Lipophilization of hydroxytyrosol-enriched fractions from Olea europaea L. by-products and evaluation of the in vitro effects on a model of colorectal cancer cells

Journal:	*Journal of Agricultural and Food Chemistry*
Manuscript ID	jf-2016-05457p.R3
Manuscript Type:	Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Bernini, Roberta; University of Tuscia, Department of Agricultural and Forestry Sciences (DAFNE) Carastro, Isabella; Universita degli Studi della Tuscia Palmini, Gaia; University of Florence, Department of Surgery and Translational Medicine Tanini, Annalisa; University of Florence, Medicina Interna Zonefrati, Roberto ; University of Florence, Medicina Interna Pinelli, Patrizia; University of Florence, Department of Pharmaceutical Sciences Brandi, Maria; University of Florence, Medicina Interna Romani, Annalisa; University of Florence, Dipartment of Pharmaceutical Science

Lipophilization of Hydroxytyrosol-Enriched Fractions from *Olea europaea* L. By-Products and Evaluation of the in vitro Effects on a Model of Colorectal Cancer Cells

Roberta **Bernini**,*[§] Isabella **Carastro**,[§] Gaia **Palmini**,[†] Annalisa **Tanini**,[†] Roberto **Zonefrati**,[†] Patrizia **Pinelli**,[#] Maria Luisa **Brandi**,[†] Annalisa **Romani** [#]

[§] *Department of Agricultural and Forestry Sciences (DAFNE), University of Tuscia, Via S. Camillo de Lellis, 01100 Viterbo, Italy*

[†] *Department of Surgery and Translational Medicine, University of Florence, Florence, Italy*

[#] *Department of Statistics, Computing, Applications "G. Parenti" (DISIA), PHYTOLAB, University of Florence, Italy*

*** Corresponding author**

Roberta Bernini, Tel. +39 0761 357452; fax +39 0761 357242. E-mail: berninir@unitus.it

Title running header: Effects of Lipophilic Hydroxytyrosol-Enriched Fractions from *Olea europaea* L. by-products on Colorectal Cancer Cells

Dedicated to the memory of Carmela Spatafora

Abstract: A hydroxytyrosol (HTyr)-enriched fraction containing HTyr 6% w/w, derived from *Olea europaea* L. by-products, obtained using an environmentally and economically sustainable technology was lipophilized under green chemistry conditions. The effects of three fractions containing hydroxytyrosyl butanoate, octanoate and oleate and unreacted HTyr, named, respectively, lipophilic fraction **5**, **6** and **7**, on the human colon cancer cell line HCT8-β8 engineered to overexpress estrogen receptor β (ERβ) were evaluated and compared to those of pure HTyr. The experimental data demonstrated that HTyr and all fractions showed an anti-proliferative effect, as it has been observed by the evaluation of the cellular doubling time under these different conditions (mean control: 32 ± 4 h; HTyr **1**: 65 ± 9 h; fraction **5**: 64 ± 11 h; fraction **6**: 62 ± 14 h; fraction **7**: 133 ± 30 h). As evidenced, the fraction **7** containing hydroxytyrosyl oleate showed the highest activity. These results were related to the link with ER-β, which was assessed through simultaneous treatment with an inhibitor of ERβ.

Keywords: *Olea europaea* L. by-products, hydroxytyrosol (HTyr), lipophilization of extracts, hydroxytyrosyl esters, colorectal cancer (CRC).

INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies that affects both men and women.[1] Several epidemiological studies have demonstrated that environmental and dietary factors are responsible for the incidence of CRC in the population. In fact, a high number of cases were recorded in Northern Europe, although this number was significantly lower in the Mediterranean area.[2,3] These data were related to the diets adopted in these two distinct geographic areas: generally high in saturated fat, meat, and protein in the first and rich in vegetables, cereals and olive oil in the second.

Olive oil is a central component of the traditional Mediterranean diet, and daily consumption is associated with numerous beneficial effects on human health,[4-6] including a chemoprotective role against colon carcinogenesis.[7-11] Recent studies have evidenced that extra virgin olive oil extracts inhibit the cell proliferation of colon cancer cells by activating the estrogen receptor β (ERβ).[12,13] In fact, it has been demonstrated that cancerous progression in human colon mucosa is characterized by a decrease in ERβ expression. [14,15] The molecules responsible for the beneficial effects of olive oil extracts are simple phenolic compounds, including tyrosol, hydroxytyrosol (HTyr), ligstroside and oleuropein derivatives (**Figure 1**).[16]

Among them, HTyr, the hydrolysis product of oleuropein,[17] has recently received particular attention for a number of biological activities related to human health demonstrated through in vitro and in vivo experiments, related mainly to its high antioxidant activity.[18]

Despite these properties, HTyr shows a low bioavailability in a cellular environment because of its hydrophilic character, which limits its passage across cell membranes. This property, also common to phenolic acids, flavonoids and tocopherols, can be conveniently modified through lipophilization.[19] Lipophilization is a strategy that can be carried out by covalently introducing a hydrophobic moiety into these compounds to produce novel derivatives with enhanced biological properties. For example, lipophilic HTyr derivatives were synthesized through the selective carboxymethylation,[20,21] etherification, [22] esterification of HTyr[23] and conjugation with other

naturally occurring phenolic moieties.[24] These compounds exhibited antioxidant and antitumor activities;[25,26] recently, they have also been used as active ingredients for antioxidant food packaging.[27]

To the best of our knowledge, despite the studies performed on the biological activities of several pure lipophilic HTyr derivatives, the effects of lipophilized natural extracts containing HTyr so far have not yet been evaluated. In consideration of this lack in the literature, in this study we investigated the antiproliferative activities of a selected fraction derived from *Olea europaea* L. by-products (pitted olive pulp), enriched in HTyr,[28-30] appropriately lipophilized through an esterification reaction performed with butanoyl, octanoyl and oleoyl chloride using an eco-friendly procedure. The effects of these fractions were evaluated on the human colon cancer cell line HCT8-β8 engineered to overexpress estrogen receptor β (ERβ) to investigate their possible role against CRC.

MATERIALS AND METHODS

Chemicals. 2-Iodoxybenzoic acid, tyrosol, butanoyl chloride, octanoyl chloride, oleoyl chloride, fulvestrant (ICI 182,780), 17β-estradiol (17β-E2), dimethyl carbonate, ethyl acetate, dichloromethane, methanol, formic acid, acetonitrile, dimethyl sulfoxide, fetal bovine serum (FBS), sodium pyruvate, L-glutamine, penicillin, streptomycin, geneticin, phenol red, trypsin, ethylenediaminetetraacetic acid (EDTA), sodium chloride, anhydrous sodium sulfate, CD_3COCD_3 (99.8% in deuterium) or $CDCl_3$ (99.8% in deuterium) were purchased from Sigma-Aldrich (Milan, Italy) of high analytical grade. RPMI 1640 medium was furnished by Lonza Group (Basel, Switzerland). Silica gel 60 F254 plates and silica gel 60 were purchased from Merck (Milan, Italy).

Cell line. The human colon cancer HCT8 cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA).

Methods. 1-Hydroxy-1-oxo-1*H*-1λ^5-benz[*d*][1,2]iodoxol-3-one (2-iodoxybenzoic acid, IBX) was prepared before use according to the safe procedure reported in the literature.[31] Cells overexpressing

human ERβ (HCT8-β8) (**Figure 2**) were established via stable transfection with the mammalian expression vector pCXN2-hERβ.[32] 17β-E2 was used as an internal positive control and dissolved in ethanol; ICI 182,780, an inhibitor of ER-β, and the lipophilic fractions were solubilized in dimethyl sulfoxide. All solutions were diluted in cell culture media to their final concentrations before testing.

Instrumental analyses. A 400 MHz nuclear magnetic resonance spectrometer (Bruker) was used to record the ^{1}H- and ^{13}C-NMR spectra of the synthesized compounds, which had been previously solubilized in CD_3COCD_3 or $CDCl_3$. All chemical shifts were expressed in parts per million (δ scale) and referenced to either the residual protons or carbon atoms of the solvent. An HP 1200 liquid chromatograph equipped with a diode array detector (Agilent Technologies, Palo Alto, CA, USA) was used for the HPLC analyses of the HTyr-enriched fraction and lipophilic fractions using an analytical column (LiChrosorb RP-18, 250 x 4.60 mm, 5 μm i.d.; Merck Darmstadt, Germany) and a four-step linear solvent gradient starting from 100% H_2O adjusted to pH = 3.2 with HCOOH (solvent A) to 100% CH_3CN (solvent B) over 88 min at a flow rate of 0.8 mL min^{-1}. A Bürker hemocytometer was used for cell proliferation evaluation.

Synthesis of hydroxytyrosol (1). HTyr was synthesized through the IBX oxidation of tyrosol according to a previously optimized procedure.[33] The final product was isolated as a colorless oil. The spectroscopic data were in agreement with those already reported.[33]

Synthesis of hydroxytyrosyl butanoate (2), hydroxytyrosyl octanoate (3) and hydroxytyrosyl oleate (4). The esterification reaction was performed by solubilizing fresh substrate (154 mg, 1.0 mmol) in dimethyl carbonate (DMC, 3.0 mL) and adding a slight excess of butanoyl, octanoyl or oleoyl chloride (1.2 mmol). The reaction mixture was kept under magnetic stirring at room temperature and monitored by thin-layer chromatography on silica gel plates using mixtures of dichloromethane and methanol (9.8/0.2 or 9.5/0.5) as eluents. After 24 h, the reaction was stopped. The solvent was evaporated by distillation under reduced pressure; then, the residue was recovered with ethyl acetate (3x20 mL), washed with a saturated solution of NaCl (10 mL) and dried over

Na_2SO_4. After filtration, the solvent was evaporated under reduced pressure. The crude was purified by flash chromatography on silica gel by elution with mixtures of dichloromethane and methanol (9.8/0.2 or 9.5/0.5) as eluents, affording the HTyr esters **2**, **3** and **4** in 60-64% yields.

Hydroxytyrosyl butanoate (2). Colorless oil. Yield: 62%. Spectroscopic data were in agreement with those reported in the literature.[34]

Hydroxytyrosyl octanoate (3). Colorless oil. Yield: 60%. Spectroscopic data were in agreement with those reported in the literature.[34]

Hydroxytyrosyl oleate (4). Colorless oil. Yield: 64%. Spectroscopic data were in agreement with those reported in the literature.[34]

Preparation and characterization of the HTyr-enriched fraction. The HTyr-enriched fraction was obtained from *Olea europaea* pitted olive pulps using a sustainable extraction technology followed by membrane separation, according to a patented procedure.[28] In brief, the entire treatment consisted of water extraction of the vegetal material followed by selective fractionation in three steps: microfiltration (MF), nanofiltration (NF), and reverse osmosis (RO). The different filtration steps were characterized by different molecular weights, with cut-off and filtration degrees. During the manufacturing process, the MF step was carried out with tubular ceramic membranes in titanium oxide and the NF, and RO steps were performed with spiral wound module membranes in polyethersulfone.[29,30] The fraction exiting from NF and RO steps, and subjected to a final concentration by using a heat pump evaporator (Vacuum Evaporators - Scraper Series, C&G Depurazione Industriale srl, Firenze, Italy) was recovered and used for the lipophilization procedure.

It was characterized in terms of polyphenolic compounds by HPLC/DAD analysis at λ=280 nm (Table 1). Hydroxytyrosol and derivatives (glucoside and glicole), secoiridoids (oleuropein, oleoside and elenoic acid), and small amounts of caffeic acid derivatives were characterized and quantified, using pure standards (tyrosol, oleuropein and caffeic acid, respectively).

Lipophilization of the HTyr-enriched fraction. A sample of the HTyr-enriched fraction containing 25 mg (0.16 mmol) of HTyr **1** was solubilized with DMC (5.0 mL), and a slight excess of butanoyl chloride, octanoyl chloride or oleoyl chloride was then added (0.19 mmol, 40-200 μL). The mixture was kept under magnetic stirring at room temperature. After 24 h, the reaction was stopped. The solvent was evaporated by distillation under reduced pressure. The residue was recovered with ethyl acetate and washed with a saturated solution of NaCl; then, the organic phases were collected and dried over Na_2SO_4. After filtration, the solvent was evaporated under reduced pressure to collect the corresponding lipophilic fractions **5**, **6** and **7**. HTyr **1** and HTyr esters **2**, **3** and **4** present in the fractions were identified and quantified by HPLC/DAD analysis at λ= 280 nm using previously synthesized standards.

Cell culture. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), sodium pyruvate (1 mM), L-glutamine (2 mM), penicillin (100 μg/ml), streptomycin (100 μg/ml) and geneticin (280.25 μg/ml), but without phenol red, at 37°C with 5% CO_2 humidified air. Confluent cell cultures were detached with a trypsin/ethylenediaminetetraacetic acid (EDTA) solution and plated at the desired density in the appropriate medium.

Cell proliferation analysis. HCT8-β8-expressing cells were plated on 6-well plates at a density of $5x10^3$ cells/well. After 24 h, the medium was replaced with RPMI 1640 medium (phenol red-free medium supplemented with 2% FBS, penicillin (100 μg/ml) and streptomycin (100 μg/ml)) and stimulated with HTyr **1**; the lipophilic fractions **5**, **6** and **7** (5, 10, 25, 50 μM); or 17β-E2 (10 nM) as a positive control. The effects of **1**, **5**, **6** and **7** were also assessed in the presence of ICI 182,780 (1 μM), which is an inhibitor of ER-β. All the stimuli were dissolved in the medium to obtain the final tested concentrations. Cells without stimuli were used as a negative control. Cells were detached with trypsin/EDTA and evaluated using a Bürker hemocytometer every 24 h for 5 days during the *log phase* of cell growth. Measurements for each dose at each time point were collected in triplicate and averaged.

Statistical analysis. Statistical differences observed in cell proliferation were analyzed in Microsoft Excel (Microsoft, Redmond, WA, USA) using an elaboration of linear and non-linear regression that was brought back to the same y-intercept with the calculus of the cell population doubling time. From the experiments a set of independent linear and non-linear comparisons between the control and each single treatment (5, 10, 25, 50 μM) of HTyr and of the three lipophilic fractions were obtained. Consequently, the linearity of each regressions and the comparison among the regressions were analyzed by one-way ANOVA followed by a post-hoc procedure for multiple comparisons with control (Dunnett's Test). A $P\ value < 0.05$ was considered significant. All data are expressed as a mean of the cellular doubling time ± standard error (SE).

RESULTS AND DISCUSSION

Chemistry

The aim and the novelty of our research was to study the effect of several lipophilic fractions derived from *Olea europaea* L. by-products (pitted olive pulp) on the human colon cancer cell line HCT8 engineered to overexpress ER-β. In particular, we investigated three fractions containing HTyr **1** and hydroxytyrosyl butanoate **2**, hydroxytyrosyl octanoate **3**, hydroxytyrosyl oleate **4**, named fractions **5**, **6** and **7**, respectively. To evaluate the *effective* role of the HTyr ester in the lipophilic fraction containing HTyr, pure HTyr was tested.

Several procedures have been described in the literature to prepare HTyr esters, including the reaction of HTyr with free acids under Mitsunobu conditions[35] or with acyl chlorides in the presence of cerium(III) chloride,[36] as well as acid- and lipase-catalyzed transesterification using carboxylic fatty acids.[37-41] In this paper, we describe the synthesis of the selected esters by treating fresh HTyr with the appropriate acyl chloride in dimethyl carbonate (DMC), an eco-friendly chemical widely used in our laboratories as both a reagent and a solvent.[17,42.43] Specifically, HTyr **1** was solubilized in DMC, and a slight excess of butanoyl chloride, octanoyl chloride or oleoyl chloride was then added. Pure samples of the corresponding hydroxytyrosyl esters **2**, **3** and **4** were isolated after

chromatography in 60-64% yields (**Figure 3**), along with unreacted HTyr. The chemoselectivity of acylation has been already observed by our group [44] and related to DMC. In fact, we hypothesized that DMC emphasizes the higher nucleophilicity of the alcoholic moiety compared to the phenolic moieties in the acylation reaction of phenethyl alcohols, driving the selective esterification.

Then, these experimental conditions were extended to an *Olea europaea* L. phenolic fraction obtained by an environmentally and economically sustainable process using pitted olive pulp as plant materials as described in the Experimental Section.[28-30] Table 1 reports the HPLC/DAD quantitative data of phenolic compounds. As showed, the HTyr content in the fraction was 6.0% w/w.

Three samples of this fraction, containing HTyr 6% w/w, were solubilized with DMC and treated with butanoyl, octanoyl and oleoyl chloride, respectively, at room temperature for 24 h. After the work-up, the corresponding lipophilic fractions **5**, **6** and **7** were analyzed by HPLC/DAD analysis (**Figure 4**); HTyr **1** and HTyr esters **2**, **3** and **4** were identified and quantified in the corresponding fractions by comparison with previously synthesized standards. The analytical data show that fraction **4** contained HTyr **1** and HTyr butanoate **2** at 45 and 48%, respectively; fraction **5** contained HTyr **1** and HTyr octanoate **3** at 47% and 40%; and fraction **6** contained HTyr **1** and HTyr oleate **4** at 30% and 64%.

Biology

Cell counts of HCT8-β8-expressing cells cultured with HTyr **1**; fractions **5**, **6** and **7** (5, 10 and 25 μM); 17β-E2 (10 nM); and ICI 182,780 (1 μM) were performed every 24 h for 5 days to assess cell proliferation. For 17β-E2, used as a positive internal control, the results show that this treatment inhibited cell growth in every experiment. In particular, HTyr **1** significantly reduced the proliferation of HCT8-β8-expressing cells in a concentration-dependent manner (**Figure 5A**). The data obtained for the HCT8-β8-expressing cells after treatment with the lipophilic fractions **5, 6** and **7** show that all the lipophilic fractions significantly reduced the proliferation of HCT8-β8-

expressing cells in a concentration-dependent manner (**Figure 5B-D**). For HTyr **1** and the lipophilic fractions **5, 6** and **7**, concentrations lower than 5 μM did not have any type of effect (data not shown). Furthermore, the fractions **5** and **6** had an effect similar to that of HTyr **1** at the lowest active concentration (5 μM), whereas fraction **7** had a different and major effect on the cell proliferation at the same concentration. In fact, while the doubling time of the HCT8-β8-expressing cells was calculated to be approximately 65 ± 9 h (mean ± SE) after treatment with HTyr **1** *vs*. 35 ± 6 h for the control without stimuli, this time was calculated to be approximately 64 ± 11 h *vs*. 31 ± 4 h for the control, 62 ± 14 h *vs*. 30 ± 3 h for the control and approximately 133 ± 30 h *vs*. 42 ± 4 h for the control after treatment with the fractions **5**, **6** and **7**, respectively. Hence, we observed that HTyr **1** and the fractions **5**, **6** and **7** induced increases in the doubling time of approximately 85%, 106%, 114% and 224%, respectively, compared to the control without stimuli.

In addition, to demonstrate that the effects of HTyr **1** and the lipophilic fractions **5**, **6** and **7** are dependent on ER-β, we assessed their effects in the presence of the ER-β inhibitor known as ICI 182,780 (1 μmol/L). The experimental data showed that the inhibitory effects observed for HTyr **1** and the lipophilic fractions **5, 6** and **7** disappeared under these conditions (**Figure 6**).

The data obtained have shown the anti-proliferative effects of HTyr **1** on HCT8-β8-expressing cells, comparable to the anti-proliferative effect induced by 17β-E2. This is in agreement with observations from other studies on CRC.[7,10] Furthermore, in this study, we have demonstrated that all the lipophilic fractions tested have an inhibitory effect, similar to that of HTyr **1**. In detail, we have determined that lipophilic fraction **7** has a greater anti-proliferative effect than the others. Additionally, from the experiments with ICI 182,780, we have also demonstrated that the observed inhibition of cell growth totally disappeared in the presence of an ER-β inhibitor, specifically ICI 182,780. Taken together, our data suggest not only that the inhibition of cell growth depends on the binding of these polyphenolic molecules to ER-β but also that the HTyr fraction enriched with HTyr oleate (fraction **7**) has a greater anti-proliferative effect than the other fractions. This effect could be related to the facility with which this molecule passes through the cell membrane, thanks to the

major lipophilicity given to the molecule by the presence of the long chain of the oleate. Future experiments, especially on the molecular effects of this link between fraction **7** and ER-β, will be necessary to understand if this fraction can be a useful tool against CRC.

ACKNOWLEDGMENT

The authors would like to thank Dr. Francesca Santoni for the preparation of a sample of HTyr and HTyr esters. Thanks also to the Complex Equipment Center (University of Tuscia) for the availability of the NMR 400 MHz Bruker spectrometer.

REFERENCES

1) Boyle, P.; Langman, J. S. ABC of colorectal cancer: epidemiology. *Brit. Med. J.* **2000**, *321*, 805-808.
2) Trichopolou, A.; Lagoiu, P.; Kuper, H.; Trichopoulos, D. Cancer and Mediterranean dietary traditions. *Cancer Epidemiol. Biomarkers Prev.* **2000**, *9*, 869-873.
3) Fazio, C.; Ricciardiello, L. Components of the Mediterranean diet with chemopreventive activity toward colorectal cancer. *Phytochem. Rev.* **2014**, *13*, 867-879.
4) Stark, A. H.; Madar, Z. Olive oil as a functional food: epidemiology and nutritional approaches. *Nutr. Rev.* **2002**, *60*, 170-176.
5) Rigacci, S.; Stefani, M. Nutraceutical properties of olive oil polyphenols. An itinerary from cultured cells through animal models to humans. *Int. J. Mol. Sci.* **2016**.
6) Rosignoli, P.; Fuccelli, R.; Sepporta, M.V.; Fabiani, R. In vitro chemopreventive activities of hydroxytyrosol: the main phenolic compound present in extra-virgin olive oil. *Food Funct*. **2016**, *7*, 301-307.
7) Hashim, Y. Z. H-Y.; Gill, C. I. R.; McGlynn, H.; Rowland, I. R. Components of olive oil and chemoprevention of colorectal cancer. *Nutr. Rev.* **2005**, *63*, 374-386.
8) Gill, C. I. R.; Boyd, A.; McDermott, E.; McCann, M.; Servili, M.; Selvaggini, R.; Taticchi, A.; Esposto, S.; Montedoro, GF; McGlynn, H.; Rowland, I. Potential anti-cancer effects of virgin olive oil phenols on colorectal carcinogenesis models in vitro. *Int. J. Cancer* **2005**, *117*, 1-7.
9) Hashim, Y.Z.; Rowland, I.R.; McGlynn, H.; Servili, M.; Selvaggini, R.; Taticchi, A.; Esposto,S.; Montedoro, G.; Kaisalo, L.; Wahala, K.; Gill, C. I. Inhibitory effects of olive oil phenolics on invasion in human colon adenocarcinoma cells in vitro. *Int J Cancer*. **2008**, *122*, 495-500.

10) Notarnicola, M.; Pisanti, S.; Tutino, V.; Bocale, D.; Rotelli, M. T.; Gentile, A.; Memeo, M.; Bifulco, M.; Perri, P.; Caruso, M. G. Effects of olive oil polyphenols on fatty acid synthase gene expression and activity in human colorectal cancer cells. *Genes Nutr.* **2011**, *6*, 63-69.

11) Corona, G.; Deiana, M.; Incani, A.; Vauzour, D.; Dessì, M. A. Spencer, J. P. E. Inhibition of p38/CREB phosphorylation and COX-2 expression by olive oil polyphenols underlies their anti-proliferative effects. *Biochem. Biophys. Res. Comm.* **2007**, *362*, 606-611.

12) Pampaloni, B.; Mavilia, C.; Fabbri, S.; Romani, A.; Ieri, F.; Tanini, A.; Tonelli, F.; Brandi, M. L. In vitro effects of extracts of extra virgin olive oil on human colon cancer cells. *Nutr. Cancer* **2014**, *66*, 1228-1236.

13) Pampaloni, B.; Palmini, G.; Mavilia, C.; Zonefrati, R.; Tanini, A.; Brandi, M. L. In vitro effects of polyphenols on human colon cancer cells. *World J. Gastrointest. Oncol.* **2014**, *6*, 289-300.

14) Martineti, V.; Picariello, L.; Tognarini, I.; Carbonell Sala, S.; Gozzini, A.; Azzari, C.; Mavilia, C.; Tanini, A.; Falchetti, A.; Fiorelli, G.; Tonelli, G.; Brandi, M. L. ERβ is a potent inhibitor of cell proliferation in the HCHT8 human colon cancer cell line through regulation of cell cycle components. *Endocr-Relat. Cancer* **2005**, *12*, 455-469.

15) Terzuoli, E. ; Giachetti, A. ; Ziche, M. ; Donnini, S. Hydroxytyrosol, a product from olive oil, reduces colon cancer growth by enhancing epidermal growth factor receptor degradation. *Mol. Nutr. Food Res.* **2016**, *60*, 519-529.

16) Tripodi, E.; Giammanco, M.; Tabacchi, G.; Di Majo, D.; Giammanco S.; La Guardia, M. The phenolic compounds of olive oil: structure, biological activity and beneficial effects on human health. *Nutr. Res. Rev.* **2005**, *18*, 98-112.

17) Gambacorta, A.; Tofani, D.; Bernini, R.; Migliorini, A. High-yielding preparation of a stable precursor of hydroxytyrosol by total synthesis and from the natural glycoside oleuropein. *J. Agric. Food Chem.* **2007**, *55*, 3386-3391.

18) Bernini, R.; Merendino, N.; Romani, A.; Velotti, F. Natural occurring hydroxytyrosol: synthesis and anticancer potential. *Curr. Med. Chem.* **2013**, *20*, 655-670.

19) Laguerre, M.; Bayrasy, C.; Lecomte, J.; Chabi, B.; Decker, E. A.; Wrutniak-Cabello, C.; Cabello, G.; Villeneuve, P. How to boost antioxidants by lipophilization? *Biochimie* **2013**, *95*, 20-26.

20) Bernini, R.; Mincione, E.; Crisante, F.; M. Barontini, M.; Fabrizi, G.; Gentili, P. Chemoselective and efficient carboxymethylation of the alcoholic chain of phenols by dimethyl carbonate (DMC). *Tetrahedron Lett.* **2007**, *48*, 7000-7003.

21) Bernini, R.; Mincione, E.; Crisante, F.; Barontini, M.; Fabrizi, G. A novel use of the recyclable polymer-supported IBX: an efficient chemoselective and regioselective oxidation of phenolic compounds. The case of hydroxytyrosol derivatives. *Tetrahedron Lett.* **2009**, *50*, 1307-1310.

22) Madrona, A.; Pereira-Caro, G.; Mateos, R.; Rodriguez, G.; Trujillo, M.; Fernandez-Bolanos, J.; Espartero, J. L. Synthesis of hydroxytyrosol ethers from olive oil waste waters. *Molecules* **2009**, *1*, 1762-1772.

23) Bouallagui, Z.; Bouaziz, M.; Lassoued, S.; Engasser, J. M.; Ghoul, M.; Sayadi, S. Hydroxytyrosol acyl esters: biosynthesis and activities. *Appl. Biochem. Biotechnol.* **2011**, *163*, 592-599.

24) Tassano, E.; Alama, A.; Basso, A.; Dondo, G.; Galatini, A.; Riva, R.; Banfi, L. Conjugation of hydroxytyrosol with other natural phenolic fragments: from waste to antioxidants and antitumour compounds. *Eur. J. Org. Chem.* **2015**, 6710-6726.

25) Calderón-Montaño, J. M.; Madrona, A.; Burgos-Moron, E.; Orta, M. L.; Mateos, S.; Espartero, J. L.; Lopez-Lazaro, M. Selective cytotoxic activity of new lipophilic hydroxytyrosol alkyl ether derivatives. *J. Agric. Food Chem.* **2013**, *61*, 5046-5053.

26) Bernini, R.; Gilardini Montani, M. S.; Merendino, N.; Romani, A. Velotti, F. Hydroxytyrosol-derived compounds: a basis for the creation of new pharmacological agents

for cancer prevention and therapy *J. Med. Chem.* **2015***, 58,* 9089-9107 and references therein.

27) Fortunati, E.; Luzi, F.; Dugo, L.; Fanali, C.; Tripodo, G.; Santi, L.; Kenny, J. M.; Torre, L.; Bernini, R. Effect of hydroxytyrosol methyl carbonate on the thermal, migration and antioxidant properties of PVA based films for active food packaging. *Polym. Int.* **2016**, 65, 872-882.

28) Pizzichini, D.; Russo, C.; Vitagliano, M.; Pizzichini, M.; Romani, A.; Ieri, F.; Pinelli, P.; Vignolini, P. Phenofarm S.r.l. Process for producing concentrated and refined actives from tissues and byproducts of *Olea europaea* with membrane technologies. EP 2338500 A1.

29) Romani, A.; Pinelli, P.; Ieri, F.; Bernini, R. Sustainability, innovation and green chemistry in the production and valorization of phenolic extracts from *Olea europaea* L. *Sustainability*, **2016**, *2*, 1002.

30) Romani, A.; Scardigli, A.; Pinelli, P. An environmentally process for the production of extracts rich in phenolic antioxidants from *Olea europaea* L. and *Cybara scolymus* L. matrices. *Eur. Food Res. Technol.* **2016**, 1-10.

31) Frigerio, M.; Santagostino, M.; Sputore, S. A user-friendly entry to 2-iodoxybenzoic acid (IBX). *J. Org. Chem.* **1999**, *64*, 4537-4538.

32) Picariello, L.; Fiorelli, G.; Benvenuti, S.; Brandi, M. L.: Galli, G.; Malentacchi, C.; Montali, E.; Bigozzi, U.; Ficari, F.; Tonelli F. In vitro bioeffects of the antiestrogen LY117018 on desmoid tumors and colon cancer cells. *Anticancer Res.* **1997**, *17*, 2099-2104.

33) Bernini, R.; Mincione, E.; Barontini M.; F. Crisante. Convenient synthesis of hydroxytyrosol and its lipophilic derivatives from tyrosol or homovanillyl alcohol. *J. Agr. Food Chem.* **2008**, *56*, 8997-8904.

34) Tofani, D.; Balducci, V.; Gasperi, T.; Incerpi, S.; Gambacorta, A. Fatty acid hydroxytyrosyl esters: structure/antioxidant activity relationship by ABTS and in cell-culture DCF assays. *J. Agric. Food Chem.* **2010**, *58*, 5292-5299.

353 35) Appendino, G.; Minassi, A.; Daddario, N.; Bianchi, F.; Tron, G. C. Esterification of
354 phenolic acids and alcohols. *Org. Lett.* **2002**, *4*, 3839-3841.
355 36) Torregiani, E.; Seu, G.; Minassi, A.; Appendino, G. Cerium(III) chloride-promoted
356 chemoselective esterification of phenolic alcohols. *Tetrahedron Lett.* **2005**, *46*, 2193-2196.
357 37) Trujillo, M.; Mateos, R.; Collantes de Teran, L.; Espartero, J. L.; Cert, R.; Jover, M.;
358 Alcudia, F.; Bautista, J.; Cert, A.; Parrado, J. Lipophilic hydroxytyrosyl esters. Antioxidant
359 activity in lipid matrices and biological systems. *J. Agric. Food Chem.* **2006**, *54*, 3779-3785.
360 38) Mateos, R.; Trujillo, M.; Pereira-Caro, G.; Madrona, A.; Cert, A.; Espartero, J. L. New
361 lipophilic tyrosyl esters. Comparative antioxidant evaluation with hydroxytyrosyl esters. *J.*
362 *Agric. Food Chem.* **2008**, *56*, 10960-10966.
363 39) Grasso, S.; Siracusa, L.; Spatafora, C.; Renis, M.; Tringali, C. Hydroxytyrosol lipophilic
364 analogues: enzymatic synthesis, radical scavenging activity and DNA oxidative damage
365 protection. *Bioorg. Chem*. **2007**, *35*, 137-152.
366 40) Trujillo, M.; Mateos, R.; Collantes de Teran, L.; Espartero, J. L.; Cert, R.; Jover, M.;
367 Alcudia, F.; Bautista, J.; Cert, A.; Parrado, J. Lipophilic hydroxytyrosyl esters. Antioxidant
368 activity in lipid matrices and biological systems. *J. Agric. Food Chem.* **2006**, *54*, 3779-3785.
369 41) Mateos, R.; Trujillo, M.; Pereira-Caro, G.; Madrona, A.; Cert, A.; Espartero, J. L. New
370 lipophilic tyrosyl esters. Comparative antioxidant evaluation with hydroxytyrosyl esters. *J.*
371 *Agric. Food Chem.* **2008**, *56*, 10960-10966.
372 42) Bernini, R.; Mincione, E.; Barontini, M.; Crisante, F.; Fabrizi, G.; Gambacorta, A. Dimethyl
373 carbonate: an environmetally friendly solvent for hydrogen peroxide
374 (H_2O_2)/methyltrioxorhenium (CH_3ReO_3) catalytic oxidation. *Tetrahedron* **2007**, *63*, 6895-
375 6900.
376 43) Bernini, R.; Crisante, F.; Ginnasi M. C. A convenient and safe O-methylation of flavonoids
377 with dimethyl carbonate (DMC). *Molecules* **2011**, *16*, 1418-1425.

44) Bernini, R.; Crisante, F.; Barontini, M.; Tofani, D.; Balducci, V.; Gambacorta, A. Synthesis and structure/antioxidant activity relationship of novel catecholic antioxidants structurally analogues to hydroxytyrosol and its lipophilic esters. *J. Agric. Food Chem.* **2012**, *60*, 7408-7416.

383 **LIST OF FIGURE CAPTIONS**

384 **Figure 1.** Chemical structures of the main phenolic compounds present in olive oil.

385

386 **Figure 2**. Observation in phase contrast of islets of the HCT8-β8 line. Original magnification: 10X.

387

388 **Figure 3.** Synthesis of HTyr esters **2**, **3** and **4**.

389

390 **Table 1.** HPLC/DAD quantitative analyses of *Olea* fraction used for the lipophilization raection.
391 Data are the mean values of triplicate samples (±SD) and are expressed as mg g^{-1}.

392

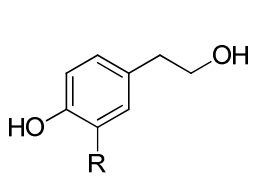
393 **Figure 4**. HPLC chromatograms of the lipophilic fractions **4**, **5** and **6**.

394

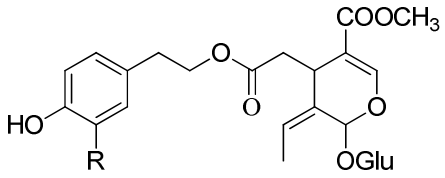
395 **Figure 5**. **A)** Growth of HCT8-β8-expressing cells in the presence of HTyr **1** (5, 25 and 50 μM) and
396 17β-E2 (10 nM). Values are the means of triplicates. **B**) Growth of HCT8-β8-expressing cells in the
397 presence of the fraction **5** and 17β-E2. Values are the means of triplicates. **C)** Growth of HCT8-β8-
398 expressing cells in the presence of the fraction **6** and 17β-E2. Values are the means of triplicates. **D)**
399 Growth of HCT8-β8-expressing cells in the presence of the fraction **7** and 17β-E2. Values are the
400 means of triplicates. *P < 0.05 *vs* control; **P < 0.01 *vs* control.

401

402 **Figure 6**. Growth of HCT8-β8-expressing cells in the presence of HTyr **1** (5 μM), the lipophilic
403 fractions **5**, **6**, **7** (5 μM) and ICI 182,780 (fulvestrant, 1μM). *P < 0.05 vs control; **P < 0.01 vs
404 control.



R=H, Tyrosol
R=OH, Hydroxytyrosol (HTyr)



R=H, Ligstroside
R=OH, Oleuropein

Figure 1

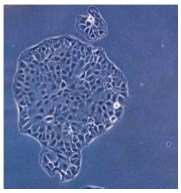
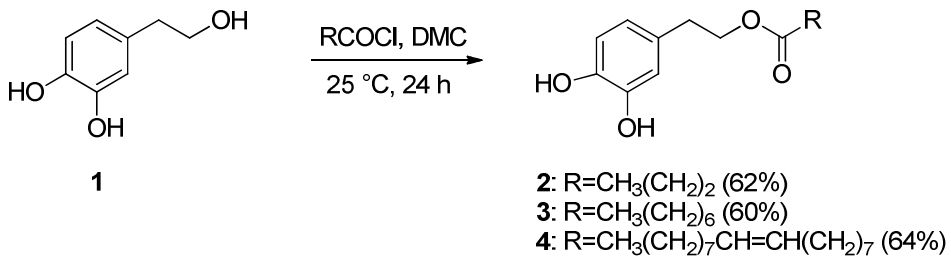


Figure 2



1

2: R=$CH_3(CH_2)_2$ (62%)
3: R=$CH_3(CH_2)_6$ (60%)
4: R=$CH_3(CH_2)_7CH{=}CH(CH_2)_7$ (64%)

Figure 3

Table 1. HPLC/DAD quantitative analyses of *Olea* fraction, used for the lipophilization reaction. Data are the mean values of triplicate samples (±SD) and are expressed as mg g^{-1}.

Phenolic compounds	
Hydroxytyrosol (HTyr)	60.53 ± 0.41
Hydroxytyrosol derivatives	11.38 ± 0.41
Tyrosol	4.35 ± 0.33
Secoiridoids	21.61 ± 0.97
Caffeic acid derivatives	0.25 ± 0.03
Total polyphenols	98.14 ± 2.43

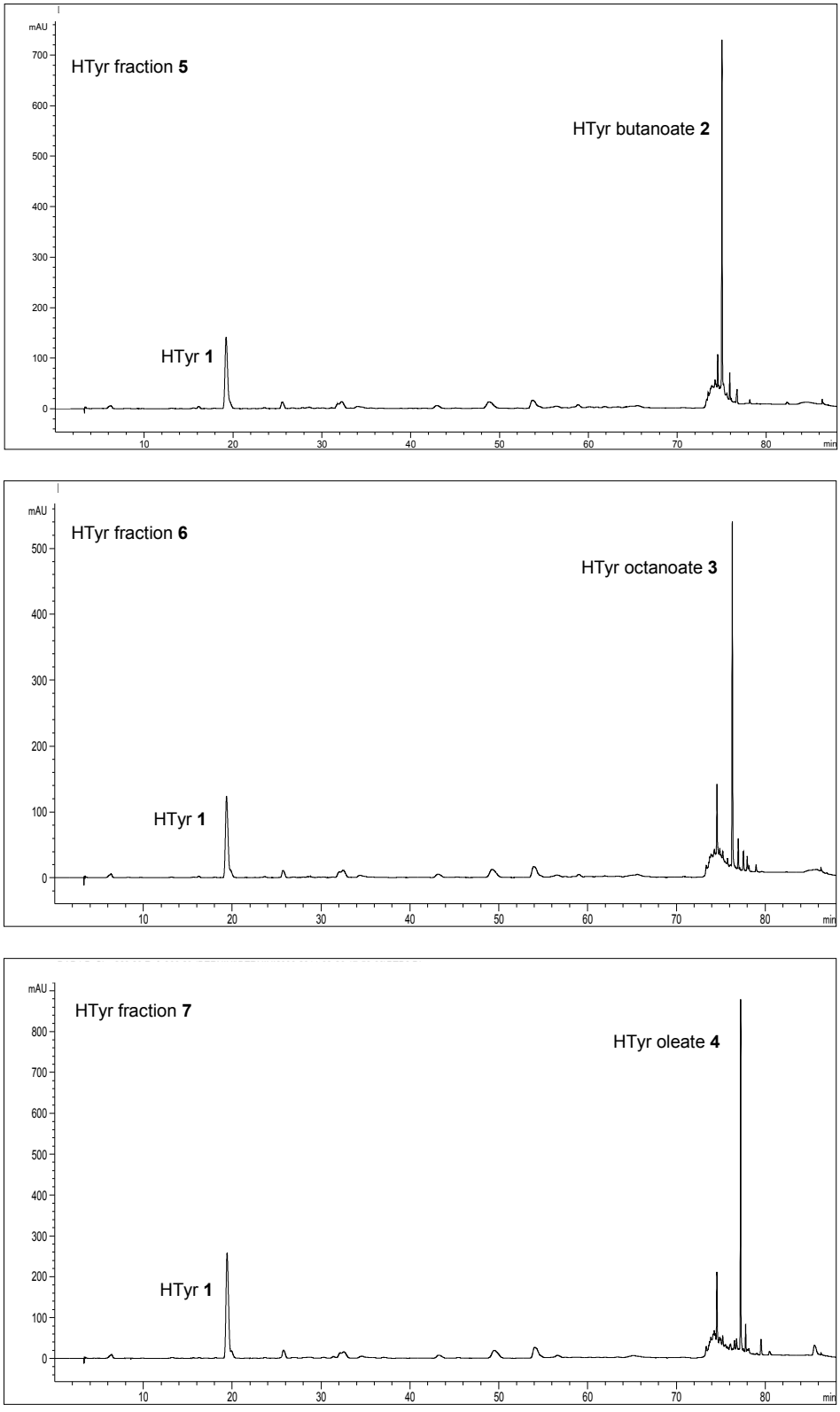

mAU
HTyr fraction 5
HTyr butanoate 2
HTyr 1
min
HTyr fraction 6
HTyr octanoate 3
HTyr 1
min
mAU
HTyr fraction 7
HTyr oleate 4
HTyr 1
min

Figure 4

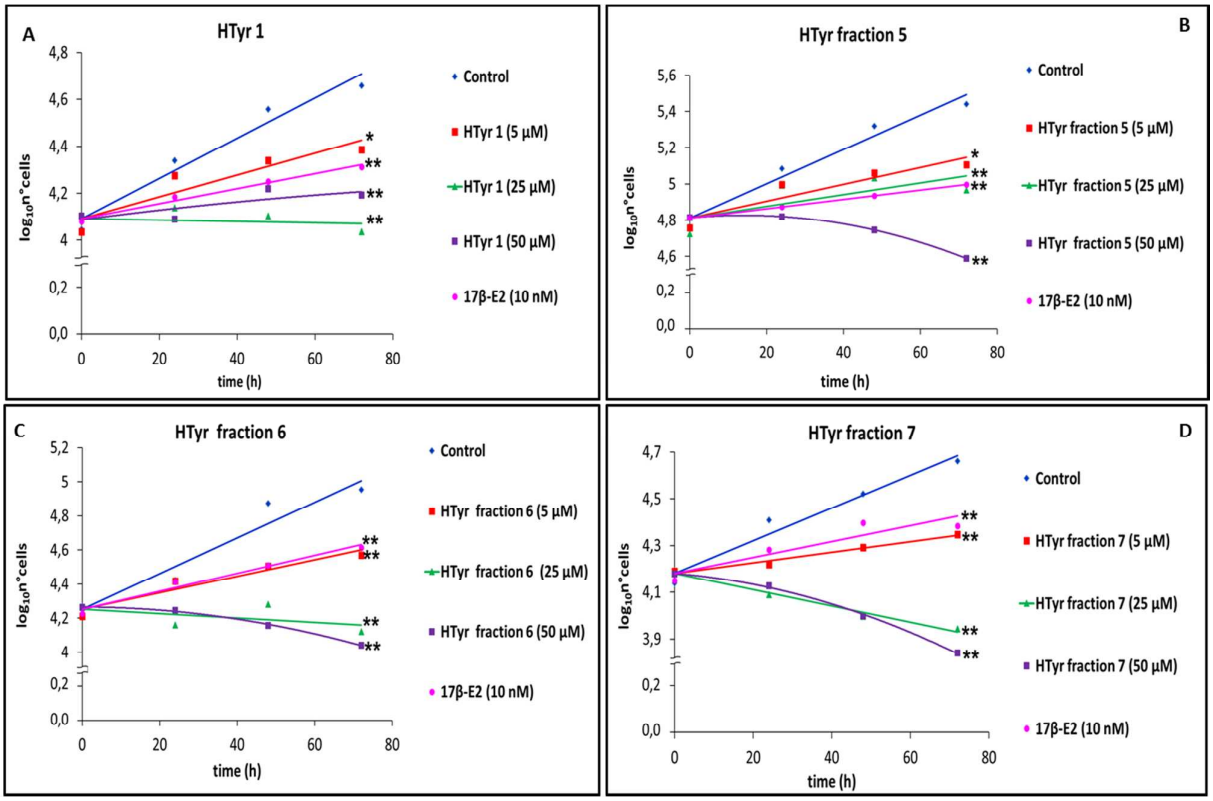

A
HTyr 1
log10n°cells
time (h)
Control
HTyr 1 (5 µM)
HTyr 1 (25 µM)
HTyr 1 (50 µM)
17β-E2 (10 nM)
B
HTyr fraction 5
HTyr fraction 5 (5 µM)
HTyr fraction 5 (25 µM)
HTyr fraction 5 (50 µM)
C
HTyr fraction 6
HTyr fraction 6 (5 µM)
HTyr fraction 6 (25 µM)
HTyr fraction 6 (50 µM)
D
HTyr fraction 7
HTyr fraction 7 (5 µM)
HTyr fraction 7 (25 µM)
HTyr fraction 7 (50 µM)

Figure 5

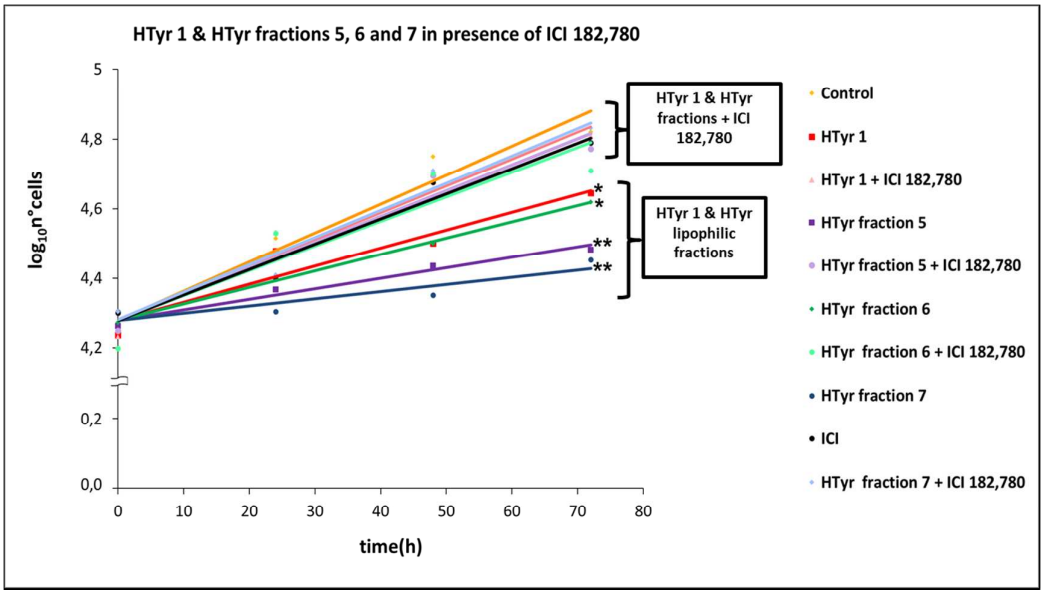

HTyr 1 & HTyr fractions 5, 6 and 7 in presence of ICI 182,780
log10n°cells
time(h)
HTyr 1 & HTyr fractions + ICI 182,780
HTyr 1 & HTyr lipophilic fractions
Control
HTyr 1
HTyr 1 + ICI 182,780
HTyr fraction 5
HTyr fraction 5 + ICI 182,780
HTyr fraction 6
HTyr fraction 6 + ICI 182,780
HTyr fraction 7
ICI
HTyr fraction 7 + ICI 182,780

Figure 6

TABLE OF CONTENTS GRAPHIC

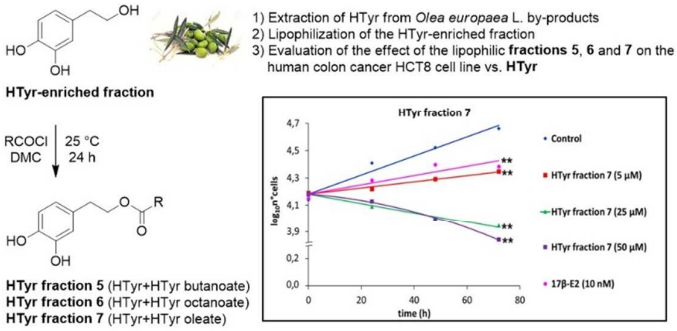


Figure 1. Chemical structures of the main phenolic compounds present in olive oil.

Figure 2. Observation in phase contrast of islets of the HCT8-β8 line. Original magnification: 10X.

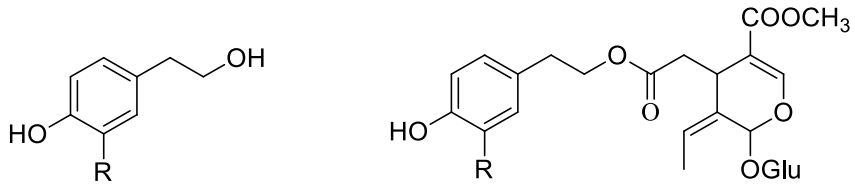
Figure 3. Synthesis of HTyr esters **2**, **3** and **4**.

Table 1. HPLC/DAD quantitative analyses of *Olea* fraction used for the lipophilization raection. Data are the mean values of triplicate samples (±SD) and are expressed as mg g^{-1}.

Figure 4. HPLC chromatograms of the lipophilic fractions **4**, **5** and **6**.

Figure 5. **A)** Growth of HCT8-β8-expressing cells in the presence of HTyr **1** (5, 25 and 50 μM) and 17β-E2 (10 nM). Values are the means of triplicates. **B**) Growth of HCT8-β8-expressing cells in the presence of the fraction **5** and 17β-E2. Values are the means of triplicates. **C)** Growth of HCT8-β8-expressing cells in the presence of the fraction **6** and 17β-E2. Values are the means of triplicates. **D)** Growth of HCT8-β8-expressing cells in the presence of the fraction **7** and 17β-E2. Values are the means of triplicates. *P < 0.05 *vs* control; **P < 0.01 *vs* control.

Figure 6. Growth of HCT8-β8-expressing cells in the presence of HTyr **1** (5 μM), the lipophilic fractions **5**, **6**, **7** (5 μM) and ICI 182,780 (fulvestrant, 1μM). *P < 0.05 vs control; **P < 0.01 vs control.



R=H, Tyrosol
R=OH, Hydroxytyrosol (HTyr)

R=H, Ligstroside
R=OH, Oleuropein

Figure 1

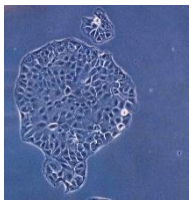
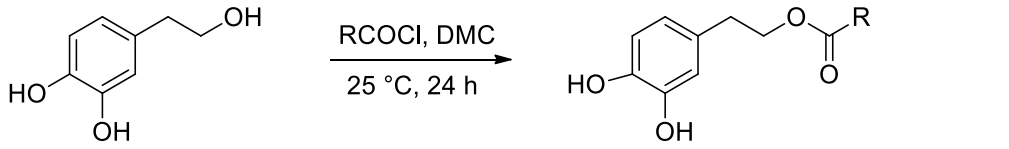


Figure 2



1

2: R=$CH_3(CH_2)_2$ (62%)
3: R=$CH_3(CH_2)_6$ (60%)
4: R=$CH_3(CH_2)_7CH{=}CH(CH_2)_7$ (64%)

Figure 3

Table 1. HPLC/DAD quantitative analyses of *Olea* fraction, used for the lipophilization reaction. Data are the mean values of triplicate samples (±SD) and are expressed as mg g^{-1}.

Phenolic compounds	
Hydroxytyrosol (HTyr)	60.53 ± 0.41
Hydroxytyrosol derivatives	11.38 ± 0.41
Tyrosol	4.35 ± 0.33
Secoiridoids	21.61 ± 0.97
Caffeic acid derivatives	0.25 ± 0.03
Total polyphenols	98.14 ± 2.43

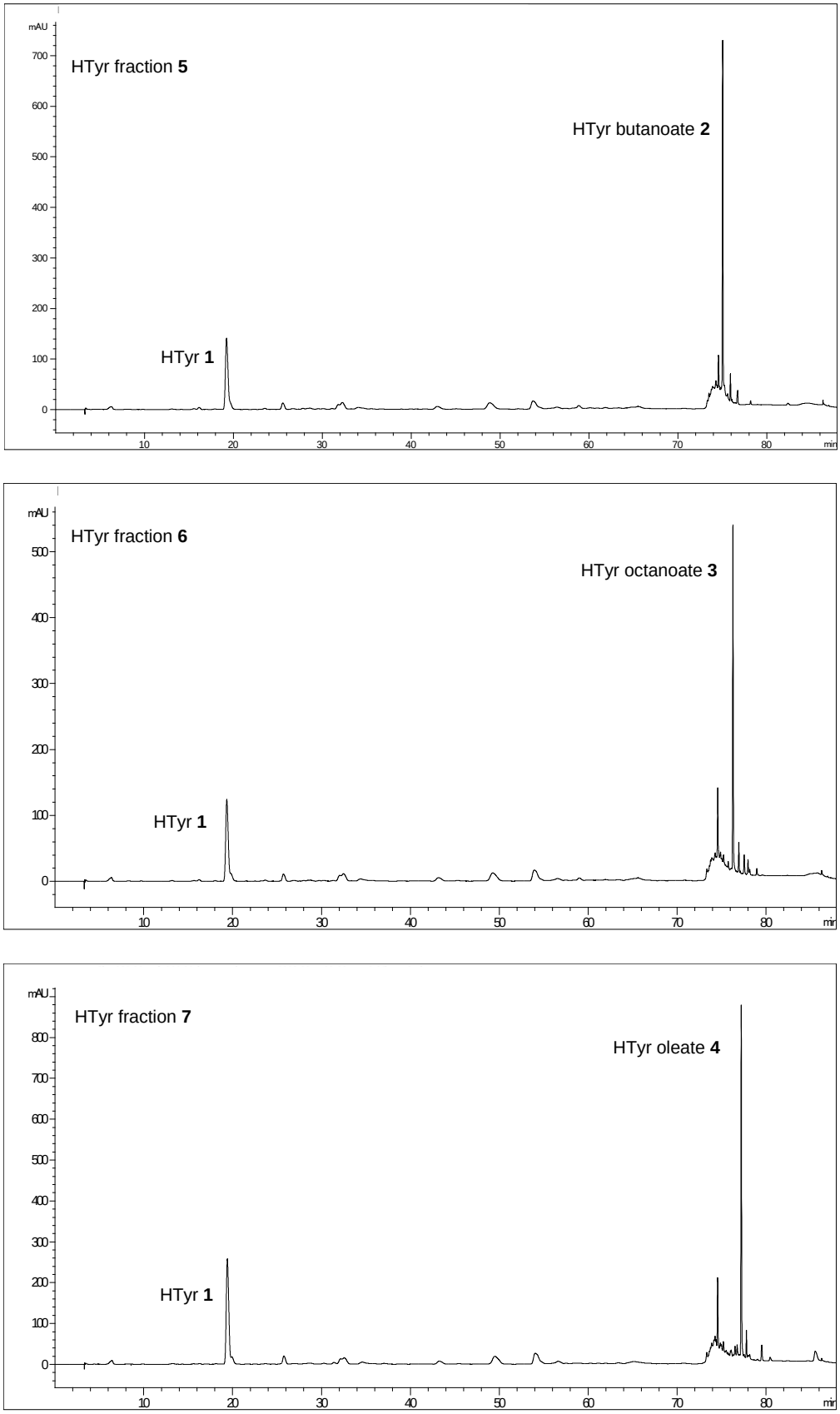

mAU
HTyr fraction 5
HTyr butanoate 2
HTyr 1
HTyr fraction 6
HTyr octanoate 3
HTyr 1
HTyr fraction 7
HTyr oleate 4
HTyr 1
min

Figure 4

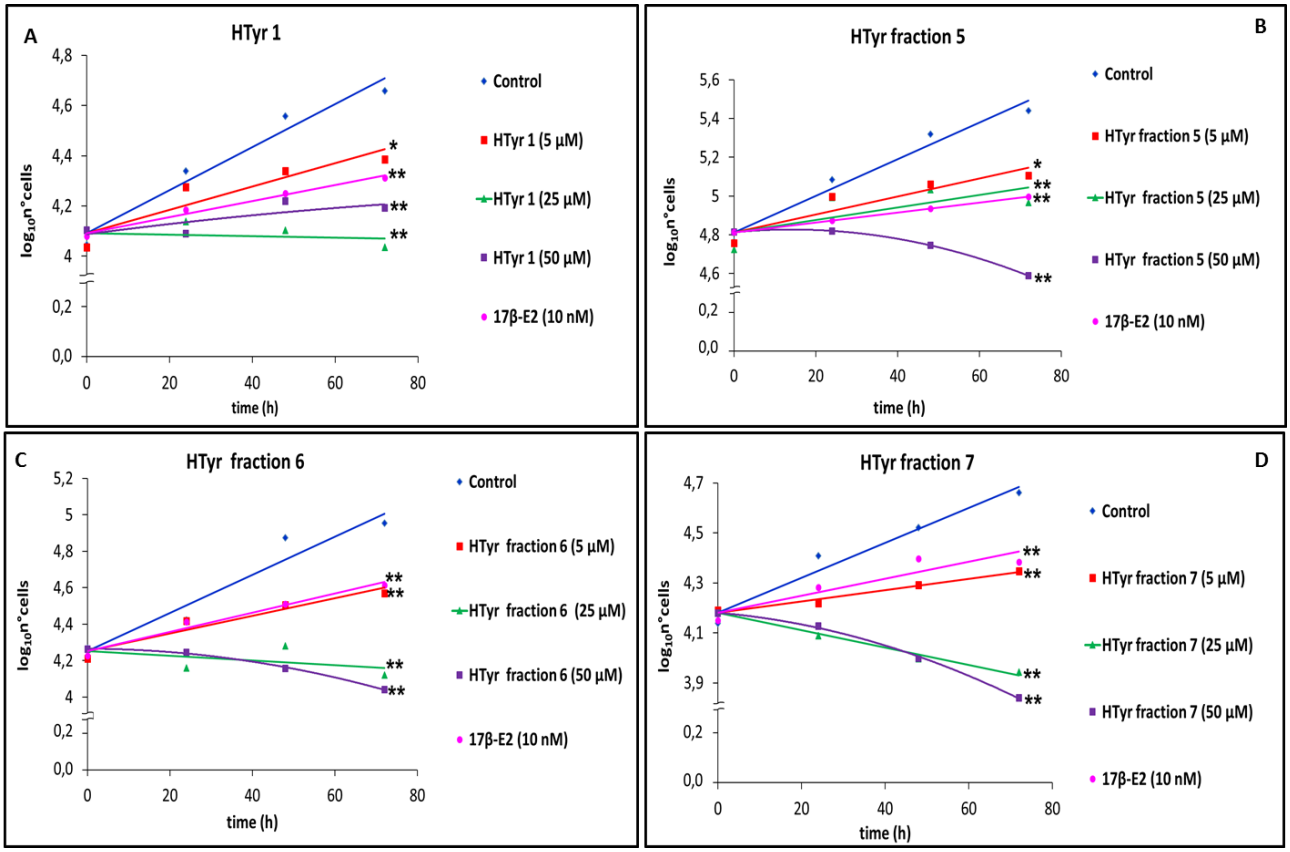

A
HTyr 1
log10n°cells
time (h)
Control
HTyr 1 (5 µM)
HTyr 1 (25 µM)
HTyr 1 (50 µM)
17β-E2 (10 nM)
B
HTyr fraction 5
HTyr fraction 5 (5 µM)
HTyr fraction 5 (25 µM)
HTyr fraction 5 (50 µM)
C
HTyr fraction 6
HTyr fraction 6 (5 µM)
HTyr fraction 6 (25 µM)
HTyr fraction 6 (50 µM)
D
HTyr fraction 7
HTyr fraction 7 (5 µM)
HTyr fraction 7 (25 µM)
HTyr fraction 7 (50 µM)

Figure 5

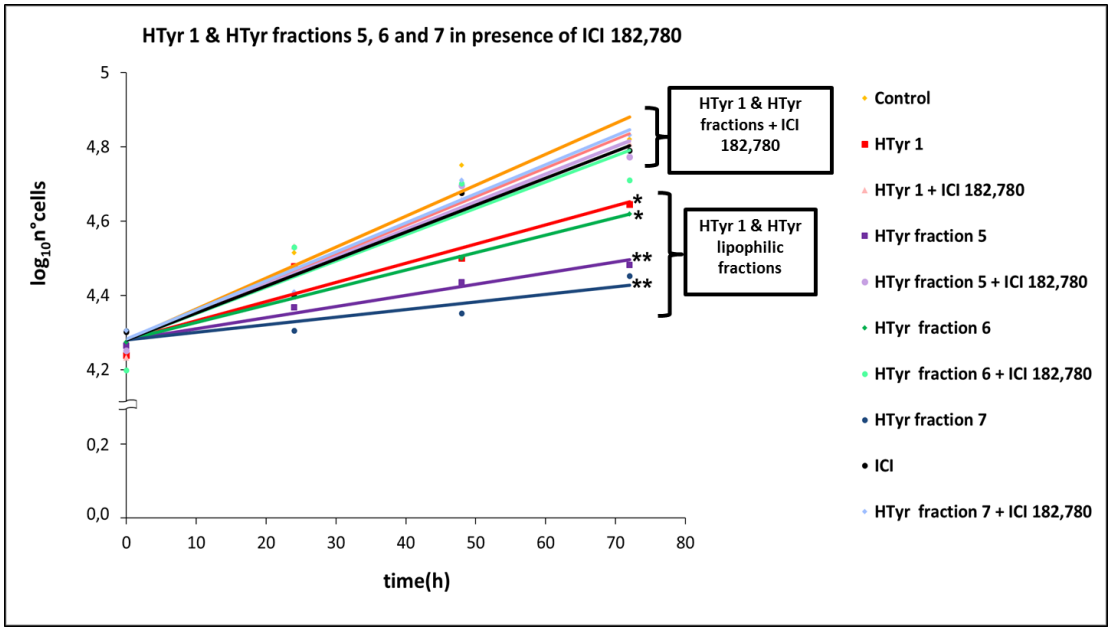

HTyr 1 & HTyr fractions 5, 6 and 7 in presence of ICI 182,780
log10n°cells
time(h)
5
4,8
4,6
4,4
4,2
0,2
0,0
0
10
20
30
40
50
60
70
80
HTyr 1 & HTyr fractions + ICI 182,780
HTyr 1 & HTyr lipophilic fractions
*
*
**
**
Control
HTyr 1
HTyr 1 + ICI 182,780
HTyr fraction 5
HTyr fraction 5 + ICI 182,780
HTyr fraction 6
HTyr fraction 6 + ICI 182,780
HTyr fraction 7
ICI
HTyr fraction 7 + ICI 182,780

Figure 6