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A two-phase olive mill by-product (pâté) as a convenient source of phenolic compounds: Content, stability, and antiaging properties in cultured human fibroblasts

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ABSTRACT

Pâté is a new olive mill by-product potentially suitable for human consumption. This work aimed to characterize the phenolic profile of pâté samples from four crop seasons (2013–2016) in fresh, dried and stored samples, applying HPLC-DAD-MS-TOF, and to evaluate the antiaging effect in a cell senescence model. The dried pâté contains high levels of hydroxytyrosol, oleuropein derivatives and other phenolic compounds and is stable for several months. A diluted hydroalcoholic extract showed antiaging effects *in vitro,* comparable to those of pure hydroxytyrosol. Pâté can thus be proposed as an additional economical and environment-friendly source of olive bioactive phenolic compounds, particularly hydroxytyrosol: 1 g of pâté provides a daily intake comparable to that derived from 200 g of a typical virgin olive oil. This work lays the basis for a possible use of this food by-product as a natural ingredient for innovative foods or food supplements, contributing to a healthier lifestyle.

1. Introduction

According to the Food and Agriculture Organization of the United Nations (FAOSTAT, 2006), 2.7 million tons of olive oil are produced annually worldwide, 76% of which in Europe, with Spain (35.2%), Italy (23.1%) and Greece (16.1%) being the highest olive oil producers. The production of olive oil yields a considerable amount of olive mill waste (OMW), which have a negative impact on land and water environment, being phytotoxic and powerful pollutants.

Although the high phenol, lipid and organic acid content is responsible for phytotoxicity, these by-products also contain valuable resources such as a great amount of interesting phytochemicals that could be recovered (Roig, Cayuela, & Sánchez-Monedero, 2006). In fact, although the olive fruit is very rich in phenolic compounds (Cecchi, Migliorini, Cherubini, Innocenti, & Mulinacci, 2015), the largest portion of these compounds is lost in the olive mill by-products during the milling process. In particular, the phenolic fraction in olive oil is below 2% of the total phenolic content of the olive fruits, with the remaining 98% being lost in OMW (Ciriminna, Meneguzzo, Fidalgo, Ilharco, & Pagliaro, 2016; Rodis, Karathanos, & Mantzavinou, 2002).

Since the importance of natural products, particularly from olives, has been highlighted (Ahmad Farooqi et al., 2017; Waltenberger, Mocan, Šmejkal, Heiss, & Atanasov, 2016), these huge quantities of olive mill by-products are potential rich sources of phenolic compounds, endowed with a wide array of biological activities. The most extensively studied is the antioxidant action, but also antimicrobial and a diverse range of other bioactivities have been demonstrated both for OMW as such and for phenolic compounds, which have been reported to be present in this wastes (Obied et al., 2005). In 2011, EFSA stated that “Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress” (European Food Safety Authority, 2011). Among these activities, much attention has focused on the antiaging properties:

Abbreviations: OMW, olive mill waste; VOO, virgin olive oil; EVOO, extra virgin olive oil; NHDFs, neonatal human dermal fibroblasts; DMEM, Dulbecco’s modified Eagle’s medium; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium:inner salt; SA-β-gal, Senescence-Associated β-galactosidase; PBS, phosphate-buffered saline

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studies in rodent models of normal and accelerated aging have shown improvement in age-related dysfunctions upon administration of olive oil phenolic compounds, which have been proposed as candidates to counteract age-associated neurodegeneration (Casamenti & Stefani, 2017). Mechanistic studies indicate that these compounds are able to act at different sites, modulating cellular pathways relevant to the aging process, interfering with protein function and gene expression modulation (Giovannelli, 2013). Hydroxytyrosol is the most studied among olive phenolic compounds, and showed several superior biological activities, some of which demonstrated in humans (Granados-Principal, Quiles, Ramirez-Tortosa, Sanchez-Rovira, & Ramirez-Tortosa, 2010; Visioli & Bernardini, 2011).

The technology for olive oil extraction has progressed significantly since the beginning of the 1970s, when the three-phase centrifugation system proved to be more efficient than the traditional pressing used for many centuries. In the 1990s, the two-phase centrifugation system was introduced in Spain as a more ecological approach for olive oil production, drastically reducing the amount of added water and producing a semi-solid by-product named alperujo, or olive pomace.

Recently, Pieralisi S.p.A. developed an innovative two-phase decanter named Leopard. This decanter produces a dehydrated husk similar to the one obtained from a three-phase decanter, but it also separates the pulp (pâté) from the husk directly after the malaxation step (Leopard Series, Pieralisi Group S.p.A. Jesi, Italy), so reducing the possible oxidation processes. This by-product, named pâté, consists of a wet homogeneous pulp free from residuals of kernel, peculiarity making it a suitable ingredient for possible commercial applications after drying. It is potentially suitable for various uses, including animal feeding, but also for human consumption in the form of food supplement or food ingredient. The possibility to use the fresh pâté was evaluated in one study (Luciano et al., 2013), who demonstrated that the inclusion of olive cake into a concentrate-based diet for lambs could be proposed as a strategy to improve the nutritional quality of meat without compromising its oxidative stability. Indeed, the inclusion of this pâté in the animal diet increased the concentration of vitamin E in muscle and extended meat oxidative stability.

To the best of our knowledge, reports on the use of this particular pâté for food formulations to be used in the human diet are not available so far. Clearly, the possibility of turning a by-product into a valuable resource, particularly for human consumption, would represent an important benefit for the miller. Recently, one study focused on the qualitative and quantitative characterization of this pâté (Lozano-Sánchez et al., 2017); the authors analyzed one sample recovered in 2015 from a mill in the Marche region (Italy) and concluded that this particular by-product can be used as source of bioactive hydrophilic and lipophilic compounds. The authors highlighted the high oxidative stability of the pâté, even if the high moisture content could be a serious technological processing problem for long-term storage of this by-product.

The aim of the present work was to characterize pâté samples obtained from different crop seasons from the Leopard decanter during the production of VOO. The samples collected over four years (2013–2016) from two productive mills were characterized in terms of phenolic compounds, and the proximate composition was determined for a subset of these. A further aim was to apply drying technologies to evaluate the shelf life of the pâté. The thermal effects of an industrial spray-drying process, as well as the changes of the phenolic profile of several lyophilized pâté samples during storage in different conditions have been evaluated by HPLC-DAD-MS-TOF. Finally, the antiaging effect of a phenolic extract from this pâté was carried out in cultured human fibroblasts, a well-known and widely applied model of cell senescence, and the results compared with those obtained with hydroxytyrosol used as reference compound.

2. Materials and methods

2.1. Chemicals

All chemicals for analysis were of analytical grade. Formic acid, methanol and hexane were from Sigma Aldrich (Steinheim, Germany). Acetonitrile of HPLC grade was from Panreac (Barcelona, Spain). The Milli-Q-system (Millipore SA, Molsheim, France) was used to produce deionized water. Syringic acid from Sigma Aldrich (Steinheim, Germany) was used as internal standard; caffeic acid and tyrosol from Sigma Aldrich (Steinheim, Germany) and oleuropein from Extrasynthese (Genay, France) were used as external standard. Stock solution of all the standards were prepared in hydroalcoholic solution. Sulfuric acid (96%, Sigma Aldrich, Steinheim, Germany) was used to prepare 1 M sulphuric acid.

2.2. Samples and sample preparation

Pâté samples of typical Tuscan cultivars (Frantoio, Moraiolo and Leccino) from two olive-mills located in two different provinces of Italy (Ancona and Livorno) were collected during the four crop seasons 2013–2016. In particular, the 2013 and 2014 samples were collected from Monteschiavo (M13 and M14) olive-mill (Maiolati Spontini, Ancona, Latitude: 43.509164; Longitude: 13.166397) and obtained by processing an olive batch (about 0.5 ton) of Leccino and Frantoio as prevalent cultivars. The 2015 and 2016 samples were collected from Terre dell'Etruria (TE 15 and TE16) olive-mill (Castagneto Carducci, Livorno, Latitude: 43.166798; Longitude: 10.580778) and obtained by processing olives from Frantoio and Moraiolo as prevalent cultivar (each close to 0.5 ton).

Samples were divided into several aliquots: one was immediately freeze-dried (FD), one was immediately frozen and then stored at −22 °C for four months before the freeze-drying process (SFD), and other aliquots were stored for one, two or four months at room temperature, i.e. in non-fully controlled conditions, and then freeze-dried (1FD, 2FD, 4FD). For the pâté of the 2013 campaign, a further sample was immediately frozen, stored at −22 °C for four months and then dried using a spray-drier (M13-SD). All the analyzed samples are summarized in Table 1.

2.3. Phenolic compounds

2.3.1. Extraction

All the samples listed in Table 1 and quantified using the external standard (ES) or the internal standard (IS) methods were extracted in triplicate. The phenolic extracts from the 2013 pâté were obtained as follows: 1 g dried sample was extracted with 2 × 10 mL of EtOH:H_2O 8:2 v/v, under magnetic stirring for 2 h, and then filtered. The obtained extract was defatted with *n*-hexane, dried under vacuum (−0.1 MPa) at 40 °C for 1 h on a Rotavapor® R-100 (from Büchi), re-dissolved in an exact volume of the same extractive mixture, and then analyzed by HPLC-DAD-MS-TOF.

The following procedure was applied for the IS method: 3 g of dried sample were extracted twice with 35 mL of EtOH:H_2O 8:2 v/v, in presence of 0.5 mL of internal standard solution (syringic acid, 3.25 mg/mL); each extraction was carried out under magnetic stirring for 1 h. The obtained mixture was centrifuged for 10 min at 10 °C and 5000 rpm, then the supernatant was defatted twice with hexane (35 mL), dried under vacuum at 40 °C for 1 h and re-dissolved in 1.5 mL of the extractive solution. An aliquot of this solution was centrifuged for 4 min at room temperature (RT) and 14,000 rpm and immediately used for the chromatographic analysis.

2.3.2. Hydrolysis of the hydroalcoholic extracts

The hydroalcoholic extracts, obtained from the immediately freeze-drying pâté samples, were submitted to a hydrolytic process in acidic

Table 1
List of the analyzed pâté samples.

Sample	Year	Origin	Storage treatment	Analysis conditions	Quantitation
M13-FD	2013	Monteschiavo (AN)	Freeze-dried	LiChrosorb RP-18 (Merck)	ES method
M13-S4FD	2013	Monteschiavo (AN)	Stored at RT for 4 months + freeze-dried	LiChrosorb RP-18 (Merck)	ES method
M13-SFD	2013	Monteschiavo (AN)	Stored at −22 °C for 4 months + freeze-dried	LiChrosorb RP-18 (Merck)	ES method
M13-SD	2013	Monteschiavo (AN)	STORED at −22 °C for 4 months + spray-dried	LiChrosorb RP-18 (Merck)	ES method
M13-FD	2013	Monteschiavo (AN)	freeze-dried	Poroshell 120 EC-C18 (Agilent Tech)	IS method
M14-FD	2014	Monteschiavo (AN)	Freeze-dried	Poroshell 120 EC-C18 (Agilent Tech)	IS method
TE15-FD	2015	Terre dell'Etruria (LI)	Freeze-dried	Poroshell 120 EC-C18 (Agilent Tech)	IS method
TE15-S4FD	2015	Terre dell'Etruria (LI)	Stored at RT for 4 months + freeze-dried	Poroshell 120 EC-C18 (Agilent Tech)	IS method
TE16-FD	2016	Terre dell'Etruria (LI)	Freeze-dried	Poroshell 120 EC-C18 (Agilent Tech)	IS method
TE16-S1FD	2016	Terre dell'Etruria (LI)	Stored at RT for 1 month + freeze-dried	Poroshell 120 EC-C18 (Agilent Tech)	IS method
TE16-S2FD	2016	Terre dell'Etruria (LI)	Stored at RT for 2 months + freeze-dried	Poroshell 120 EC-C18 (Agilent Tech)	IS method
TE16-S4FD	2016	Terre dell'Etruria (LI)	Stored at RT for 4 months + freeze-dried	Poroshell 120 EC-C18 (Agilent Tech)	IS method

ES, external standard; IS, internal standard; RT, room temperature.

medium. This method was previously proposed to evaluate the total content of free and bound tyrosol and hydroxytyrosol in VOO (Mulinacci et al., 2006). Briefly, 300 µL of hydroalcoholic extract were treated with 300 µL of H_2SO_4 1 M for 2 h at 80 °C in a vial for HPLC. Then, 400 µL of EtOH were added, the obtained sample was centrifuged at 14,000 rpm for 5 min and the solution was immediately used for the chromatographic analysis.

2.3.3. Identification and quantification

The analysis was carried out using a HP 1200 liquid chromatograph equipped with a DAD detector coupled to a TOF-MS with an ESI interface (all from Agilent Technologies), under the following conditions: gas temperature 300 °C, nitrogen flow rate 12 L/min, nebulizer pressure 20 psi, capillary voltage 3800 V. The mass spectra were acquired in the *m/z* range 100–1000 Th in negative ion mode, setting the fragmentation energy between 80 and 180 V. Data acquisition and evaluation were performed using MassHunter software: acquisition module (Acq) B.05.01, qualitative analysis module (Qual) B.06.00.

The column LiChrosorb RP-18 250 × 4.6 mm (5 µm) (Merck) was used to analyze the extracts obtained from the pâté 2013. The eluents were H_2O at pH 3.2 by formic acid (A) and acetonitrile (B) and the analyses were carried out applying the following multistep gradient: from 100% A to 89% A in 23 min followed by a 10 min plateau; 8 min to 87% A followed by a 4 min plateau; 10 min to 80% A and a 13 min plateau; 2 min to 75% A and a 5 min plateau; 10 min to 65% A and a 3 min plateau; 8 min to 55% A and a 3 min plateau; to 100% B within 4 min, and a final plateau of 7 min. Total time of analysis 117 min; oven temperature 26 °C; flow rate 0.8 mL min^{-1}.

A Poroshell 120 EC-C18 150 × 3 mm, 2.7 µm (Agilent Technologies) column was used to analyze the samples listed in Table 1. A six-step linear solvent gradient was used, starting from 95% H_2O adjusted to pH 3.2 by formic acid (A) up to 100% acetonitrile (B), as follows: from 95% A to 60% A in 40 min followed by a 5 min plateau; to 100% B within 5 min and a final plateau of 3 min. Total time of analysis 53 min, flow rate 0.4 mL min^{-1}.

The quantitative evaluation of all the 2013 samples was performed through the use of three external standards, each with a five-point regression curve: tyrosol ($Y = 1548.2 * X + 6.7$; $R^2 \geq 0.999$), oleuropein ($Y = 531.8 * X + 18.3$; $R^2 \geq 0.999$) and caffeic acid ($Y = 12514.0 * X + 33.3$; $R^2 \geq 0.999$), according to our previous works (Oliveras-López et al., 2007; Romani et al., 2007). Tyrosol and hydroxytyrosol derivatives were evaluated at 280 nm using tyrosol as reference; secoiridoids at 280 nm with oleuropein as standard; verbascoside and the cinnamoyl derivatives at 330 nm with caffeic acid as surrogate standard.

The immediately freeze-dried 2013 sample, the samples from 2014, 2015 and 2016 crop season, and all the samples obtained with the acidic hydrolytic protocol (Section 2.4), were quantified applying the internal standard method and following the same approach used by the official International Olive Council method for the analysis of olive oil biophenols (COI/T.20/Doc No 29, 2009). Syringic acid was used as internal standard, and tyrosol as external standard; consequently, quantitative data were expressed as mg_{tyr}/kg of dried pâté.

2.4. Proximate composition and dietary fiber analyses

The proximate composition was evaluated for the two samples collected from Terre dell'Etruria in 2015 and 2016. The fat content was gravimetrically determined after Soxhlet extraction, according to the ISS protocol (ISS., 1996). Proteins were evaluated using the Kjeldhal method and applying the formula: (g/100 g) = N * 6.25, where N is total nitrogen. Dietary fiber content was determined according to the AOAC method 991.43 (AOAC., 1995).

2.5. Biological assays in human fibroblasts

For these tests, the hydroalcoholic extract obtained from the 2013 pâté (freeze-dried within 3 days, M13-FD) was used. This extract, analyzed by the external standard method (Table 1) contained 0.58 mg/mL hydroxytyrosol (corresponding to 3.8 mM) and 5.4 mg/mL total phenolic compounds.

2.5.1. Cell cultures and experimental conditions

Neonatal Human Dermal Fibroblasts are a primary cell line derived from neonatal human dermal tissue (NHDFs Clonetics, Lonza). They were maintained in Dulbecco's modified Eagle's medium (DMEM, Lonza) supplemented with 10% FBS (Gibco, NY), 100 units/mL of penicillin G, 0.1 mg/ml streptomycin and 2 mM glutamine (PAN-Biotech GmbH, Germany), at 37 °C in a humidified incubator containing 5% CO_2. Culture medium was changed every 2–3 days until cells reached 90–95% confluence. They were then sub-cultivated by trypsinization (Trypsin-EDTA 1x in PBS, Euroclone) and the attained population doubling level (PDL) was calculated according to the equation: PDL = 3.32 × logN/No (where N and No are the recovered and seeded cell numbers, respectively). The experiments were conducted starting from pre-senescent (PDL = 24) to senescent fibroblasts (PDL = 35), as described previously (Menicacci, Cipriani, Margheri, Mocali, & Giovannelli, 2017).

For short-term viability assay, NHDFs cells were seeded into 96-well plates (3000 cells per well) and grown for 72 h in the presence of M13-FD at three different dilutions: 1:100, 1:1000, 1:10,000. Control cultures were treated with ethanol 0.8%, i.e. the concentration corresponding to the lower dilution, or DMEM only.

Cell viability was then assessed by means of MTS cytotoxicity assay (see below). Cytotoxicity was evaluated by measuring the absorbance in two independent experiments and data were expressed as percentage of

control.

For long-term experiments, cell cultures were treated continuously with M13-FD 1:1000, 1:5000, 1:10,000 or 1 μM hydroxytyrosol until senescence. The culture medium was replaced every 2 days to maintain the treatment concentration relatively constant over time. At each passage, the number of cells recovered after trypsinization for each treatment was measured in a Burker chamber to evaluate cell growth over time.

2.5.2. MTS viability assay

The Cell Titer 96 Non-Radioactive Cell Proliferation Assay Kit (Promega) was used. The assay is based on the bioreduction of MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium:inner salt] into formazan by NADH and NADPH produced by dehydrogenase enzymes only in active and viable cells. The reagent was added to each well and 96-well plates were incubated at 37 °C in humidified 5% CO_2 atmosphere until color development had occurred (1–2 h). The formation of a purple formazan product was then measured spectrophotometrically at 490 nm. The measured absorbance value is a function of the amount of formazan produced and is proportional to the number of viable cells.

2.5.3. SA-β-galactosidase assay

The senescence-associated β-galactosidase (SA-β-gal) assay is designed to detect β-galactosidase activity at pH 6, typically expressed in senescent cells. Cells were washed twice in phosphate- buffered saline (PBS) and fixed for 5 min at room temperature in 2% formaldehyde/0.2% glutaraldehyde, washed twice in PBS and then incubated at 37 °C, with fresh SA-β-gal staining solution (1 mg/mL 5-bromo-4-chloro-3-indolyl-D-galactoside, 40 mmol/L citric acid/sodium phosphate dibasic at pH 6, 150 mmol/L NaCl, 2 mmol/L $MgCl_2$, 5 mmol/L $K_3[Fe(CN)_6]$ and 5 mmol/L $K_4[Fe(CN_6)]3H_2O$). Staining was evident in 2–4 h and maximal in 12–16 h. The next day hematoxylin was used to counterstain the cells. Cells were finally manually counted to determine the percentage of SA-β-gal positive cells over the total.

2.5.4. LDH release

Cell damage was evaluated by the lactate dehydrogenase (LDH) release assay. LDH is a stable cytoplasmic enzyme present in all cells and rapidly released into the cell culture supernatant upon damage of the plasma membrane. LDH activity was determined in an enzymatic test (Cytotoxicity Detection Kit, Roche), based on the reduction of NAD^+ to $NADH/H^+$ by the LDH-catalyzed conversion of lactate to pyruvate, and on the further diaphorase/$NADH/H^+$-mediated reduction of the tetrazolium salt INT to formazan.

NHDFs were seeded into 96-well plates (3000 cells per well). The next day, DMEM was replaced in each well with 200 μL of red phenol-free DMEM containing the different treatments. After 24 h the medium was withdrawn, centrifuged at 250 RCF for 5 min, and the supernatant used for enzyme assay. To determine LDH activity in 100 μL of supernatant, 100 μL of freshly prepared Reaction Mixture were added to each well and incubated for up to 30 min at room temperature. Finally, the absorbance at 490–492 nm was measured spectrophotometrically.

2.5.5. Western blot analysis

After washing the wells with PBS, cellular proteins were extracted in RIPA buffer [50 mm Tris–Cl (pH 7.5), 150 mm NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS] containing 1% protease and phosphatase inhibitor Cocktail (Sigma–Aldrich Chemicals) with the aid of a cell scraper. Cell lysates were then sonicated (Microson XL 2000; Misonix, Farmingdale, NY, USA), clarified by centrifugation and supernatants collected and stored at − 20 °C. Protein content was measured by using the Bio-Rad DC protein assay kit (Bio-Rad).

Forty-fifty micrograms of proteins for each sample were subjected to 4–12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis separation (Bis-Tris Plus BOLT, Invitrogen, Thermo Fisher Scientific, San Giuliano Milanese, Italy) and transferred to polyvinylidene fluoride membranes ([PVDF] Millipore, Billerica, MA, USA). Total amounts of p16 and GAPDH protein (the latter used as loading control) were determined by immunostaining with primary rabbit polyclonal antibodies: anti p16 (N-20), sc-467 (Santa Cruz Biotechnology); anti-GAPDH, 14C10 (Cell Signaling Technology) and suitable peroxidase-conjugated secondary antibodies (Sigma–Aldrich Chemicals Co.). Proteins were visualized using the enhanced chemiluminescence procedure with Immobilon Horseradish Peroxidase Substrate (Millipore) and immune-reactive bands were quantified by densitometric analysis using the Quantity-One software (Bio-Rad Laboratories). Each density measure was normalized by using the corresponding GAPDH level as an internal control.

2.6. Statistical analysis

All the quantitative data were expressed as the mean ± standard error. Statistical analyses of data were performed by one-way ANOVA and F-Test ($P < .05$); Fisher's LSD test was then applied to data on phenolic composition and Bonferroni's post-hoc test to data from biological assays (DSAASTAT software v. 1.1).

3. Results and discussion

Differently from wastewaters or solid olive residue (pomace), pâté is a new olive by-product of particular interest in that it is recovered during and not after the milling process of an edible fruit by an exclusively mechanical treatment. To the best of our knowledge, only one recent publication is focused on the composition of this by-product (Lozano-Sánchez et al., 2017).

One main goal of the present work was the evaluation of content and stability of the phenolic fraction in pâté after drying treatments and after different storage times. The dried pâté appears homogeneous, devoid of woody parts and non-hygroscopic also after several months of storage (Supplementary 1). To date, we know that the phenolic fraction extracted from olive into olive oil never exceeds 2%, remaining almost completely in the milling by-products (Jerman Klen, Golc Wondra, Vrhovšek, Sivilotti, & Vodopivec, 2015). However, consumption of table olives does not guarantee a significant intake of these phenolic compounds, as they are almost completely degraded during the common industrial processes used to remove olive bitterness.

In this context, we aimed at evaluating the possibility of using the dried pâté as a natural ingredient for formulating innovative foods or food supplements to improve the daily intake of these bioactive natural compounds, thus adding value to this by-product. First, several samples were collected starting from 2013 campaign to evaluate the composition of the pâté. Successively, the phenolic content was determined in other pâté samples harvested over four years in two productive mills (Table 1).

The water content of all samples ranged between 78 and 80%, in agreement with recent data on a pâté recovered in Italy during the 2015 season (Lozano-Sánchez et al., 2017). As the pâté production is concentrated in a few months and in view of its high water content, we decided to investigate the effect of a room temperature (18–20 °C) storage of the fresh pâté in plastic closed tanks for several months. This model was chosen to simulate a simple storage process, directly applicable in the mill, and aimed to maintain the fresh pâté before the drying process for several weeks. The freeze-drying procedure was chosen as an elective method to stabilize the fresh pâté samples over time, and all the phenolic extracts were derived from the treatment of these dried samples.

The phenolic compounds were identified by HPLC-DAD-MS-TOF studying the typical profiles at 280 nm and 330 nm. The detected molecules are those typical of olive oil and olive fruit and already known as derived by *Olea europaea* L. Two different quantitative approaches were applied as discussed below and summarized in Tables 1, 2 and

Table 2
Phenolic content of pâté samples quantified by the internal standard method according to the IOC protocol for biophenols from olive oils; data are the mean (SD) of three independent measurements, expressed as mg_{tyr}/kg on dry matter basis. In each column, different letters indicate significant differences at $p < 5\%$. M, mill of Monteschiavo; TE, mill of Terre dell'Etruria; FD, freeze-dried; S1FD, S2FD, S4FD, freeze-dried after 1, 2, 4 months of storage in non-controlled conditions.

Sample	Hydroxytyrosol									Tyrosol								
	Free			Glucoside			Total			Free			Glucoside			Total		
M13-FD	12,074	(156)	B	1394	(62)	E	13,468	(218)	C	383	(10)	B	1336	(18)	F	1719	(28)	C
M14-FD	2883	(43)	A	371	(17)	A	3253	(60)	A	85	(8)	A	85	(8)	A	170	(16)	A
TE15-FD	4103	(131)	A	1532	(68)	E	5635	(199)	B	382	(5)	B	844	(24)	E	1226	(29)	B
TE15-S4FD	21,499	(2888)	D	1682	(203)	F	23,181	(3090)	E	3175	(373)	G	770	(68)	D	3945	(441)	F
TE16-FD	11,317	(415)	B	844	(36)	C	12,161	(451)	C	840	(23)	C	710	(21)	D	1550	(44)	C
TE16-S1FD	12,383	(154)	B	1056	(79)	D	13,438	(233)	C	1814	(42)	D	594	(64)	C	2408	(107)	D
TE16-S2FD	15,130	(414)	C	1011	(25)	D	16,141	(440)	D	2457	(58)	E	270	(9)	B	2727	(67)	E
TE16-S4FD	16,886	(319)	C	565	(12)	B	17,451	(331)	D	2847	(60)	F	62	(2)	A	2909	(63)	E

Sample	Verbascoside			Caffeic acid			β-OH acteoside 1			β-OH acteoside 2			Luteolin			Oleuropein derivatives			Total phenols		
M13-FD	3380	(15)	C	90	(3)	A	292	(12)	D	343	(8)	C	456	(8)	A	9200	(186)	D	49,550	(421)	BCD
M14-FD	118	(13)	A	nd	49	(7)	A	69	(11)	AB	1203	(43)	B	2767	(79)	A	27,695	(524)	A		
TE15-FD	6702	(168)	E	219	(30)	A	1272	(16)	F	1405	(43)	D	1020	(102)	B	13,232	(357)	E	52,969	(2019)	CD
TE15-S4FD	5006	(914)	D	4038	(502)	C	1616	(86)	G	1812	(134)	E	3234	(530)	D	3695	(571)	B	75,748	(6276)	E
TE16-FD	2509	(89)	B	3070	(75)	B	420	(4)	E	310	(6)	C	1186	(111)	B	5225	(214)	C	45,573	(1525)	B
TE16-S1FD	546	(22)	A	4982	(69)	D	202	(11)	C	151	(12)	B	1685	(49)	C	3514	(366)	B	48,507	(1154)	BC
TE16-S2FD	379	(17)	A	5409	(156)	E	122	(12)	B	97	(15)	AB	1650	(54)	C	3474	(87)	B	52,367	(923)	CD
TE16-S4FD	154	(10)	A	4907	(121)	D	44	(6)	A	33	(9)	A	1703	(40)	C	3550	(81)	B	51,123	(850)	CD

Supplementary 2.

3.1. Preliminary evaluation on pâté 2013

The 2013 pâté was used for a preliminary screening primarily focused on evaluating the suitability of the sample to be dried by an industrial spray drier, widely used for treating liquid and semi-liquid formulations for foods or pharmaceutical products. At the same time, we investigated the changes in the phenolic content and phenolic profile of the fresh pâté after a four months storage in plastic tank. The antiaging efficacy of the phenolic extract from this pâté was then evaluated choosing cultured human fibroblasts as a suitable cellular model to study the modulation of senescence.

Firstly, we used an analytical method previously applied by our research group to determine the phenolic compounds in EVOOs (Mulinacci et al., 2013; Oliveras-López et al., 2007; Oliveras Lopez et al., 2008; Romani et al., 2007). This method was recognized as particularly suitable to investigate the composition of the secoiridoid fraction, mainly constituted by oleuropein derivatives. These molecules are known to be sensitive to oxygen during the milling process and consequently the successive management of the fresh pâté can affect the phenolic concentration. Fig. 1 shows the effect of the different storage treatments on the phenolic content of pâté 2013. The principal components of this by-product were hydroxytyrosol, tyrosol, dialdehydic form of elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA) and verbascoside. As expected, the storage for four months at −22 °C of the fresh pâté before the freeze-drying process (M13-SFD) did not modify the phenolic profiles. On the opposite, the storage in non-controlled conditions at room temperature (18–20 °C) for the same amount of time induced an almost complete hydrolysis (presumably enzyme-mediated) of the oleuropein derivatives, with a corresponding increase of free tyrosol and hydroxytyrosol (M13-S4FD). Upon spray-drying of pâté (over 100 °C for a few seconds) after addition of maltodextrins, the phenolic fraction was stable (data not shown); the final sample appeared as a fine and non-hygroscopic powder. Nevertheless, the high viscosity of the pâté resulted to be the limiting factor to apply the spray-dryer technology on a larger scale to dry the fresh pâté, because during the process the small holes of the spray-cone were partially or completely obstructed. Nevertheless, the test allowed verifying that the olive phenolic compounds are not too sensitive to thermal degradation up to around 100 °C, and this information can be useful for further studies aiming to find sustainable and alternative drying processes to treat the fresh pâté.

3.2. Phenolic profile in pâté samples over years

After the first evaluation of pâté 2013, other samples derived from different cultivars and two different geographical areas were collected during the years 2014–2016. To carry out this comparative analysis of the phenolic profiles, a more efficient method, based on a next generation column (Poroshell 120 EC-C18 Agilent Technologies) was applied. This approach allowed to strongly reduce the required time for analysis and the costs, improving the efficiency of the chromatographic separation. All the extracts from the pâté samples harvested during the four years were quantified using the internal standard method, according to the IOC protocol for biophenols in olive oils, with the final data expressed as mg_{tyr}/kg dried pâté. Chromatographic profiles at 280 nm of the hydroalcoholic extracts of pâté 2015 are compared in Fig. 2, while the quantitative results are summarized in Table 2.

HPLC-DAD-MS-TOF allowed to detect 21 phenolic compounds, some of which previously described in olive, olive oil and other olive oil waste (Kanakis et al., 2013; Rubio-Senent, Rodríguez-Gutiérrez, Lama-Muñoz, & Fernández-Bolaños, 2013). The analyses showed that pâté contains appreciable amounts of phenolic compounds as reported in other studies (Lozano-Sanchez et al., 2011; Lozano-Sánchez et al., 2017). As expected, the lowest value was reported for the extract from pâté 2014 (M14-FD sample) deriving from olives of the 2014 campaign, severely damaged by *Bactrocera oleae* infestation (Cecchi, Migliorini, Cherubini, Trapani, & Zanoni, 2016).

Considering the samples freeze-dried within 3 days (FD), the total phenolic content ranged from 45,573 (TE16-FD sample) to 52,969 (TE15-FD sample) mg_{tyr}/kg dried matter basis (DM), with a comparable content in pâté from the same mill. Hydroxytyrosol was the most abundant phenol for the extracts from 2013, 2014 and 2016 campaigns while for the year 2015 the major compounds were the oleuropein derivatives.

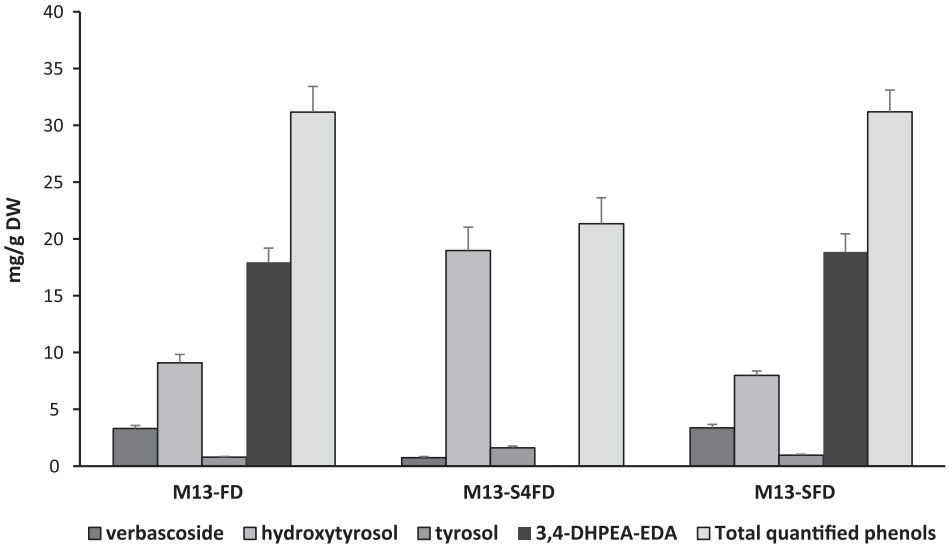

Fig. 1. Effect of different storage treatments on the phenolic content of pâté 2013; data are expressed in mg/g dried weight (DW) as a mean of three independent analysis. **M13-FD** freeze-dried within 3 days; **M13-S4FD**, freeze-dried after 4 months of storage in not controlled conditions; **M13-SFD**, freeze-dried after 4 months of storage at −22 °C.

The effect of four months storage in non-fully controlled conditions (10–20 °C in plastic tank) on the phenolic content was also evaluated for the 2015 sample. As showed in Table 2 and Fig. 2, the phenolic profile varied, with hydroxytyrosol and tyrosol considerably increased. Regarding hydroxytyrosol (free + glucoside) the amount ranged from 5635 to 23,181 mg_{tyr}/kg DM. At the same time, the verbascoside and the oleuropein derivatives showed a reduction of their content after the four months of storage.

Finally, for the samples from 2016, the study was carried out considering the effect of storage at 18–20 °C also for shorter times (one and two months). As expected, the trend observed for the 2015 sample was confirmed: the amount of hydroxytyrosol and tyrosol increased gradually and proportionally to the storage time; verbascoside content again showed a drastic reduction, from 2509 to 154 mg_{tyr}/kg DM; oleuropein derivatives content decreased from 5225 to 3550 mg_{tyr}/kg DM. The decrease of verbascoside and oleuropein derivatives is associated to increase in hydroxytyrosol, the main product of the spontaneous hydrolysis, taking place during the fresh pâté storage. During the four months of storage, no apparent fermentative process was noticed, and the smell of samples remained similar to that of fresh pâté. Nevertheless, further studies will need to further explore this aspect. To complete and simplify the analytical determination, an acid hydrolysis was applied on the phenolic extracts of the four pâté collected in this study (Table 3), aimed to evaluate the actual hydroxytyrosol and tyrosol content, including both the free forms and those linked to the secoiridoid nucleus. This approach, previously proposed for the determination of these phenolic compounds in EVOOs (Mulinacci et al., 2006), allows to simplify their determination avoiding

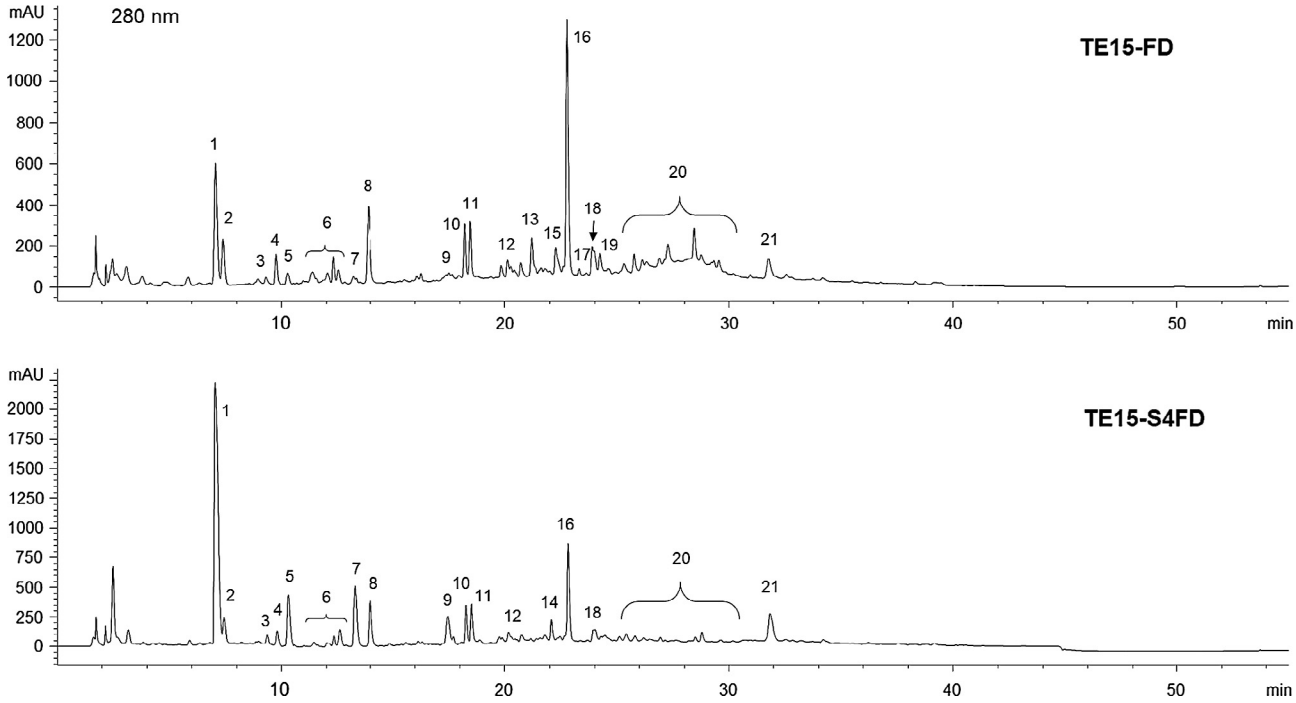

Fig. 2. Comparison of the phenolic profile at 280 nm of the pâté 2015 (TE15-FD) and the corresponding sample maintained for 4 months at 18–20 °C (TE15-S4FD). 1, hydroxytyrosol; 2, hydroxytyrosyl glucoside; 3, unknown 1; 4, tyrosyl glucoside; 5, tyrosol; 6, unknown 2; 7, caffeic acid; 8, syringic acid (internal standard); 9, p-coumaroyl derivative; 10, β-OH acteoside 1; 11, β-OH acteoside 2; 12, flavonoid 1; 13, flavonoid 2; 14, caffeoyl derivative 1; 15, flavonoid 3; 16, verbascoside; 17, caffeoyl derivative 2; 18, flavonoid 4; 19, caffeoyl derivative 3; 20, oleuropein derivatives; 21, luteolin.

Table 3
Quantitation of total tyrosol and hydroxytyrosol (free and bound forms) after acid hydrolysis. Data are the mean (SD) of three independent measurements, expressed as mg_{tyr}/kg on dry matter basis. In each column, different letters indicate significant differences at $p < 5\%$.

Sample	Hydroxytyrosol (mg/Kg)			Tyrosol (mg/Kg)			Sum of tyr + OH-tyr (mg/Kg)		
M13-FD	29,145	(316)	D	4283	(66)	C	33,428	(382)	D
M14-FD	5920	(86)	A	569	(28)	A	6489	(114)	A
TE15-FD	23,667	(196)	C	5522	(81)	D	29,189	(277)	C
TE16-FD	18,867	(157)	B	4023	(73)	B	22,890	(230)	B

underestimations of the minor derivatives; only one peak for tyrosol and one for hydroxytyrosol were obtained after the hydrolysis. The total amount of both these phenolic compunds is considerably lower if determined at the start of the storage (Table 2, t0) without applying the acid hydrolysis (Table 3). When comparing the samples of 2015 and 2016, similar results were obtained. The content of hydroxytyrosol after four months of storage at 18–20 °C was almost the same obtained after the acidic hydrolysis of the phenolic fraction of 2015 pâté, showing values of 23,181 ± 3090 mg_{tyr}/kg DM and 23,667 ± 196 mg_{tyr}/kg DM, respectively. Similarly, for the 2016 pâté the hydroxytyrosol amount in TE16-S4FD was 17,451 ± 331 mg_{tyr}/kg DM, close to the value in the hydrolyzed extract (TE16-FD): 18,827 ± 157 mg_{tyr}/kg DM.

Regarding the tyrosol content in pâté of 2015 and 2016, the amounts evaluated after the hydrolysis were consistently higher (+28%) of those measured in the samples after the four month-storage without hydrolysis. This discrepancy can be attributed to underestimation of tyrosol derivatives present in lower concentration in the extract with respect to hydroxytyrosol derivatives.

In view of a possible use of the pâté for human consumption, we carried out the proximate analysis on the pâté samples recovered in 2015 and 2016 in Tuscany: the amount of fat and proteins was similar (from 10 to 15%) and the dietary fiber content was close to 50% of the dry pâté, with a 6% of fermentable fiber, in both the 2015 and 2016 samples.

This dry pâté was stable over time, as confirmed by the analysis of TE15-FD after 12 months of storage in a dark closed bottle at room temperature (Supplementary 3): the total phenolic content was the same than t_0. This finding confirms that the dried pâté can be proposed as an additional source of the olive bioactive phenolic compounds, particularly of free and bound hydroxytyrosol. It is worth noting that the total phenolic compounds in 1 g of dry 2015 pâté (evaluated by internal standard method, according to the IOC method for olive oils) can guarantee a daily intake close to 40 mg of total phenolic compounds, of which 25 mg of hydroxytyrosol, i.e. an amount comparable to that derived by the daily consumption of 200 g of a EVOO with a total phenolic content close to 200 mg/kg.

3.3. Toxicity testing and anti-aging activity in human fibroblasts

3.3.1. Short term toxicity

To define the working dilutions of the M13-FD extract, we first carried out short-term toxicity experiments evaluating cell viability with the MTS method. The results showed that the 1:100 dilution reduced cell viability upon 72 h incubation and was discarded, whereas the 1:1000 and 1:5000 dilutions were not toxic (Fig. 3). For the long term experiments, the 1:1000 and 1:5000 dilutions were used.

3.3.2. Long term effects on cell proliferation, survival, and markers of cell senescence

When cells were continuously treated with either M13-FD or hydroxytyrosol, the 1:1000 dilution of the extract showed slowing effects on cell proliferation, starting at about 10 days of treatment. These

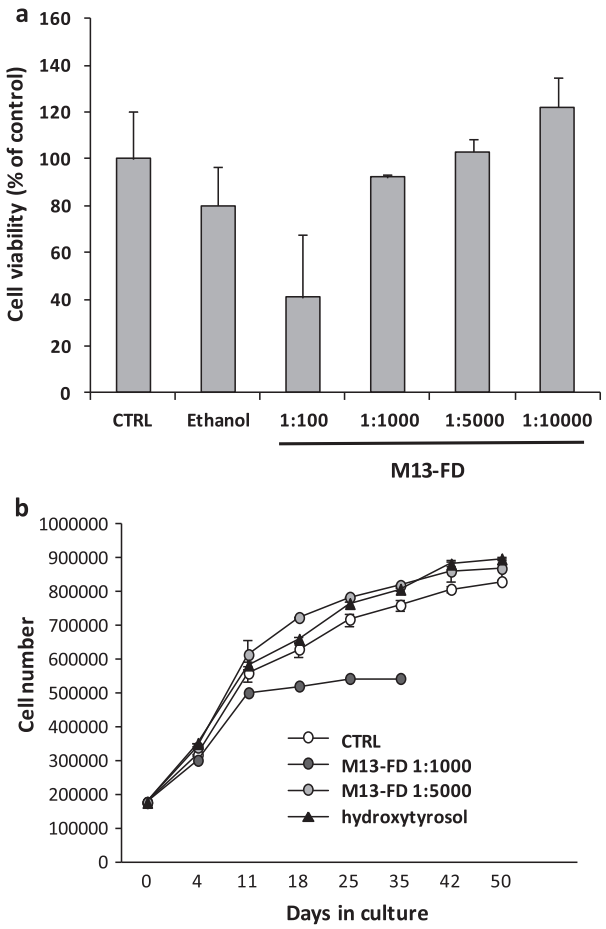

Fig. 3. Panel a: NHDF viability upon short-term (72 h) treatment with the indicated dilutions of the extract (M13-FD) and the vehicle (ethanol, 0.8%) measured with MTS. Data are expressed as the percentage absorbance value of the control, untreated fibroblasts (mean ± SE of three experiments). Panel b: Number of NHDFs recovered at each passage upon long-term treatment with the indicated dilutions of the extract (M13-FD) and hydroxytyrosol (1 μM). Data from one representative experiment (out of three experiments) are expressed as the mean ± SE of technical duplicates.

cultures were terminated at 35 days. On the contrary, both 1 μM hydroxytyrosol and 1:5000 M13-FD were able to increase the number of cells recovered at each passage, and although the differences were small the effect was statistically significant (Fig. 4).

This effect of hydroxytyrosol and M13-FD 1:5000 was paralleled by reduced LDH release measured at senescence, indicating that the two treatments strongly reduced cell death at this time point (Fig. 4).

The expression of the senescence marker protein p16, involved in cell cycle arrest at senescence, was found increased at the end of the experiment as expected, and strongly reduced by the treatment with both hydroxytyrosol and M13-FD 1:5000 (Fig. 4C). Finally, the expression of the enzymatic activity of SA-β-gal was markedly increased in senescent fibroblasts, and this effect was counteracted by the two treatments, which brought about a 30% reduction of the percentage of positive cells at the end of the treatment (Fig. 4B, D, E).

Testing the antiaging activity of a compound *in vivo* involves time- and animal consuming complex experiments in which specific functions affected by age, such as cognitive, motor, autonomous or metabolic, are measured. The aging process *per se* can be investigated at the cellular level in a widely used simple *in vitro* model, that has repeatedly shown predictive value towards *in vivo* effects: the senescent primary cell culture. Cellular senescence is characterized by complex modifications in the protein expression profile of the cell, leading to replicative arrest and cell dysfunction. Senescent human fibroblasts, the most widely used cell type in this context, can be protected against replicative

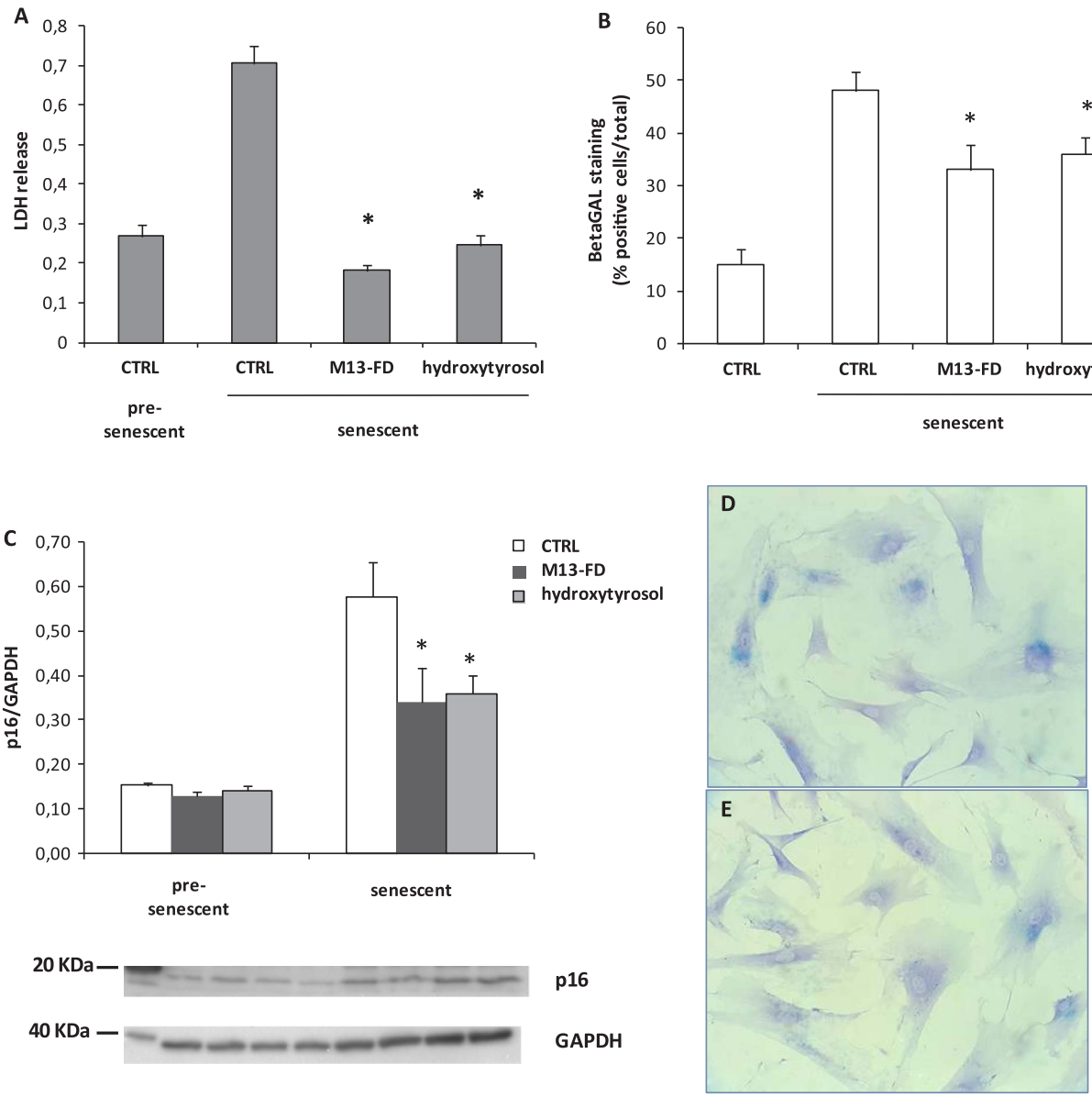

Fig. 4. (A) LDH release in the NHDF culture medium at the beginning and at the end of long-term treatment with the 1:5000 dilution of the extract (M13-FD) and hydroxytyrosol (1 μM). **(B)** SA-β-gal staining in NHDFs at the beginning and at the end of long-term treatment with the 1:5000 dilution of the extract (M13-FD) and hydroxytyrosol (1 μM): cell counts. **C**: P16 protein expression evaluated by western blot at the beginning and at the end of long-term treatment with the 1:5000 dilution of the extract (M13-FD) and hydroxytyrosol (1 μM). Representative images of the blotted membranes are shown below the graph. Data are the mean ± SE of three experiments. $^{*}p < .05$ statistically significant difference from senescent control fibroblasts. (D and E) representative microphotographs of senescent control (D) and M13-FD-treated (E) NHDFs.

senescence upon long-term treatment with natural compounds such as resveratrol (Giovannelli et al., 2011) and epigallocatechingallate (Han et al., 2012) as well as different plant extracts (Ding et al., 2017); among the latter, an extract from *Olea europaea* delayed senescence in IMR90 and WI38 human fibroblasts (Katsiki, Chondrogianni, Chinou, Rivett, & Gonos, 2007). Hydroxytyrosol (1 μM) has been shown to lengthen the chronological lifespan of cultured human fibroblasts (Sarsour et al., 2012). Recently, we have shown that 1 μM hydroxytyrosol is able to counteract replicative senescence and the associated inflammatory phenotype in long-term treated cultured human fibroblasts, showing reduced beta-galactosidase activity and p16 expression, along with enhanced cell protection (Menicacci et al., 2017). In the present work, we have evaluated the antiaging activity of the FD extract by comparison with 1 μM hydroxytyrosol in the same model of replicative senescence.

The final concentration of hydroxytyrosol in the 1:5000 dilution of the hydroalcoholic extract, effective in the antiaging tests, was 0.76 μM. This dilution induced the same protective effects than pure hydroxytyrosol at 1 μM concentration, reasonably due to the presence of other phenolic compounds in the extract. Thus, these experiments indicate that the extract is as active as the pure reference compound, hydroxytyrosol, in delaying the senescence process upon a long-term exposure.

4. Conclusions

In conclusion, we studied a particular two-phase olive by-product (pâté), obtained directly after the malaxation step and characterized by a wet homogeneous pulp free from residuals of kernel. Our data show that pâté is a convenient source of phenolic compounds and that the main components of this by-product are hydroxytyrosol, tyrosol, 3,4-DHPEA-EDA and verbascoside.

Regarding the storability of fresh pâté, storage in non-controlled conditions leads to a strong increase of hydroxytyrosol content over

time. After freeze-drying, the dry pâté is a homogeneous powder, that resulted stable for several months.

Biological assays carried out on a simple hydroalcoholic extract of pâté proved to have antiaging activity in cultured human cells upon long-term exposure, with a potency comparable to that of pure hydroxytyrosol. Thus, pâté can be proposed as an environment-friendly source of olive bioactive phenolic compounds, particularly of free and bound hydroxytyrosol: 1 g of pâté is able to provide a daily intake comparable to that derived from 200 g of an EVOO with a phenolic content of 200 mg/kg.

As a whole, this work lays the basis for the possibility of using this food by-product as a natural ingredient for formulating innovative foods or food supplements contributing to a healthier lifestyle.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jff.2017.12.018.

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