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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

Table 1
Summary of results obtained in the *Drosophila* wing spot test (SMART) with Alperujo, hydroxytyrosol, tyrosol and verbascoside.

Compounds	Number of wings	Small spots (1–2 cells) $m=2$	Large spots (more than two cells) $m=5$	Twin spots $m=5$	Total spots $m=2$
Negative control (H_2O)	297	0.23 (69)	0.01 (4)	0.01 (3)	0.26 (76)
Positive control (H_2O_2)	272	0.46 (126)	0.04 (12)	(0)	0.51 (138)+
Simple treatment					
Alperujo (μL/mL)					
3.75	38	0.23 (9)	0.05 (2)	(0)	0.29 (11)–
30	40	0.20 (8)	0.05 (2)	0.02 (1)	0.28 (11)–
Hydroxytyrosol (μM)					
6.25	40	0.20 (8)	0.12 (5)	(0)	0.32 (13)i
100	40	0.15 (6)	0.02 (1)	(0)	0.18 (7)–
Tyrosol (μM)					
17	40	0.25 (10)	(0)	(0)	0.25 (10)–
140	40	0.17 (7)	0.02 (1)	(0)	0.20 (8)–
Verbascoside (μM)					
29	40	0.20 (8)	(0)	(0)	0.20 (8)–
240	38	0.23 (9)	(0)	(0)	0.24 (9)–
Combined treatment					
Alperujo (μL/mL)					
3.75 + H_2O_2	40	0.25 (10)	0.02 (2)	(0)	0.30 (12)i
30 + H_2O_2	40	0.15 (6)	(0)	(0)	0.15 (6)–
Hydroxytyrosol (μM)					
6.25 + H_2O_2	40	0.25 (10)	0.02 (1)	(0)	0.28 (11)–
100 + H_2O_2	40	0.40 (16)	(0)	(0)	0.40 (16)i
Tyrosol (μM)					
17 + H_2O_2	38	0.42 (15)	(0)	(0)	0.39 (15)i
140 + H_2O_2	40	0.17 (7)	(0)	(0)	0.18 (7)–
Verbascoside (μM)					
29 + H_2O_2	40	0.15 (6)	0.02 (1)	(0)	0.18 (7)–
240 + H_2O_2	40	0.17 (7)	(0)	0.02 (1)	0.20 (8)–

Statistical diagnoses according to Frei and Würgler (1988): + (positive), – (negative) and i (inconclusive). m: multiplication factor. Significance levels $\alpha=\beta=0.05$, one-sided test without Bonferroni correction.

enzymatic activity present in the cellular lysate and that in the culture medium in a 340 nm absorbance automated microplate reader (TECAN, Salzburg, Austria). The results were compared with the corresponding controls by one-way ANOVA using Turkey's multi-comparison procedures.

3. Results

3.1. Genotoxicity assessment of AL, hydroxytyrosol, tyrosol and verbascoside

The genotoxic effect of AL, HT, TY and VE supplementation in the control and H_2O_2-treated larvae are showed in Table 1. Frequency of total spots per wing in the negative control was 0.26. These data are in accordance with the frequency of basal spots obtained previously in the ST cross [38]. The genotoxicity rates induced by AL, HT, TY and VE were similar with no differences with the control flies at both concentrations tested. Only HT at the lower concentration assessed (6.25 μM) showed an inconclusive result.

3.2. Protective effects of AL, hydroxytyrosol, tyrosol and verbascoside against H_2O_2-induced damage

The protective effect produced by the four substances assessed against the H_2O_2 induced damage is also showed in Table 1 (combined treatment). The genotoxicity rate observed in flies used as positive control increased significantly (0.51 spots/wing). When larvae were grown in AL supplemented media, the genotoxic rate significantly returned to the water control level at the higher concentration (30 μL/mL). AL protected the larvae against the injury induced by H_2O_2, showing inhibition percentages of 41.17% (3.75 μL/mL) and 70.58% (30 μL/mL).

Conversely, the chronic co-treatment of ST larvae with HT (6.25 and 100 μM) plus H_2O_2 decreased the spot frequency at the lower concentration tested in a wider extent than those observed at the highest concentration. These results were confirmed by the percentages of inhibition determined (47.05% at 6.25 μM and 21.56% at 100 μM).

Tyrosol showed a protective pattern similar than the observed in AL. At the lowest concentration (17 μM) it did not modify significantly the frequency of mutated spots, inhibiting the H_2O_2 induced damage in a 20% only. Conversely, the larvae reared in a medium supplemented with 140 μM (higher concentration) showed a significantly lower genotoxicity rate than positive control and an inhibition rate close to 66%.

Finally, VE was the only compound that counteracted the oxidative stress caused by H_2O_2 reducing the genotoxicity rates to the negative control level in both concentrations tested. Furthermore, this compound showed almost the same inhibition activity against H_2O_2 induced genotoxicity at the lower (29 μM; 66.66%) and at the higher concentrations (240 μM; 60.78%) assessed.

3.3. Effects of AL, hydroxytyrosol, tyrosol and verbascoside on cell viability

Fig. 1 shows the results of the assessment of the *in vivo* cytotoxicity activity of the studied compounds against HL60 cells. A clear cytotoxic effect was observed when the culture medium was supplemented with AL concentrations of 40 μL/mL or higher. The IC50 value was observed when the culture medium was supplemented with 45 μL/mL. The same pattern was observed in VE treated cells, in which the viability fall to less than 10% using lower supplementations (approx. 100 μM), showing an IC_{50} value located at 60 μM.

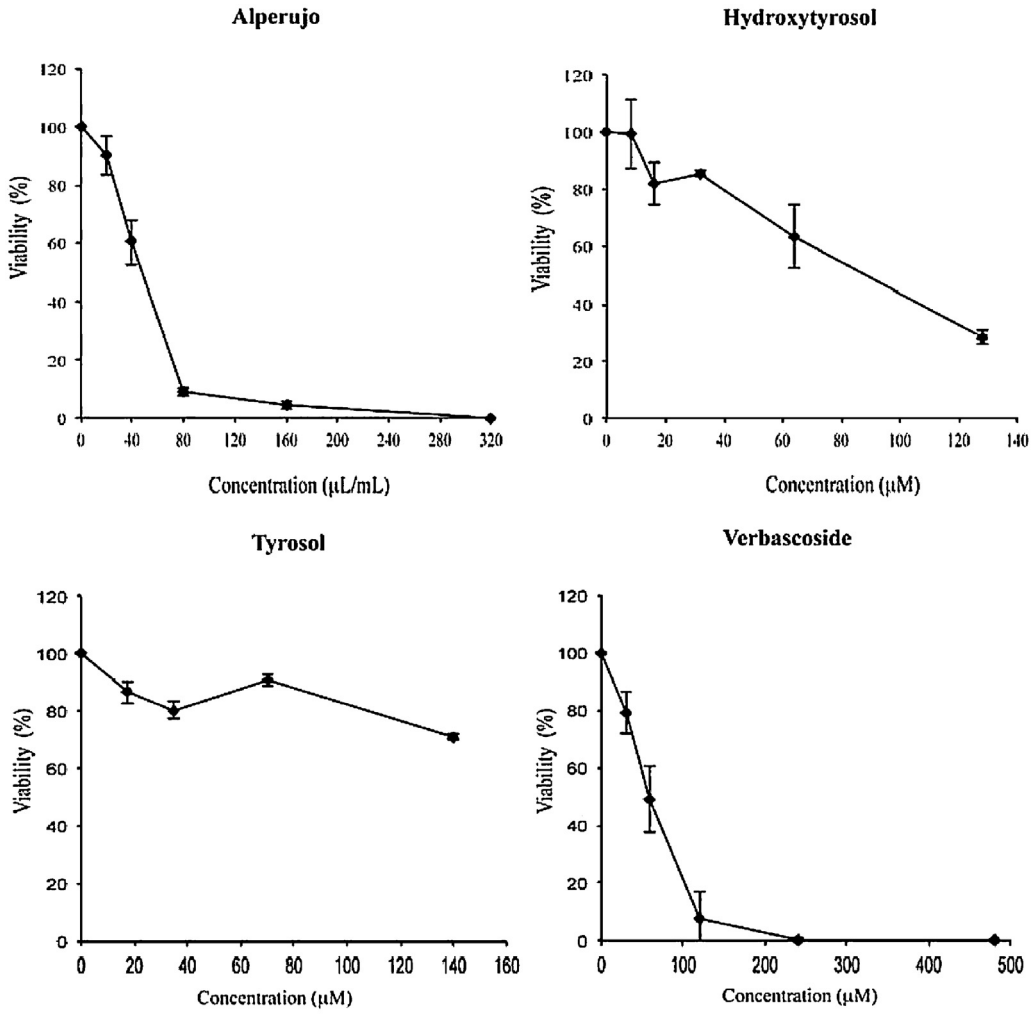

Fig. 1. Effect of alperujo and three phenolic compounds in the viability of HL60 human leukaemic cells after 72 of co-culture. Alperujo (20–320 μL/mL), hydroxytyrosol (8–128 μM), tyrosol (8.75–140 μM) and verbascoside (30–480 μM). Cell number was counted using a hemocytometer and cell viability was evaluated by trypan blue exclusion.

HT supplementation showed a lower inhibition activity against HL60 cells. Only using the higher concentration, the inhibition percentage raises to 78%. The IC_{50} value for this compound was also achieved using an elevated supplementation (90 μM). Conversely, the use of TY as medium supplement did not produced a significant inhibition on the HL60 cell growing without achieving the IC_{50} at any of the assessed concentrations.

3.4. Effects of AL, hydroxytyrosol, tyrosol and verbascoside on internucleosomal DNA fragmentation

The apoptosis process is recognized by morphological changes and the appearance of DNA fragments of 200 base pairs. A ladder pattern of the DNA fragmentation, as detected by electrophoresis, appeared as early as 5 h after cells were exposed to AL (Fig. 2), indicating that HL60 cells underwent apoptosis. However, no ladder pattern was observed, under identical conditions, in the control and cells treated with HT, TY and VE, even after 5 h.

3.5. Analysis of apoptosis and necrosis in HL60 cells by fluorescence microscopy with ethidium bromide/acridine orange double staining

Fig. 3 shows the results of EB/AO staining. HL60 cells appeared viable and intact with bright green nucleus after 2 h treatment with HT, TY and VE. Nevertheless, cells treated with AL showed early morphological changes (2 h) which were followed by moderate chromatin condensation and generation of apoptotic bodies (6 h). Apoptotic process was fully established at late stage (24 h) when EB stained cell nuclei were detected. On the contrary, TY did not show any significant sing of apoptosis, showing uniformly green cells with normal morphology at 2, 6 and 24 h. HT effect was moderate but sufficient to induce the formation of apoptotic bodies and morphological change in cells in the late stages of incubation. After 24 h of culture, the morphology of cells changed after showing an orange nucleus. The same growing profile was observed in the VE-treated cells.

3.6. LDH release

The increase of LDH activity indicates an increase in the number of dead or plasma membrane-damaged cells. LDH release was low (5%) in HL60 untreated control cells during all studies time points (6, 12, 24 and 48 h) (Fig. 4). AL and HT induced a significant increase of LDH release only after 12 h of co-incubation. However, the pro-necrotic effect of VE was already significant at 6 h, being drastically increased at 24 and 48 h of culture. Following the pattern observed in the previous experiments, no statistically significant increase in LDH release was observed in cells treated with TY.

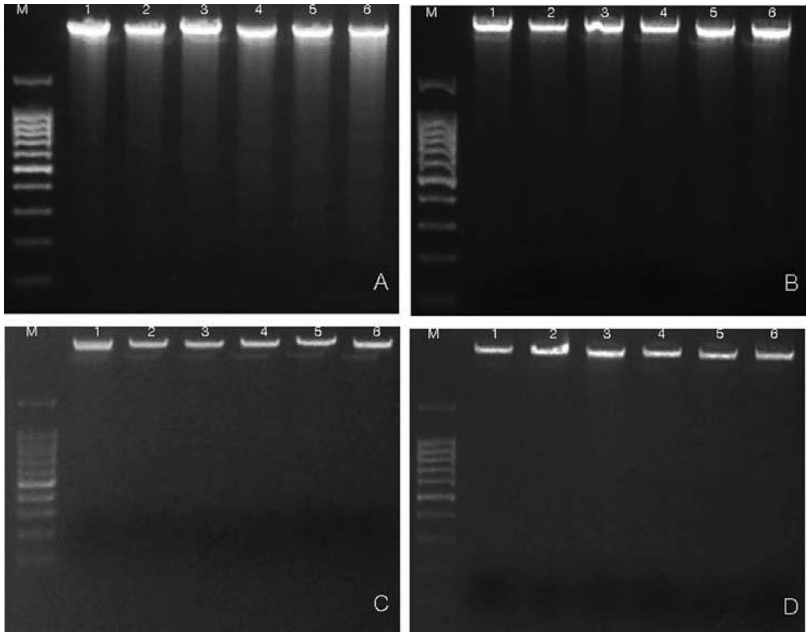

Fig. 2. Effects of alperujo, hydroxytyrosol, tyrosol and verbascoside on DNA fragmentation in HL60 cells. (A) alperujo: lane 1, control; lane 2, 20 μL/mL; lane 3, 40 μL/mL; lane 4, 80 μL/mL; lane 5, 160 μL/mL; and lane 6, 320 μL/mL. (B) Hydroxytyrosol: lane 1, control; lane 2, 8 μM; lane 3, 16 μM; lane 4, 32 μM; lane 5, 64 μM; and lane 6, 128 μM. (C) Tyrosol: lane 1, control; lane 2, 8.75 μM; lane 3, 17 μM; lane 4, 35 μM; lane 5, 70 μM; and lane 6, 140 μM. (D) Verbascoside: lane 1, control; lane 2, 30 μM; lane 3, 60 μM; lane 4, 120 μM; lane 5, 240 μM; and lane 6, 480 μM. Lane M, DNA size markers.

3.7. *Activity of caspase 3*

Caspase-3 activation is another key marker of cellular apoptosis. The induction of apoptosis by AL (160 μL/mL) and HT (128 μM) was already observed at 6 h and declined later (Fig. 5) when necrotic process appeared evident as described above (Fig. 4). Our results also indicated that VE (240 μM) increased caspase-3 activity in HL60 cells at 24 h. Caspase-3 activation was not observed in TY-treated cells.

4. Discussion

Alperujo is one of the by-products produced in the Spanish food industry in largest quantities, averaging 790,000 tons by year [2]. By this reason, there is an increased interest in finding potential uses of this kind of natural by-products as a proper way to reduce the waste produced and to increase the overall benefits obtained from olive trees. However, such applications, and even more if they are intended to be employed as pharmaceutical or nutritional compounds, must be extensively studied using both, *in vitro* and *in vivo* models to avoid any potential derived health problem [39]. In this study, we complemented previous evidences that promote the AL as a promising compound with anticarcinogenic properties due to its phenolic profile [40].

The absence of genotoxicity was also found by Pierantozzi et al. [41], by demonstrating that AL does not induce chromosomal aberrations in *Allium cepa* neither reversal mutations in the *Salmonella* Ames test. It is in agreement with the lack of genotoxic activity observed in the SMART test in our study in AL and their major phenolic compounds, which were also assessed individually. Among them, HT was previously characterized as non genotoxic nor in *Salmonella* and *Escherichia coli* prokaryotic systems [42]; TY was non-genotoxic nor in the SMART test [10] and in Jurkat cells [43], and more recently, VE was also characterized as non-genotoxic in highly bioactivated and standard genetic backgrounds also using the wing spot test [44]. On the contrary, some toxic effects associated have been described in agronomics experimental assays using barley and onion [41,45]; but they were only observed using relatively high concentrations and were easily counteracted with a thermal treatment. Conversely, our results do not show a significant level of toxicity in the *in vivo* SMART animal model of *Drosophila melanogaster*, even when the same phenolic concentration as in the genotoxicity assays was used (data not shown).

The lack of genotoxicity leaded us to test the possible DNA protective effects of AL against oxidative stress. H_2O_2 is a very well-known inducer of genotoxicity through oxygen free radical-based mechanisms. Due to that, the beneficial properties of a compound can be referred to its effect on H_2O_2-induced genotoxicity in SMART [38], in which, AL was able to inhibit significantly the oxidative damage. This in agreement with previous bioassays demonstrating their efficacy for scavenging oxi-radical and protecting DNA [40].

This highly valuable antioxidant properties observed in this by-product would be mainly attributed to its phenolic components. Alburquerque et al. [2] reported that water-soluble phenols are present in AL at 14.2 g/Kg dry weight, being HT and TY the two major compounds [9]. Verbascoside is also present in AL but in a lower concentration range of 20.22–55.02 mg/Kg, depending on the extraction method used.

In our study, HT significantly decreased the genotoxicity rate only at the highest-tested dose (100 μM). Conversely, the protection against H_2O_2 induced damage was significantly better at the lower concentration tested (6.25 μM). This in agreement with previous studies in which HT was characterized as an efficient scavenger of peroxyl radicals that prevented olive oil auto-oxidation [46,47] and inhibited DNA oxidative damage in different cell types [48,49]. It was also demonstrated that HT protects human liver-derived HepG2 cells against the genotoxicity of acrylamide by reducing the generation of ROS [50] and Jurkat cells from H_2O_2-induced damage [43]. It also exerts a protective effect against ROS-induced damage in epithelial Caco-2 intestinal cells, melanoma cells and in erythrocytes [51–53]. It was noteworthy the lower protective effective against H_2O_2 induced damage observed using higher HT concentrations. This pattern agrees with previous studies in which antioxidant activity of phenolic compounds

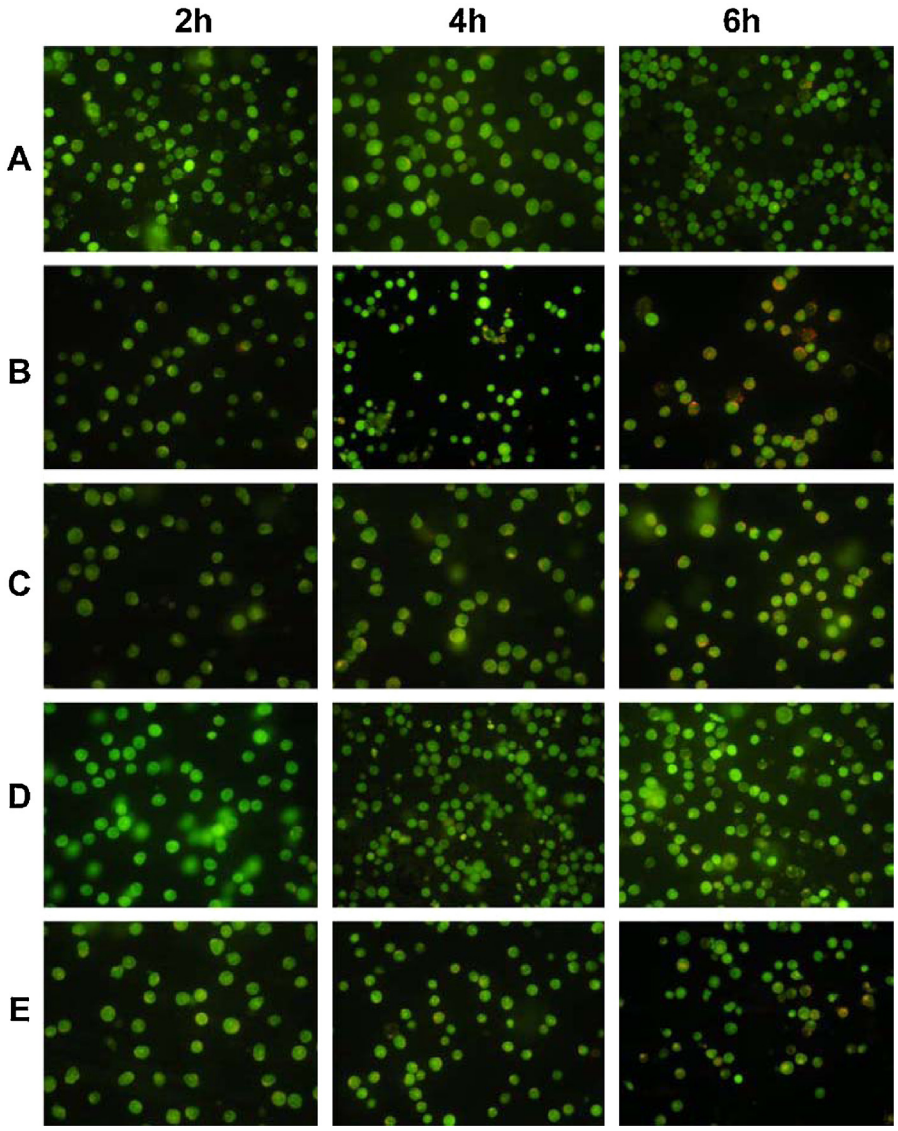

Fig. 3. Time-lapse cell viability determination using acridine orange/ethidium bromide staining. HL60 cells (A) Control, (B) alperujo (160 μL/mL), (C) hydroxytyrosol (128 μM), (D) tyrosol (140 μM) and (E) verbascoside (240 μM). Apoptotic cells showed intense green dots (DNA condensation) at early apoptotic stages and an orange nucleus in late apoptotic stays. Necrotic cells were stained uniformly orange and normal cells were stained uniformly green.

intimately depends on their concentration [54]. In this sense, it was demonstrated that antioxidant molecules can also display pro-oxidant activities under certain conditions, such as at higher doses, by enhancing the generation of superoxide radicals [55,56]. This same effect was also described in flavonoids, which protected rat H4IIE cells against H_2O_2-induced cytotoxicity and apoptosis at low concentrations (10–25 μM), whereas cytotoxicity, DNA damage and apoptosis were observed at high concentrations (50–250 μM) [57].

Tyrosol showed antigenotoxic activity against H_2O_2 induced damage only at high doses. This finding is in agreement with our previous results related to the modulation of genotoxicity by extra-virgin olive oil phenols [10]. This phenol was characterized as an efficient hydroxyl radical scavenger [58,59] and also preventing Caco-2 cells from ox-LDL induced injury [23]. It has been also reported that TY has moderate but stable antioxidant activity [60], exerting its effect only as a hydroxyl radical scavenger or, at most, as an α-tocopherol regenerator [58]. This limitations agree with the highly dose-dependent effect observed in our experimental design, in which, the treatment supplemented with lower doses of TY showed the lowest protective effect against H_2O_2 induced damage.

Verbascoside also acted as an antigenotoxic compound toward H_2O_2-induced damage and decreases the genotoxicity rates in the wing spot test. This phenylethanoid glycoside polyphenolic compound inhibits lipid peroxidation [20] and hydroxyl radicals scavenging properties [21]. It was also reported that it exhibits anti-inflammatory effects [61] and repairing properties against DNA damage induced by oxidative stress [62], which is in agreement with our results.

To determine the potential chemopreventive effect of AL and its compounds on human health, they were assessed in a specific human cancer cell model. AL decreased the survival rate of HL60 leukemia cells in a dose-dependent manner. This result agree with the DNA fragmentation and caspase-3 activation (6 h) observed, indicating an increased apoptosis rate and also agree with the results observed in EB/OA staining (nuclear DNA condensation and formation of apoptotic bodies). However, necrotic cell death measured by LDH release was only significant at later stage (12 h). These results confirm and support the hypothesis that

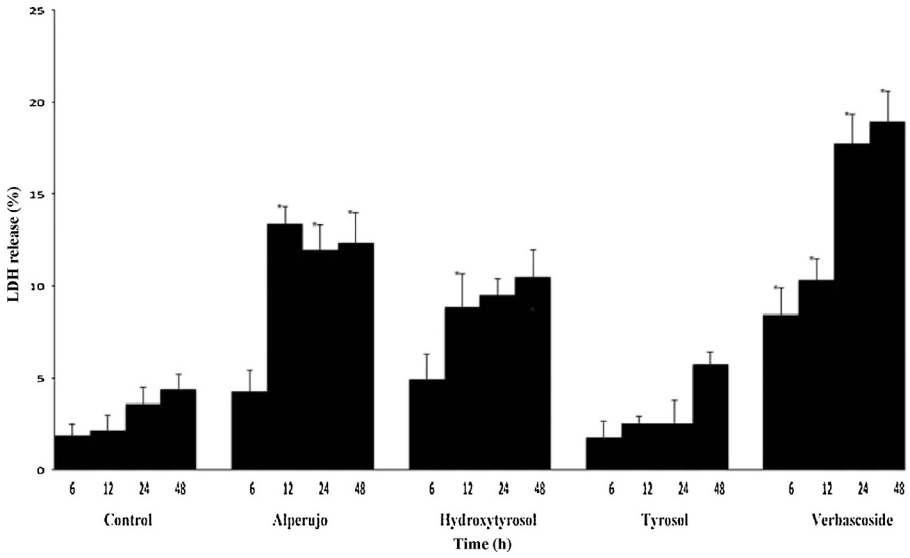

Fig. 4. Effects of alperujo and its three main phenolic compounds on lactate dehydrogenase (LDH) activity in HL60 cells. Aperujo (160 μL/mL), hydroxytyrosol (128 μM), tyrosol (140 μM) and verbascoside (240 μM). Data are the mean ± SD of 3 experiments. *Key*: (*) $P < 0.05$ when compared with control by one-way ANOVA using Turkey's multi-comparison procedures.

cell growth inhibition evidenced under the experimental conditions was correlated to an early signal of apoptosis, which is a key requirement of a candidate for anti-cancer drug [63]. Furthermore, our study agree with the preliminary assessment performed by Ramos et al. [64], which describes a potential chemopreventive activity of methanol/water extracts of dry mill residues against breast cancer cell line.

Our results presented here showed that HT also inhibited cell growth, and increased cell shrinkage and apoptotic bodies' formation, which correlated to significant increase of caspase-3 activation by acting isolately. Hydroxytyrosol is not toxic on normal kidney cells [65] and can even exert protection against oxidative damage of erythrocytes [66]. Furthermore it is cytotoxic on HepG2 cancer cells and, but at the same time, it is able to protect against the oxidative damage in this cancer cell line by decreasing the reduced glutathione [67]. The cytotoxicity observed in HL60 cells by Fabiani et al. [68] was accompanied by internucleosomal DNA fragmentation. These activities are congruent with our results of cytotoxicity in the HL60 cell line with the exception that we did not observed DNA fragmentation at the assayed concentrations.

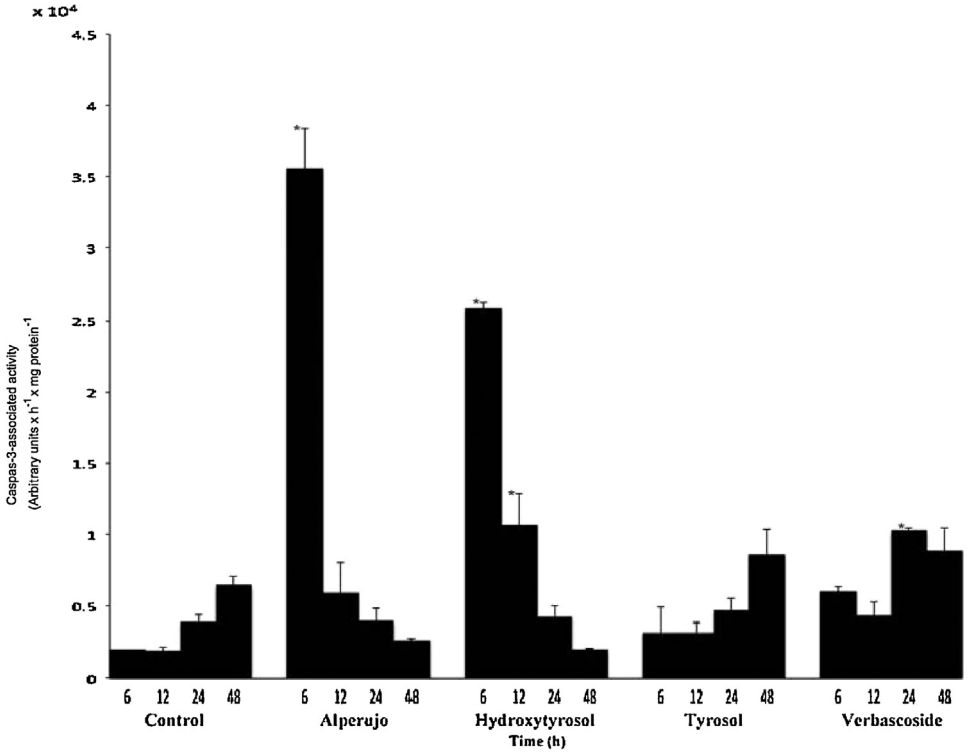

Fig. 5. Caspase 3 activities in HL60 cells treated with alperujo and its three major phenolic compounds. Alperujo (160 μL/mL), hydroxytyrosol (128 μM), tyrosol (140 μM) and verbascoside (240 μM). Values represent means of 3 separate experiments. *Key*: (*) $P < 0.05$ when compared with control by one-way ANOVA using Turkey's multi-comparison procedures.

As shown in this study, TY did not strongly modify cell viability and cell death in HL60. Babich and Visioli [69] used carcinoma cells from the salivary gland and concluded that the TY cytotoxic level was very low. This lack of toxicity was also described by Loru et al. [65] in kidney cells. On the other hand, TY is able to prevent oxidative alterations in Caco-2 cells [23]. All these studies in several types of cells indicate that TY is not cytotoxic and are in agreement with ours.

Finally, we observed that verbascoside declined significantly HL60 cell viability, and potently induced LDH release. However, we were unable to detect DNA fragmentation at the studied concentrations, and caspase-3 was moderately increased only after 24 h. This could suggest that VE induced cell death through the necrotic pathway mainly, as was proposed by Fiers et al. [70], but also inducing apoptosis of the target cells [71]. This is in agreement with the EB/AO staining pattern, in which symptoms of apoptosis and, to a lesser extent, necrosis was only observed after 24 h of treatment. Due to this, we suggest that the effect produced by VE may have developed more slowly, as was described previously by Santoro et al. [72], in which, the peak of the VE cytotoxic effect was detected only after 48 h of co-culture, regardless the concentration employed.

In conclusion, we demonstrated that AL and its major phenolic compounds (HT, TY and VE) are safe, induced a clear antimutagenic effect in the wing spot assay and may acts as inducer of cell death in HL60 cells in different extent. Alperujo and HT were representative of the caspase 3-dependent cell death. However, VE showed a pro-necrotic pathway and TY did not induce cell death. Consequently, further studies of these apoptotic or necrotic pathways will advance our knowledge and understanding the efficacy of the chemopreventive effects of these compounds, some of which may become very potent therapeutic drugs of the future.

Conflict of interest

The authors declare that there is no conflict of interest.

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