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Evaluation of potential antigenotoxic, cytotoxic and proapoptotic effects of the olive oil by-product "alperujo", hydroxytyrosol, tyrosol and verbascoside

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ABSTRACT

Olive oil is an integral ingredient of the "Mediterranean diet". The olive oil industry generates large quantities of a by-product called "alperujo" (AL) during the two-phase centrifugation system developed in the early nineties. AL could be a potent exploitable source of natural phenolic antioxidants. Our results showed that AL and its distinctive phenols hydroxytyrosol, tyrosol and verbascoside were not genotoxic in the Somatic Mutation and Recombination Test (SMART) of *Drosophila melanogaster* and exerted antigenotoxic activity against DNA oxidative damage generated by hydrogen peroxide (H_2O_2). Alperujo and hydroxytyrosol also exhibited notable antiproliferative and caspase 3-dependent proapoptotic effects toward the human tumoral cell line HL60. AL can provide a cheap and efficient source of chemopreventive phenolic compounds with strong antioxidant properties, becoming a promising and potent therapeutic drug in the future.

1. Introduction

Alperujo (AL) is one of the most important by-products produced by the European olive oil industry. This semi-solid derivative is collected in large quantities in the olive-mills during the two-phase extrusion process [1]. This system, widely implemented since the beginning of the nineties, has reduced the production of contaminant by-products and the energy and water consumption compared with the previous three-phase system [2].

This by-product was characterized as a rich source of phenolic antioxidant substances [3]. However, they were normally lost in the wastewater of the olive-mill in the older three-phase system due to their hydrophilic properties [4]. With the implementation of the two-phase extraction, the concentration of antioxidants detected in AL is up to 100-times higher than in olive oil [5]. Due to that, several extraction models have been proposed for an efficient recovering of these valuable AL compounds [6–9].

The antioxidant activity of the phenols was related to several biological activities, including antigenotoxicity and cytotoxicity [10], anti-allergenic [11], antimicrobial [12], cardioprotective and anti-inflammatory properties [13]. Among the different phenolic compounds detected in the AL, hydroxytyrosol (HT) is one of the most important molecules due to their biological activity [9]. This phenol counteracts the oxidation of low-density lipoproteins [14], protects different cells from hydrogen peroxide induced cytotoxicity [15] and reduces lactate dehydrogenase activity [16]. In addition, two other phenols have also been proposed as molecules with major biological activities [5]. Verbascoside (VE), a phenylethanoid glycoside, also displays various biological effects such as an important anti-oxidative and anti-bacterial activity [17–19] a reduction in the concentration of free radicals, an inhibition of the lipid peroxidation [20] and a key role scavenging hydroxyl radicals [21]. Conversely, tyrosol (TY) have been linked to a reduction in the reactive oxygen species (ROS) production [22], a protection of CaCo-2 cells against cytotoxic and apoptotic effects of oxidized-LDL [23], an inhibition in the activity of the leukocyte 5-lipoxygenase [24] and an antimicrobial activity against several bacterial strains [25].

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Mutations in somatic cells play a well-established role in cancer initiation and other stages of the carcinogenic process [26]. To detect this induction of this abnormal cellular process, one of the most commonly used techniques is the Somatic Mutation and Recombination Test (SMART) in *Drosophila* wings [27]. This procedure, which is based in the detection of induced genetic alterations and the loss of heterozygosity in somatic cells of the flies, is widely employed as a tool for assessing genotoxicity and antigenotoxicity of simple and complex substances in eukaryotic cells [10,28]. Similarly, the selective inhibition and the promotion of the cytotoxicity in *in vitro* growing tumoral cells are also excellent approaches to study the health-promoting properties of the natural and chemical products [29]. This can be assessed by detecting the induction of morphological and biochemical cell death features like apoptosis or necrosis [30].

Therefore, the aim of this research was to investigate the genotoxic/antigenotoxic effects of the AL and its three major phenolic compounds: HT, TY and VE. In addition, the cytotoxic effect and induction of apoptosis or necrosis by these compounds have also been investigated using molecular, enzymatic and cellular approaches.

2. Materials and methods

2.1. Extraction procedure and determination of phenol compounds from alperujo

Ten grams of AL from drupes of *Picual* cultivar were placed in the extraction cell of a superheated-liquid extractor. After assembling the cell and locating it in the oven, this was pressurized with 10 bar and brought up the working temperature to 200 °C by 12 min for static extraction. After that, the dynamic extraction starts by opening the inlet valve and controlling the outlet restrictor to keep the pressure meanwhile the extractant is pumped for 15 min at 1 mL/min. The overall extract is collected and concentrated in a rotary-evaporator.

Determination of HT, TY and VE in the concentrated extract was carried out using an Agilent 1100 liquid chromatograph connected to a diode array detector following the procedure described by Japon-Lujan and Luque de Castro [31].

2.2. Phenolic compounds

The commercial phenols used in this study, tyrosol (CAS number 501-94-0, Fluka, Sigma–Aldrich, Spain), verbascoside (CAS number 61276-17-3, Apin Chemicals) and hydroxytyrosol (CAS number 10597-60-1, Sigma), were dissolved in the culture media.

2.3. Somatic mutation and recombination test (SMART)

The assay was essentially performed as described by Graf, Wurgler, Katz, Frei, Juon, Hall and Kale [27] using two *Drosophila* strains: the multiple wing hairs strain with genetic constitution (*mwh*/*mwh*) and the flare-3 strain (*flr*^{3}/*In* (*3LR*), *TM3 Bd*^{S}). Briefly, three day old larvae, obtained from the standard cross between virgin females and males were washed and transferred to culture vials with 0.85 g of *Drosophila* Instant Medium (Formulas 4–24, Carolina Biological Supply, Burlington, NC, USA), wetted with 4 mL of corresponding concentrations of AL (3.75 and 30 μL/mL), HT (6.25 and 100 μM), TY (17 and 140 μM) or VE (29 and 240 μM). These concentrations were selected according to those obtained in the chemical characterization of the AL. Concurrent negative controls treated with distilled water and positive controls with H_2O_2 (0.12 M) were also run for each experiment. The antigenotoxic capacity of each substance was assessed as the obtained using a combined treatment of the hydrogen peroxide (0.12 M) and the different concentrations of AL and phenols. After the growing period, (10–12 days) emerging adult flies were collected and stored in 70% ethanol. After that, wings were removed and mounted on slides using Faure's solution. Mutant spots were assessed in both dorsal and ventral surfaces of the wings in a photonic microscope at 400× magnification as described by Lindsley and Zimm [32].

For evaluation of the genotoxic effects recorded, the frequencies of spots per wing of a treated series were compared to negative control series as described by Frei and Wurgler [33] and Frei and Wurgler [34]. To avoid false positive and negative results, the negative controls data were summed. Statistical analyses were done for single, large, twin and total number of spots recovered. The inhibition percentages (IP) in combined treatments were calculated for the total spots per wing using the procedure described by Abraham [35]:

$$\mathrm{IP} = \frac{\text{Genotoxin alone} - \text{Genotoxin plus phenol}}{\text{Genotoxin alone}} \times 100$$

2.4. Cell culture and viability assay

The cytotoxic effect of the compounds was assessed using the HL60 human promyelocytic leukemia cell line, following our standard procedures [36]. Briefly, cells were cultured in a complete RPMI 1640 medium in a culture incubator at 37 °C under a 5% CO_2 humidified atmosphere. During the exponential phase of growth cells were harvested and diluted to a final concentration of 2.5×10^5 cells/mL. Thereafter, 2 mL of the dilution were incubated with eight different treatments (four compounds at low and high concentration) in plastic dishes by 72 h, as follows: AL (20–320 μL/mL), HT (8–128 μM), TY (8.75–140 μM) or VE (30–480 μM). After culture, cell viability was determined by the trypan blue dye exclusion test in a Neubauer chamber. Growth curves were plotted as survival percentage with respect to the control growing at 72 h, and IC_{50} values were determined.

2.5. Agarose gel analysis of DNA fragmentation

To determine protective effect against chromatin fragmentation, HL60 cells were incubated by 5 h in 12-well plates at a concentration of 1.5×10^5 cell/mL using the same treatments described above and including an incubation without any supplementation served as a control. After that, DNA was extracted using a commercial kit (Dominion mbl, MBL 243) and treated with RNase for 30 min at 37 °C. Electrophoresis was performed at 50 V/cm for 2 h and then, the gel was observed and digitally imaged under UV light after staining with Ethidium Bromide (EB).

2.6. Detection of cellular apoptosis and necrosis trough AO/EB fluorescence staining

In vivo cytotoxicity was assessed trough co-incubation of the analyzed compounds with HL60 cells. Five different treatments (AL at 160 μL/mL, HT at 128 μM, TY at 140 μM, VE at 240 μM and a negative control without supplementation) were co-incubated with HL60 cells (1.5×10^5 cell/mL) at 37 °C in RPMI 1640 medium by 24 h. Samples were collected at 2, 4 and 24 h and stained according to our standard protocol [36]. Briefly, cells were washed twice in PBS to remove the remaining medium and resuspended in 1 mL of PBS. After that, they were incubated in the dark with 20 μL of ethidium bromide (100 μg/mL) for 5 m at room temperature. Thirty seconds before the end of the incubation 20 μL of acridine orange (100 μg/mL) were added. Finally, cells were re-washed twice in PBS, extended in a clean slide glass and assessed under an epifluorescence microscope at 400× magnification using a blue filter. Four different patterns were distinguished: live cells appeared uniformly stained in green; similar pattern was observed in early apoptotic cells, but they also showed bright green dots in the nuclei as a consequence of nuclear fragmentation. Late apoptotic cells also showed ethidium bromide red dots in the cytoplasm and often a fragmented nuclei. Finally, necrotic cells showed an orange staining in all the cytoplasm without any condensed DNA.

2.7. Enzymatic detection of apoptosis

2.7.1. Preparation of the cytoplasmic extracts

Two different extracts were obtained in order to determine the caspase-3 and LDH release, following the procedure described by Gonzalez-Rubio, Hidalgo, Ferrin, Bello, Gonzalez, Gahete, Ranchal, Rodriguez, Barrera, Aguilar-Melero, Linares, Castano, Victor, De la Mata and Muntane [37]. Active HL60 growing cells (250,000 cells/mL) were treated with AL (160 μL/mL), HT (128 μM), TY (140 μM), VE (240 μM) and without supplementation (negative control) in 12 well-plates by 6, 12, 24 and 48 h of culture. After that, cells were centrifuged at 15000 G for 5 min at 4 °C and the supernatant (culture medium) was stored at −80 °C. The remaining pellet was treated with 150 μL of lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 5 μg/mL aprotinin, 10 μg/mL leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 0.6% Nonidet NP-40) and incubated on *ice* for 10 min. Thereafter, cells were centrifuged to obtain cytoplasmic fraction (supernatant) and stored at −80 °C until use.

2.7.2. Caspase 3 activity

Caspase 3 activity is widely used as a marker of the cellular apoptosis. To determine its activity we firstly assessed the total protein concentration of the cytoplasmic fraction in a microtiter plate reader (GENios Microplate Reader, TECAN, Salzburg, Austria) using the Bradford method. The caspase-3 activity was measured using Ac-DEVD-AFC (100 μM, Bachem AG, Budendorf, Switzerland) as a fluorometric substrate following the manufacturer instructions. Briefly, 25 μg of protein (cytoplasmic fraction) was dissolved in the assay buffer and assessed in a spectrofluorometer TECAN (Salzburg, Austria) emitting at 505 nm. The reaction was monitored every 300 s for 2 h. The results were compared with the corresponding control by one-way ANOVA using Turkey's multi-comparison procedures.

2.7.3. Measurement of LDH release

LDH activity was measured in culture medium and cell lysate by monitoring NADH reduction during pyruvate–lactate transformation. Briefly, samples (150 μL culture medium, 5 μL cytoplasmic fraction) were incubated with 0.2 mM β-NADH and 0.4 mM pyruvic acid diluted in PBS (pH 7.4). The percentage of LDH released was calculated as percentage of the total amount, considered as the sum of the