosis was assessed after incubating the cells for 3 h with medium alone (control) or containing different concentrations of compounds ( $\blacktriangle$ , maslinic acid;  $\bullet$ , oleanolic acid). Values are means with their standard errors depicted by vertical bars.

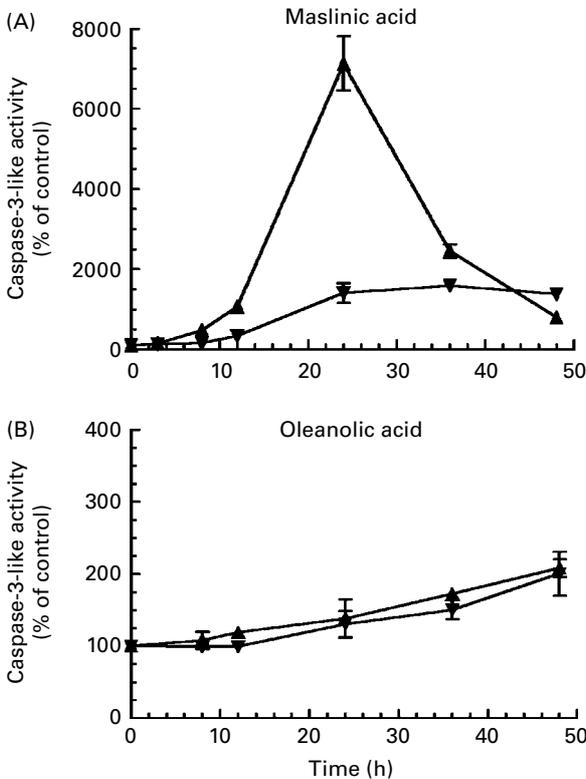
% of the cells were viable even at 250  $\mu\text{M}$  (Fig. 2 (A)). Only at 300  $\mu\text{mol/l}$  was the number of living cells reduced to 74.6 (SE 1.4) % ( $P < 0.0001$ ) within 3 h. The viability of HT-29 cells after 3 h incubation with oleanolic acid at concentrations up to 150  $\mu\text{mol/l}$  was 97.5 (SE 0.3) %. Cell viability at concentrations of 200, 250 and 300  $\mu\text{mol/l}$  was reduced to 83.6 (SE 2.2) %, 69.5 (SE 3.3) % ( $P < 0.001$ ) and 58.9 (SE 2.9) % ( $P < 0.001$ ), respectively.

*Cell proliferation*

Maslinic acid displayed dose-dependent antiproliferative activity in HT-29 cells with an  $\text{EC}_{50}$  of 101.2 (SE 7.8)  $\mu\text{mol/l}$  (Fig. 2 (B)). Oleanolic acid also inhibited the proliferation of HT-29 cells in a dose-dependent manner but to a lesser extent. Incubation of cells for 72 h with 50 and 200  $\mu\text{mol/l}$  oleanolic acid induced 16 and 62% reductions in cell number, respectively, with an overall  $\text{EC}_{50}$  of 160.6 (SE 10.6)  $\mu\text{mol/l}$  (Fig. 2 (B)).

*Caspase-3-like activity*

When HT-29 cells were exposed to 150  $\mu\text{mol/l}$  maslinic acid, caspase-3-like activity was induced in a time-dependent manner with a 14-fold activation between 24 and 48 h of exposure (Fig. 3). Maslinic acid at 250  $\mu\text{mol/l}$  resulted in a more than 60-fold activation of caspase-3 at 24 h, whereas thereafter caspase-3-like activity declined (Fig. 3 (A)).



**Fig. 3.** Time-dependent caspase-3 activity in HT-29 cells. Caspase-3-like activity at various time-points of incubation with maslinic acid (A) and oleanolic acid (B) ( $\blacktriangledown$ , 150  $\mu\text{mol/l}$ ;  $\blacktriangle$ , 250  $\mu\text{mol/l}$ ) was determined by measuring cleavage of acetyl-aspartyl-glutamyl-valyl-aspartyl-amino-4-methyl-coumarine. Values are means with their standard errors depicted by vertical bars.

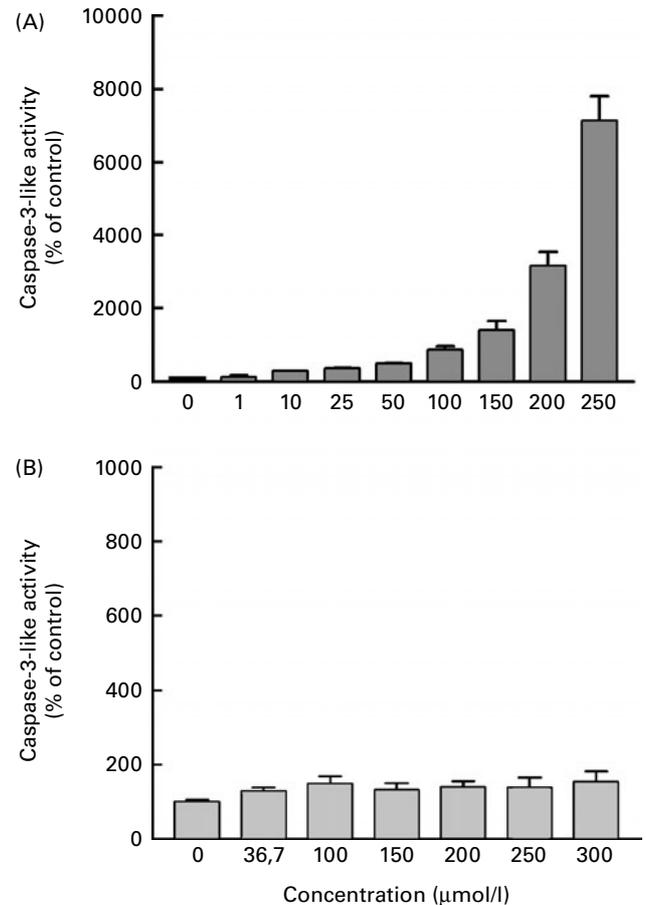
At concentrations of 150 or 250  $\mu\text{mol/l}$ , oleanolic acid, unlike maslinic acid, showed only moderate effects on caspase-3-like activities, with a maximum of two-fold activation at 48 h (Fig. 3 (B)).

When caspase-3-like activity was studied at 24 h as a function of maslinic acid concentration, we found a dose-dependent increase (Fig. 4 (A)). At 10  $\mu\text{mol/l}$ , maslinic acid stimulated caspase-3-like activity 3-fold over the control ( $P < 0.0001$ ), and higher concentrations induced a steady increase in the activation (Fig. 4 (A)).

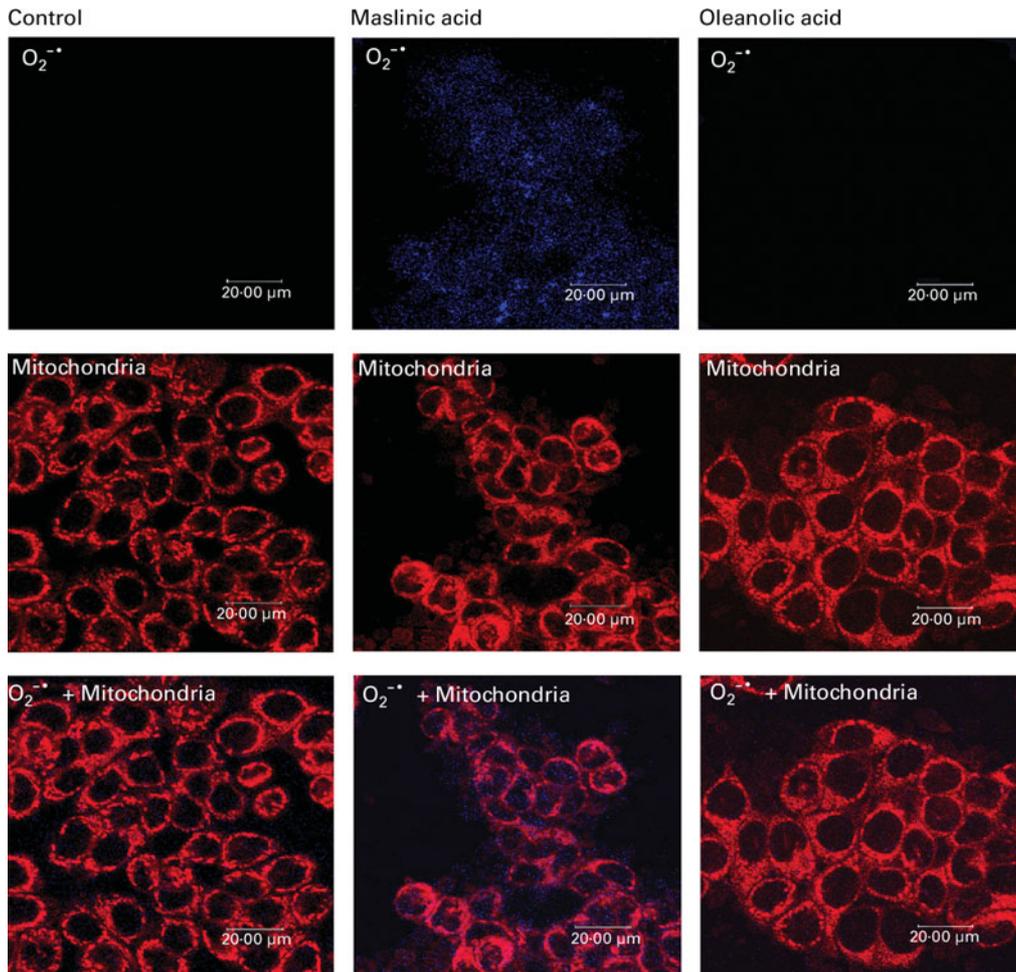
Surprisingly, oleanolic acid had no effect on caspase-3 activity at any of the times or concentrations studied in the range from 50 to 300  $\mu\text{mol/l}$  (Fig. 4).

*Generation of mitochondrial reactive oxygen species*

Caspase-3 can be activated by a death-receptor-mediated pathway but also by the mitochondrial apoptosis pathway<sup>(18)</sup>. Increased levels of mitochondrial superoxide anions have been found in many studies as an initiator of the mitochondrial apoptosis pathway<sup>(19)</sup>. Confocal microscopy analysis revealed an early increase of superoxide anion production in mitochondria of HT-29 cells treated for 4 h with 150  $\mu\text{mol/l}$  maslinic acid (Fig. 5). Maslinic and oleanolic acids clearly differed in



**Fig. 4.** Dose-dependent caspase-3 activity in HT-29 cells. Induction of caspase-3-like activity was determined after incubations with maslinic acid (A) and oleanolic acid (B) at 24 h based on the cleavage of the fluorogenic substrate acetyl-aspartyl-glutamyl-valyl-aspartyl-amino-4-methyl-coumarine. Values are means with their standard errors depicted by vertical bars.



**Fig. 5.** Detection of superoxide radicals in the mitochondria of HT-29 cells. Cells were incubated with medium alone (control), 150  $\mu\text{mol/l}$  maslinic acid and 150  $\mu\text{mol/l}$  oleanolic acid. During the last period of incubation cells were loaded with proxiylfluorescamine for the detection of  $\text{O}_2^{\bullet-}$  in combination with MitroTracker for the visualization of the mitochondria.

their ability to generate mitochondrial reactive oxygen species (ROS). HT-29 cells treated with 150  $\mu\text{mol/l}$  oleanolic acid did not display any blue fluorescence which indicated the presence of  $\text{O}_2^{\bullet-}$ , and were not different from control cells.

#### Membrane permeability

We then assessed whether activation of caspase-3 by maslinic acid induced the morphological characteristics of apoptosis. Maslinic acid (150  $\mu\text{mol/l}$ ) increased accumulation of the Hoechst dye 33342 in HT-29 cells over time (Fig. 5). After exposing HT-29 cells to 150  $\mu\text{mol/l}$  maslinic acid for 8 h, 14.8 (SE 0.7) % ( $P < 0.0001$ ) of cells showed accumulation of Hoechst 33342. This accumulation increased to 49.4 (SE 2.9) % ( $P < 0.0001$ ) following 24 h of incubation (Fig. 6 (A)).

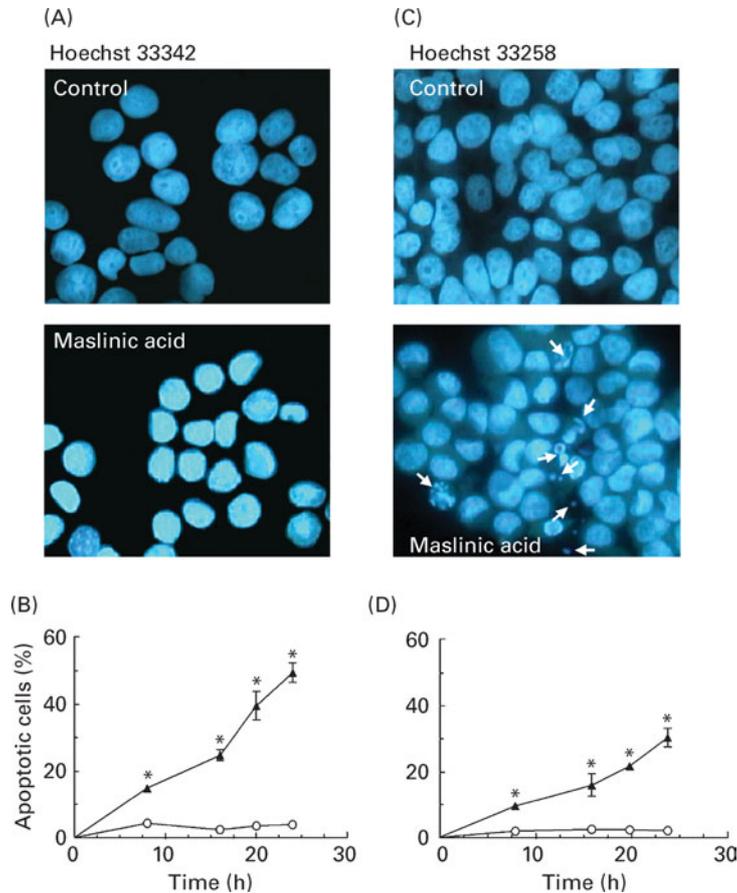
#### Nuclear fragmentation

Exposure to 150  $\mu\text{mol/l}$  maslinic acid increased the fragmentation of DNA and chromatin condensation (Fig. 6 (B)). Apoptotic bodies were detected after 8 h of incubation in 9.7 (SE 0.9) % ( $P < 0.0001$ ) of cells, and after 24 h nuclear fragmentation was detected in 30.8 (SE 2.9) % ( $P < 0.0001$ ) of HT-29 cells.

#### Discussion

The Mediterranean diet, which is rich in olives and olive oil, is believed to confer various health benefits including a decreased cancer risk<sup>(2)</sup>. Here we attempt to identify which of the triterpenic compounds of the olive fruit extract was responsible for its anti-tumour activity described previously<sup>(3)</sup>, and to assess whether there was an additive action.

Both maslinic and oleanolic acids reduced cell proliferation rates, with half-maximal effects for growth-inhibition of around 100–150  $\mu\text{mol/l}$ . However, maslinic acid did not induce necrosis even at concentrations that cause full inhibition of cell growth. Oleanolic acid reduced cell growth with lower activity and caused complete inhibition at 320  $\mu\text{mol/l}$ . This growth-inhibition may be attributed, at least in part, to cell-cycle arrest, since oleanolic acid is involved in the  $G_0/G_1$  checkpoint control and the inhibition of DNA replication in the human colon carcinoma cell line HCT15<sup>(20)</sup>. The moderate yet significant non-specific toxicity is most likely the cause of the apparent anti-proliferative activity, since at 300  $\mu\text{mol/l}$  the percentage of dead cells was 40%, which is in agreement with other results in the literature<sup>(20,21)</sup>.



**Fig. 6.** Determination of early and late apoptotic events in HT-29 at different time-points. Cells that accumulated Hoechst 33352 dye due to membrane disintegration (A) were counted and expressed as the percentage of apoptotic cells (B) under control conditions (○) or after 150 μmol/l maslinic acid treatment (▲). Cells that displayed nuclear fragmentation (C) were counted and expressed as the percentage of apoptotic cells (D) under control conditions (○) or after 150 μmol/l maslinic acid treatment (▲). Values are means with their standard errors depicted by vertical bars. Mean values were significantly different from those of the control group: \* $P < 0.0001$ .

The most promising finding of the present study was the ability of maslinic acid to induce apoptosis in colonic cancer cells. It increased the activity of caspase-3 in a time- and a dose-dependent manner, with the highest activation of 70-fold above that in control cells at a concentration of 250 μmol/l. Oleanolic acid did not activate caspase-3 even at the highest concentrations tested. The inability of this compound to induce apoptosis is in agreement with other studies<sup>(21)</sup>, and was also reported for the human colon carcinoma cell line HCT15<sup>(20)</sup>. Although oleanolic acid has been described to inhibit tumour initiation and promotion steps<sup>(22)</sup>, its overall anti-tumour activity is relatively weak<sup>(13)</sup>. For this reason, new synthetic analogues of this compound have been synthesized in order to enhance its potency<sup>(21,23)</sup>.

The present results indicate that maslinic acid can induce an activation of caspase-3 with a superior activity to that exerted by the same concentration in the complete olive fruit extract. The lowest concentration used in the present study which corresponds to 10 μmol/l maslinic acid induced an activation of caspase-3 of 300% of that in control cells. Once activated, caspases cleave a battery of cellular substrates, leading finally to morphological changes as a hallmark of apoptosis<sup>(18)</sup>. The increase in caspase-3-like activity caused by maslinic acid was followed by full execution of apoptosis

with a disintegration of the plasma membrane and finally a pronounced nuclear fragmentation.

Many anticancer drugs induce apoptosis by activating intrinsic pathways that increase cellular ROS production<sup>(24,25)</sup>. Therefore, we determined ROS levels in the mitochondria of HT-29 cells treated with maslinic or oleanolic acids, and demonstrate that maslinic acid disrupts the function of mitochondria at the early stage of apoptosis induction. HT-29 cells exposed to maslinic acid showed markedly increased levels of superoxide anion radicals in mitochondria. On the other hand, the failure to generate superoxide anions in mitochondria of oleanolic acid-treated cells may explain the lack of apoptotic activity shown by this triterpene. ROS production occurs in an early phase suggesting that maslinic acid triggers a rapid release of cytochrome *c* from mitochondria into the cytosol that in turn activates procaspase-9 and the downstream effectors, including the pro-caspases -3, -6 and -7, followed finally by the cleavage of proteins and DNA that characterize the final phase of apoptosis. ROS can affect divergent cellular functions depending on the cellular level and their compartmentation. Mitochondria are the primary cellular site of ROS production and, under certain conditions, elevated mitochondrial ROS levels can serve as pro-apoptotic signals<sup>(26)</sup>. As enzymatic and/or non-enzymatic antioxidant systems control

ROS levels, the balance of ROS production and their removal in a given cell type is most critical also for the elimination of transformed cells that when allowed to bypass apoptotic elimination can lead to solid tumours. Dietary constituents promoting mitochondrial ROS production could be as important in cancer prevention as dietary antioxidants.

Maslinic and oleanolic acids are highly lipophilic molecules. In order to overcome their water insolubility, these pentacyclic triterpenes were dissolved in 2% (v/v) DMSO, a percentage previously used<sup>(3)</sup>. Although DMSO is normally used at 1%, with very highly lipophilic compounds, the percentage of DMSO can be safely increased to 5%, given that it has been reported that it may be used without any significant cell damage, after an exposure of 2 h, up to 10% in Caco2 cells<sup>(27)</sup>. DMSO may also possess other activities, such as antioxidant activity. For this reason and in order to minimize the effect of DMSO, the same amount both in the control and in the treated cells was employed in all the assays.

Induction of apoptosis in precancerous cells may provide protection against cancer development and therefore, may provide the basis for a novel nutritional strategy for cancer prevention. The practical implication is that consumption of diets containing maslinic acid in the form of olives or olive oil may trigger precancerous cells to die by apoptosis. The concept of dietary chemoprevention is usually applied in the context of protecting normal cells from initiating events that introduce oncogenic mutations. However, substantial literature is available to show that carcinogenesis represents a progression of cellular changes<sup>(28)</sup>, and agents that disrupt this progression at any point can be considered chemopreventive. The HT-29 cells used in the present study represent a model of an advanced stage of tumour development. Therefore, we consider it especially significant that maslinic acid induced apoptosis in these cells at concentrations that may be achieved with a normal diet.

For any chemopreventive activity of a dietary constituent the intake via the relevant foods must be sufficient to attain the cellular concentrations that display the bioactivity. According to data from the literature, the mean daily consumption of table olives and olive oil in the Mediterranean area corresponds to approximately 40 g or ten medium-sized olives and 33 g oil. Consequently, in the Mediterranean basin the regular consumption of olives and olive oil could provide an average daily intake of maslinic acid of approximately 34 mg/d. There is no information indicating the metabolic fate of maslinic acid either in man or in cell culture. Recently the pharmacokinetics of oleanolic acid in rats has been reported<sup>(29)</sup>, with an absolute oral bioavailability of 0.7% for the administrations of 25 and 50 mg/kg. The extent of urinary excretion was minimal for oral doses, indicating the oral bioavailability of oleanolic acid could be due to a poor absorption, therefore, the intestinal epithelium is exposed to high concentrations of these compounds. Assuming that 30% of this compound is not absorbed in the small intestine and reaches the colon unaltered, its distribution with an assumed volume of around 250 ml would result in maslinic acid concentrations as high as 86  $\mu\text{mol/l}$  – a concentration that was proven to be sufficient to initiate apoptosis. Based on the same assumption and taking into account the amount of oleanolic acid present in olives and olive oil, this compound would reach a colonic concentration

of around 60  $\mu\text{mol/l}$ . With a normal diet, these colonic oleanolic acid concentrations would most likely not result in cytotoxicity or anti-proliferative activity. Moreover, oleanolic acid has already been shown to have a low side-effect profile as demonstrated in toxicity experiments conducted in mice and rats<sup>(22)</sup>.

In conclusion, we demonstrate that the naturally occurring pentacyclic triterpene, maslinic acid, is the responsible compound in an olive extract that was shown to inhibit colon cancer cell growth<sup>(3)</sup>. Maslinic acid inhibits cellular proliferation at non-toxic concentrations and more importantly restores apoptosis sensitivity in human colon adenocarcinoma cells. Given that apoptosis induction is arguably the most important process to remove cells that have lost growth control, maslinic acid appears as a promising new chemical entity in the diet with a cancer-chemopreventive activity. What is surprising is that the presence of one hydroxyl group in the chemical structure of maslinic acid as compared to oleanolic causes these different biological activities.

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