Olive Fruit Extracts Inhibit Proliferation and Induce Apoptosis in HT-29 Human Colon Cancer Cells

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Abstract

Olives and their derivatives represent an important component of the Mediterranean diet that has been considered to be protective against cancer. We investigated the effect on cell proliferation and apoptosis in HT-29 cells of an extract from the skin of olives composed of pentacyclic triterpenes with the main components maslinic acid (73.25%) and oleanolic acid (25.75%). Studies of the dose-dependent effects showed antiproliferative activity at an EC50 value of 73.96 ± 3.19 μmol/L of maslinic acid and 26.56 ± 2.55 μmol/L of oleanolic acid without displaying necrosis. Apoptosis was confirmed by the microscopic observation of changes in membrane permeability in 40.9 ± 3.9% and detection of DNA fragmentation in 24.5 ± 1.5% of HT-29 cells incubated for 24 h with olive fruit extract containing 150 and 55.5 μmol/L of maslinic and oleanolic acids, respectively. Caspase-3 was activated in a dose-dependent manner after incubation for 24 h. The extract containing 200 μmol/L maslinic acid and 74 μmol/L oleanolic acid increased caspase-3-like activity to 6-fold that of control cells. Programmed cell death was induced by the intrinsic pathway, as evidenced by the production of superoxide anions in the mitochondria of cells treated with olive fruit extracts containing 150 and 55.5 μmol/L of maslinic and oleanolic acids, respectively. Our results report for the first time, to our knowledge, the inhibition of cell proliferation without cytotoxicity and the restoration of apoptosis in colon cancer cells by maslinic and oleanolic acids present in olive fruit extracts.

Introduction

The diverse countries and regions in the Mediterranean basin have their own dietary traditions, which are reflected in different eating patterns. However, in the entire area, table olives and olive oil constitute a regular dietary component and a source of compounds that have important biological properties (1). The nutritional benefits of table olives are associated with monounsaturated fat and minor components (2). Triterpenoids are non-nutritive dietary microconstituents present in the skin of the fruits of Olea europaea. They protect the integrity of the fruit, as they act as insect antifeedants and antimicrobial agents (3). The main triterpenes (Fig. 1) in the skin of olive fruit are maslinic acid (2α,3β-dihidroxyolean-12ene-28oic) and oleanolic acid (3β-hydroxyolean-12-ene-28-oic) at concentrations of 681 ± 63 mg/kg and 420 ± 20 mg/kg, respectively (4). The amount of these compounds in the oil is much lower than in the fruit and depends on the oil quality. Extra virgin olive oil with acidity under 1% contains 64.2 ± 8.1 mg/kg of maslinic acid and 57.2 ± 7.4 mg/kg of oleanolic acid, depending on the fruit variety (5). These values increase to 193.9 ± 14.0 mg/kg for maslinic acid and 244.0 ± 28.1 mg/kg for oleanolic acid in virgin olive oil. This is because the hydrolytic processes that take place in the fruit during extraction facilitate the release of these triterpenes from the skin (5).

Epidemiological data suggest an inverse correlation between regular consumption of olive oil and cancer risk (6,7). This hypothesis have been supported by animal studies that showed a protective effect of olive oil against the UV induced damage of the skin (8) and its ability in preventing the colon crypts aberrant foci growth and colon carcinoma in rats (9). The protective or nonpromoting activity of olive oil is often ascribed to its high content of oleic acid 18:1(n-9), a mono-unsaturated fatty acid. However, recent evidence suggests that oleic acid 18:1(n-9) may not exclusively account for this beneficial activity, and up to now, the components of olive oil responsible for its protective role remains undefined (10,11). Therefore, the present study focuses on whether and to what extent an olive fruit extract could reduce cell growth and promote apoptosis in HT-29 human colon adenocarcinoma cells. Colorectal cancers are difficult to treat with existing therapeutic methods. Therefore, identifying dietary phytochemicals that have antitumor activities and investigating their mechanism of action may lead to major advances in the prevention of human cancer.

Materials and Methods

Chemicals and reagents. Cell culture media and supplements were from Invitrogen. Cell culture plates were from Renner and Quadraperm
wells were obtained from Merck. The fluorophores SYTOX-Green, MitoTracker Red CMXRos, and proxyl fluorescenceme were from Bioprobes. Hoechst dyes were purchased from Sigma and the fluorogenic capase-3 substrate acetyl-aspartyl-glutamyl-valyl-aspartyl-aminono-4-methyl-coumarin was obtained from Calbiochem.

**Olive fruit extract.** Olive fruit extract was provided by Dr. V. Ruiz-Gutierrez from the Instituto de la Grasa, Seville, and was obtained as follows. Olive fruits from Arbequina cultivars were treated following a method that extracted the pentacyclic triterpene acids present on the skin of olive fruits (4). Briefly, 200 g of olives were immersed in 200 mL of CHCl₃ for 1 min at room temperature. Subsequently, a further extraction was carried out by immersing the olives in 200 mL of MeOH for 1 min and the extract was washed with H₂O. The CHCl₃ and MeOH extracts were dried completely in vacuo in a rotary evaporator at 35°C. The crude extract was chromatographed on a silica gel column to separate maslinic and oleanolic acids, which were eluted with CHCl₃. The chloroform fraction was evaporated in a rotary evaporator and the extract was kept as a powder.

The extract from the skin of the fruit was analyzed by gas chromatography following the method of Perez-Camino and Cert (5). It contained 1073 ± 122 mg/kg of maslinic acid, 377 ± 43 mg/kg of oleanolic acid, and 14 ± 2 mg/kg of eriodirol with traces of maslinic acid derivatives. The percentages of pentacyclic triterpene acids were 73.25% of maslinic acid, 25.75% of oleanolic acid, and 1% of eriodirol with traces of maslinic acid derivatives. This crude extract will be referred as “olive fruit extract.” Concentrations refer to maslinic acid, which is the main compound of the skin. Every time a concentration is expressed, the amount of oleanolic acid will also be given.

**Cell culture.** HT-29 cells (passage 106) were provided by American Type Culture Collections and were used between passages 150 and 200. HT-29 cells (passage 106) were provided by American Type Culture Collections and were used between passages 150 and 200.

**Cell proliferation.** In the proliferation assay, HT-29 cells were seeded at a density of 5 x 10⁴ cells/well onto 24-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 to increasing concentrations of the olive fruit extract for 3 h. Necrotic cell death was evaluated with SYTOX-Green, which becomes fluorescent after DNA binding. The percentage of dead cells was determined by comparing SYTOX-fluorescence prior to cell lysis with the fluorescence measured after the cells were solubilized with 1% (v/v) Triton X-100 in isotonic NaCl. Cell numbers were determined on the basis of a calibration curve. The calibration curve was measured using cell numbers of between 1 x 10⁴ and 1.5 x 10⁵ cells, which had been adjusted after determining the cell numbers in a Neubauer chamber. Fluorescence of the corresponding cell numbers was measured at 538 nm after excitation at 485 nm using a fluorescence multwell plate reader (Fluoroskan Ascent, Thermo Electron).

**Necrosis.** The potential nonspecific toxicity of the olive fruit extract in HT-29 cells was assessed prior to the study. Cells were seeded at a density of 5 x 10⁴ cells/well on 24-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 to increasing concentrations of the olive fruit extract for 3 h. Necrotic cell death was evaluated with SYTOX-Green, which becomes fluorescent after DNA binding. The percentage of dead cells was determined by comparing SYTOX-fluorescence prior to cell lysis with the fluorescence measured after the cells were solubilized with 1% (v/v) Triton X-100 in isotonic NaCl. Cell numbers were determined on the basis of a calibration curve. The calibration curve was measured using cell numbers of between 1 x 10⁴ and 1.5 x 10⁵ cells, which had been adjusted after determining the cell numbers in a Neubauer chamber. Fluorescence of the corresponding cell numbers was measured at 538 nm after excitation at 485 nm using a fluorescence multwell plate reader (Fluoroskan Ascent, Thermo Electron).

**Capase-3-like activity.** Capase-3-like activity was measured according to the method described previously (12). This activity was used as an early apoptosis marker. Briefly, cells were seeded at a density of 5 x 10⁴ well onto 6-well plates and allowed to adhere for 24 h. The time course of capase-3 activation was evaluated in an initial set of experiments. Cells were exposed to 2 different concentrations of olive fruit extract for 4, 8, 12, 24, 36, and 48 h. The first dose contained 150 and 55.5 μmol/L of maslinic and oleanolic acids, respectively. The second dose contained 230 and 92.5 μmol/L of maslinic and oleanolic acids. Cells were trypsinized once the incubation had finished. Cell numbers were determined and the cells were centrifuged at 2500 x g; 10 min. Cytosolic extracts were prepared by adding 750 μL of a buffer containing: 2 mmol/L EDTA; 1.63 μmol/L 3-[4-(4-carboxybenzyl)-4-methylamino]phenylalanine; 5 μmol/L diithiothreitol; 1 μmol/L phenyl-methyl-sulfonyl-fluoride; 10 μg/mL pepstatin A; 20 μg/mL leupeptin; 10 μg/mL aprotinin; and 10 mmol/L HEPESKOH (pH 7.4) to each pellet and homogenizing with 10 strokes. The homogenate was centrifuged at 100,000 x g for 30 min at 4°C and the cytosolic supernatant was incubated with the fluorogenic capase-3 tetrapeptide-substrate Ac-DEVD-amino-4-methyl-coumarin at a final concentration of 20 μmol/L. The capase-3 substrate was cleaved. The emission at 460 nm was then determined after excitation at 390 nm using the fluorescence plate reader.

The study of the time-dependent activation of capase-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.

**Membrane permeability.** Early changes in membrane permeability were detected subsequent to incubating 3 x 10⁻⁵ HT-29 cells/well on glass slides placed in Quadrupern wells. Cells were allowed to adhere for 24 h. Cells were incubated with 150 and 55.5 μmol/L of maslinic and oleanolic acids, respectively, for 8, 16, 20, and 24 h. At the end of the incubation, cells were stained with 1 mg/mL Hoechst 33342, and the rate of accumulation of the dye in early apoptotic cells (13) was detected using an inverted fluorescence microscope (Leica DMIRBE) 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 3 independent cell batches, and images were evaluated without knowledge of their identity. Apoptotic cells were determined by the number of cells showing elevated fluorescence vs. the total cell counts.

**Nuclear fragmentation.** Nuclear fragmentation as a late marker of apoptosis was determined by staining DNA with Hoechst 33258. HT-29 cells (3 x 10⁻⁵ cells/well) were then incubated with 150 μmol/L maslinic acid and 55.5 μmol/L of oleanolic acid for 8, 16, 20, and 24 h. Cells were washed with PBS, allowed to air-dry for 30 min, and fixed with 2% paraformaldehyde before staining with 1 mg/mL Hoechst 33258. Images were evaluated without knowledge of their identity. Apoptotic cells were determined by the number of cells displaying chromatin condensation and nuclear fragmentation vs. total cell counts.

**Detection of superoxide radicals.** The production of superoxide radicals in mitochondria of HT-29 cells was visualized using a confocal laser scanning microscope (Leica TCS SP2). Cells were seeded at a density of 3 x 10⁴/well on glass slides placed in Quadrupern wells. Cells were grown for 24 h to allow adhesion to the slides. Subsequently, the medium was substituted with a fresh one containing olive fruit extract that had 150 and 55.5 μmol/L of maslinic and oleanolic acids, respectively. Cells were incubated for 4 h. A total of 50 μL/m of proxyl fluorescent was loaded into the cells for the last 2 h of incubation. The aim was to determine the production of superoxide anions in the mitochondria. Mitochondria were stained with 500 μmol/L MitoTracker Red CMXRos. This was loaded into 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. Mitochondria were visualized after excitation at 543 nm, at emissions of 590–650 nm.

**Statistical analysis.** Data were given as the mean ± SEM. We applied a nonlinear approximation model, using the least square method, to derive the EC₅₀ values for growth inhibition. This model was based on a competition curve using 1 component (Graph Pad Prism). Data were evaluated by 1-way ANOVA and post hoc Tukey’s multiple comparison tests (Graph Pad Prism). Statistical differences between time and dose were tested by 2-way ANOVA and Bonferroni’s post test. At least 3 independent experiments were carried out for each variable. A P < 0.05 level was taken as significant.
Results

Olive fruit extract does not exert nonspecific cytotoxicity. HT-29 cells were exposed to increasing concentrations of olive fruit extracts for 3 h. Cell viability was unaffected (96.8 ± 0.3%) for concentrations from 10 μmol/L and 3.7 μmol/L up to 250 μmol/L and 92.5 μmol/L of maslinic and oleanolic acid, respectively. However, the percentage of living cells dropped slightly to 90.3 ± 0.6% (*P*, *P* < 0.001) at concentrations of 300 μmol/L maslinic acid and 111 μmol/L oleanolic acid.

Olive fruit extract inhibits cell proliferation of HT-29 human colon cancer cells. The olive fruit extract induced growth inhibition in a dose-dependent manner after 72 h of exposure to increasing concentrations (Fig. 2). The concentration that induced a 50% inhibition of cell proliferation compared with controls was 73.96 ± 3.19 and 26.56 ± 2.55 μmol/L of maslinic and oleanolic acids, respectively.

Olive fruit extract is an apoptosis inducer in HT-29 cells. The time course of activation of caspase-3 was assessed by exposing HT-29 cells to 150 μmol/L maslinic acid and 55.5 μmol/L oleanolic acids (or to 250 μmol/L maslinic acid and 92.5 μmol/L oleanolic acids) at different times. Caspase-3-like activities were 400% of control cells at 24 h when the concentrations of maslinic and oleanolic acids were 150 and 55.5 μmol/L, respectively. Caspase-3-like activity of cells treated with medium alone was set at 100% (14). At 36 and 48 h, apoptosis reached 600% of control cells (Fig. 3A). After 12 h, the extract containing 250 and 92.5 μmol/L maslinic and oleanolic acids, respectively, activated caspase-3 activity. The degree of activation increased with time: at 24 h, apoptosis was 7.5-fold that of control cells, at 36 h, it was 14-fold, and at 48 h, 18-fold.

The activation of caspase-3 by 150 and 55.5 μmol/L maslinic and oleanolic acid was associated with increased accumulation
of Hoechst dye 33342 (Fig. 4A). After exposing HT-29 cells to olive fruit extracts for 8 h, 11.0 ± 1.0% (P < 0.0001) of cells became strongly fluorescent. This accumulation increased with time, reaching 40.9 ± 3.9% (P < 0.0001) after 24 h of incubation (Fig. 4B).

The dose-response effect of the olive fruit extract on the induction of apoptosis was studied at 24 h. The extract displayed a dose-dependent increase in caspase-3-like activation (Fig. 3B). Concentrations of 50 and 18.5 μmol/L of maslinic and oleanolic acids caused caspase-3-like activation that was 200% of control cells (P < 0.05). Concentrations of 300 and 111 μmol/L of maslinic and oleanolic acids increased the activity to 8.5-fold that of the control (P < 0.0001).

Hoechst 33258 staining showed apoptotic bodies after exposure of HT-29 cells to 150 and 55.5 μmol/L maslinic and oleanolic acids (Fig. 4C). Apoptotic bodies were detected in 4.9 ± 0.6% (P < 0.05) of cells after 8 h of incubation (Fig. 4D). Nuclear fragmentation increased to 24.5 ± 1.5% (P < 0.0001) of cells after 24 h of exposure to the extract.

Confocal microscopic analysis revealed that the induction of apoptosis was preceded by an early increase in superoxide production in mitochondria of HT-29 cells. These cells had been treated for 4 h with olive fruit extracts containing 150 and 55.5 μmol/L maslinic and oleanolic acids, respectively (Fig. 4E).

Discussion

Olives and olive oil occupy a central position in the Mediterranean diet. They are believed to beneficially affect numerous biological processes, including cancer (1,2). However, there are no studies on the effects of the minor components of olive fruits, such as maslinic and oleanolic acids, on colon cancer. We demonstrated that an extract of the waxy coating of olive fruits had an antiproliferative effect on HT-29 human colon cancer cells. The extract had a half-maximal effect on growth inhibition at ~75 and 25 μmol/L of maslinic and oleanolic acids. Noteworthy, high concentrations of olive fruit extracts with full inhibition of cell growth did not induce any signs of cytotoxicity. Consequently, the inhibition of cell proliferation appeared to be specifically reduced either by changes in cell cycle progression and/or the induction of apoptosis.

Our results clearly show that the extract from the skin of the olive fruit induced apoptosis in HT-29 cells. Our study used a series of in vitro assays to model different stages of programmed cell death. The activation of the effector caspase-3 was determined, as it represents the converging point of different caspase-dependent apoptosis pathways (15). Achievement of apoptosis beyond activation of caspase-3 led to the characteristic hallmarks of programmed cell death. These include: disintegration of the plasma membrane, characterized by cell-staining with Hoechst 33342 dye (16); increased fragmentation of DNA and chromatin condensation, which is shown by Hoechst 33258 staining.

Apoptosis was initiated by the intrinsic pathway, or mitochondria-mediated effector mechanism (17), as shown by the superoxide anions detected in the mitochondria. HT-29 cells exposed to 150 and 55.5 μmol/L of maslinic and oleanolic acids showed a marked increase in reactive oxygen species (ROS) levels. Flavone also enhances the production of superoxide anion radicals in mitochondria (18). Consequently, olive fruit extract induces ROS production in an early phase. This suggests that the compounds in the mixture trigger a rapid release of cytochrome c from mitochondria into the cytosol. In turn, this activates procaspase-9 and the downstream effectors, including the pro-caspases -3, -6, and -7. Finally, this is followed by the cleavage of proteins and DNA, which characterizes the final phase of apoptosis.

ROS are thought to affect divergent cellular functions, depending on the cellular level and their compartmentation. Mitochondria are the primary cellular site of ROS production. Under certain conditions, elevated mitochondrial ROS levels can serve as pro-apoptotic signals (19,20). Consequently, dietary constituents that promote mitochondrial ROS production, such as the pentacyclic triterpenic acids evaluated in the present study, could be as important in cancer prevention as dietary antioxidants.

The activity displayed by the olive fruit extract containing maslinic and oleanolic acids can be linked with the fact that triterpene compounds are used for medicinal purposes in many Asian countries. Pentacyclic triterpenoids are abundant in the plant kingdom. Compounds such as betulinic and ursolic acids have been reported as antitumor agents (21). Betulinic acid has been shown to act as a selective inhibitor of human melanoma in cell culture and animal models. It functions by induction of apoptosis (22). Recently, ursolic acid, which is the main component of the wax-like coatings of apples, pears, and other fruits, has been reported to induce apoptosis. This also occurs through the mitochondrial intrinsic pathway and caspase-3 activation in M4Beu melanoma cells (23).

Finally, we wondered whether the daily consumption of table olives and virgin olive oil provides enough of these compounds to attain the described health-protecting properties. According to data from the literature, the mean daily consumption of table olives in Mediterranean countries corresponds to ~40 g or 10 medium-size olives. If the concentration of maslinic and oleanolic acids is 681 and 420 mg/kg, respectively (4), then the estimated intake of maslinic acid is 28 mg/d and the intake of oleanolic acid is ~17 mg/d. Moreover, the contribution of virgin olive oil cannot be underestimated. The concentration of maslinic acid in virgin olive oil is 172 mg/kg and 231 mg/kg for oleanolic acid (5). The daily consumption of virgin olive oil is 33 g. Therefore, under these conditions, the total daily intake provided by habitual consumption of olives and virgin olive oil is 34 mg of maslinic and 25 mg of oleanolic acids. If the bioavailability of these compounds remains at 2.3% (24), as reported for triterpenoid 23-hydroxybuthenic acid, then the intestinal epithelium is exposed to high concentrations of these compounds. Based on the assumption that an estimated 30% of the compounds are not absorbed in the small intestine and reach unaltered the colon in a distribution phase of around 250 mL, the concentration of maslinic and oleanolic acids would be ~86 μmol/L and 66 μmol/L, respectively. These concentrations induced a 50% inhibition of cell proliferation and led to a 3-fold activation of caspase-3. Thus, the concentrations we used appear relevant to conditions in vivo. The concept of dietary chemoprevention is frequently used in the context of protecting normal cells from initiation events that introduce oncogenic mutation. Consequently, maslinic and oleanolic acids may be particularly effective in the prevention of colon carcinoma formation, as we used HT-29 cells that represent an advanced stage of tumor development.

In summary, maslinic and oleanolic acids inhibit cellular proliferation at nontoxic concentrations and restore apoptosis sensitivity in human colon adenocarcinoma cells. Apoptosis induction is arguably the most important process in removing cells that have lost growth control. Therefore, the olive extract containing 73.25% maslinic and 25.75% oleanolic acids appears...
to have cancer chemopreventive activity. Nevertheless, more work is required to single out the contribution of each of these important bioactive food components protective activities against human colorectal cancer. Such studies may lead to improved guidelines and, possibly, to formulating an appropriate diet.

**Literature Cited**


