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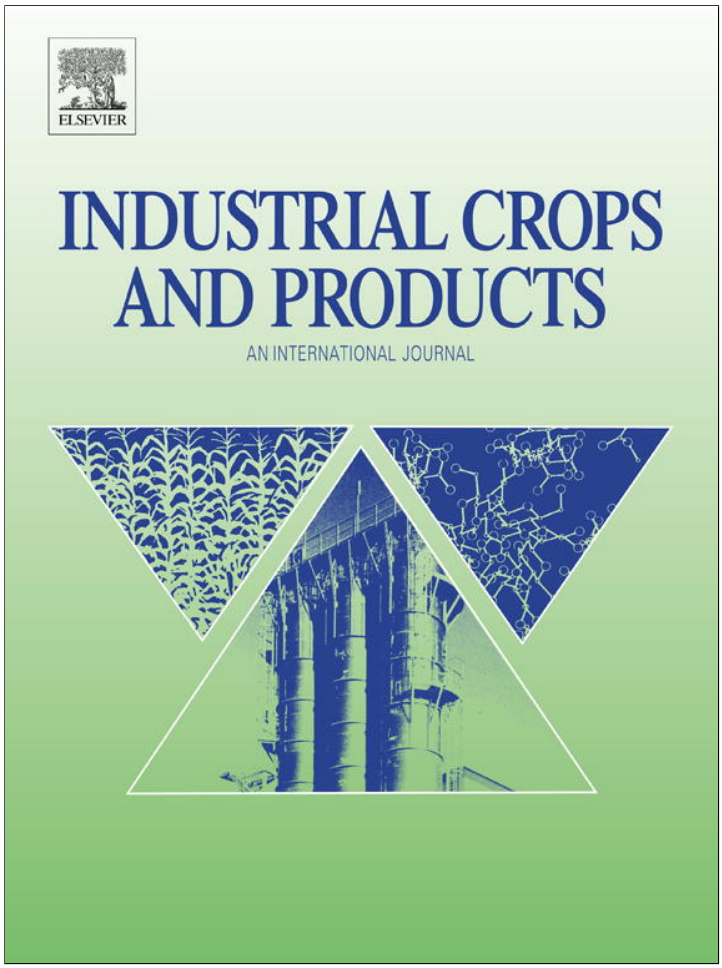

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Valorization of olive mill residues: Antioxidant and breast cancer antiproliferative activities of hydroxytyrosol-rich extracts derived from olive oil by-products

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ABSTRACT

Phenolic composition, antioxidant and breast cancer antiproliferative activities of water and methanol/water derived extracts from olive pomace (OP) and dry olive mill residue (DOR), from Portuguese industries, were studied. DOR water (DORW) extracts showed the highest extraction yield; as well as the highest total phenolic content (TPC) and hydroxytyrosol (HT) (~25 mg/g extract). HPLC–ESI-MS analysis identified HT in both OP and DOR, whereas HT-1-glucoside, tyrosol, oleuropein aglycone isomers, verbascoside and oleuropein were only detected in DOR. Additionally, a de(carboxymethyl)oleuropein aglycone isomer, in aldehyde form, was reported for the first time in DOR. DOR water extract also presented the most effective DPPH scavenging capacity and antiproliferative activity, comparatively to OP water (OPW) extract. Moreover, antioxidant potential of phenolic compounds present in DORW extract was comparable to HT, and to butylated hydroxyanisole (BHA), a widely used food industry antioxidant. Phenolic compounds present in DORW extract also showed comparable tumor antiproliferative activity on MDA-MB-231, relatively to HT and 5-fluorouracil (5-FU), a well-known cytostatic agent. MDA-MB-231 cell growth inhibition, upon 5-FU incubation was even incremented in the presence of DORW extract.

These results demonstrate DOR extracts potential as source of phenolic compounds for nutraceutical applications, as food supplements, opening new strategies for its olive mill residues valorization.

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1. Introduction

Epidemiological studies, within the Mediterranean basin, have correlated the low incidence of coronary heart disease and certain types of cancer, e.g. colorectal and breast, with the high consumption of fruits, vegetables, cereals and, in particular virgin olive oil (Vasilopoulou et al., 2005). European countries are responsible for about 75% of world olive oil production, with Spain, Italy and Greece as the main producers respectively, (56%, 25% and 16% in 2005–2010 period) (IOOC, 2010). Within the same period, Portugal was only responsible for ~2.3% of the world olive oil production. However, in 2000–2009 period, olive oil production increased ~63% in Portugal (Fernández-Bolaños et al., 2002), mainly due to intensive olive groves production, especially in the Alentejo region, which by itself represents 56% of national production (INE, 2009). In this region, olive oil extraction is predominantly carried out by the so-called two-phase centrifugation system which generates a high-water content solid residue called two-phase olive pomace (OP), composed of skin, pulp and stone pieces of olive fruit. Every year, about 400 000 tons of OP residues with high phytotoxicity are produced between October and January, causing environmental problems if not adequately treated. This pulp residue is usually dried at high temperatures (400–800 °C) and further subjected to hexane extraction in order to obtain the OP oil (~9.2% of the dry original OP) (Vlyssides et al., 2004), generating another by-product named dry olive mill residue (DOR) (Moral and Mendez, 2006). This final residue, DOR, which represents approximately 35% of the original dry OP (Vlyssides et al., 2004), is normally burned for power generation. Other less important applications of these residues include their use as organic fertilizers (Lopez-Pineiro et al., 2008), or animal feeding supplements (Martin Garcia et al., 2003). Despite the economic valorization resulting both from recovery of OP oil and from energy production, these residues could be substantially valorized if other valuable chemical components are isolated after OP oil extraction and prior to burning. This is the case of phenolic compounds which, given their wide range of bio-applications, could be an important contribution to these residues valorization, while not fully compromising the energetic valorization of the remaining residue (~80% of the initial mass).

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OP contains about 98% of olive fruit phenolic compounds which can be divided in several classes: simple phenols (e.g., tyrosol (2-(4-hydroxyphenyl)ethyl alcohol) and hydroxytyrosol (HT) (2-(3,4-dihydroxyphenyl)ethyl alcohol)); cinnamic acid derivatives (e.g., caffeic acid and verbascoside); flavonoids (e.g., apigenin, luteolin and rutin (quercetin-3-rutinoside)); and secoiridoids (e.g., oleuropein, oleuropein aglycone and de(carboxymethyl)oleuropein aglycone isomers) (Obied et al., 2007a). Some of these compounds were also identified in DOR (Sampedro et al., 2004). Based on chemical composition, these industrial olive oil by-products (OP and DOR) are potential sources of valuable phenolic compounds, given their biological potential. Indeed, several biological activities have been attributed to compounds isolated from olives or olive derived products, focused mainly on antimicrobial (Bisignano et al., 1999), antioxidant (Obied et al., 2007b) and antitumoral (Menendez et al., 2008). In this perspective, increased valorization of these olive mill residues could be achieved through their detailed characterization and their exploitation (as mixtures or after isolation of valuable compounds) for nutraceutical or biomedical applications.

The present work reports the extraction and chemical characterization of phenolic compounds from Portuguese OP and DOR by HPLC–MS/MS and HPLC–MS^n, as well as the evaluation of their antioxidant activity and antiproliferative impact on a human breast cancer cell line (MDA-MB-231) aiming at selecting the residue from conventional OP processing units with the higher potential for the production of bioactive extracts.

2. Materials and methods

2.1. Reagents and standards

Dulbecco's modified Eagle's medium (DMEM) 4.5 g/L glucose and L-glutamine, fetal bovine serum (FBS), penicillin–streptomycin mixture 10 000 units/mL, trypsin (5 g/L)/EDTA (2 g/L) and trypan blue stain 0.4% (w/v) were purchased from Lonza (Verviers, Belgium). Methanol, ethanol, phosphoric acid, sodium acetate trihydrate and anhydrous disodium hydrogen phosphate were supplied by Merck (Darmstadt, Germany). Acetonitrile (HPLC-grade solvent) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Formic acid (purity > 98%) was purchased from Fluka Chemie Fluka Chemie GmbH (Buchs, Switzerland Parent company, Sigma-Aldrich Chemical Co.) (Sigma–Aldrich Co., St. Louis, MO, USA). Folin–Ciocalteu's reagent 2 N, sodium carbonate, potassium ferricyanide, iron chloride (III), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2-*tert*-butyl-4-methoxyphenol (BHA), gallic acid (97.5–102.5%, titration), 5-fluorouracil (5-FU) (purity ≥99%) and dimethylsulfoxide (DMSO) (cell culture grade) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Hydroxytyrosol (100 mg dissolved in 2 mL of ethanol) was obtained from Cayman Chemicals (Ann Arbor, MI, USA). Sodium dihydrogen phosphate dihydrate and trichloroacetic acid were purchased from BDH Prolabo (Nogent sur Marne, France). Water was treated in a Milli-Q water purification system (Millipore, Billerica, MA, USA).

2.2. Samples

Two-phase OP and DOR samples were collected in January 2010 at Mariano Lopes & Filhos, Lda. (UCASUL), OP oil factory located in Alvito, Beja, Portugal. OP was frozen at −80 °C and DOR was kept at room temperature protected from light until further use.

2.3. Extracts preparation

The extraction procedure was adapted from literature (Romero et al., 2002). OP was dried at 40 °C for 3 days until constant weight. OP and DOR were extracted using two different conditions: methanol/water 8:2 (v/v) and water. About 2 g of dried sample was mixed with 30 mL of extraction solvent under constant stirring, protected from light, for 40 min at room temperature. After centrifugation, for 10 min, at 9000 × *g*, room temperature (Centrifuge Hermle Z323K, Hermle Labor Technik, Wehingen, Germany), the supernatant was collected into a dark bottle. The leftover solid residue was extracted five times more, using the same extraction conditions, but with successively smaller stirring time periods (30, 15, 10 and 2× 5 min). All the collected supernatants were combined in order to obtain the final extracts, which were then filtered through a 0.22 μm PES filter (Pall Life Sciences, Ann Arbor, MI, USA). Methanol was removed from the extracts in vacuum at 37–40 °C and finally water was removed by freeze-drying (FTS Systems, Inc., Stone Ridge, NY, USA). The solid extracts were kept at −80 °C protected from light until analysis.

2.4. Determination of total phenolic content

Total phenolic content was determined using Folin–Ciocalteu's reagent (Ranalli et al., 2000). Briefly, 0.2 mL of sample was mixed with 1.5 mL of Folin–Ciocalteu's reagent 0.2 N. The reaction was kept in the dark for 5 min. Then, 1.5 mL of 7% (w/v) sodium carbonate solution was added to the mixture and the reaction was kept in the dark for 1 h. The absorbance was then read at 725 nm in a double-beam UV/vis spectrophotometer (Helios alpha spectrophotometer, Thermo Scientific, Bremen, Germany). Gallic acid was used as phenolic compound standard for calibration curve (20–120 μg/mL; $y = 0.0070x - 0.0016$, where x and y represent gallic acid concentration (μg/mL) and absorbance at 725 nm, respectively; $r^2 = 0.9999$).

2.5. Identification of phenolic components by HPLC–MS

HPLC–MS/MS analyses were carried out in a Hewlett–Packard (HP) 1050 liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a Rheodyne injector with a 10 μL loop, a quaternary pumping system and a UV detector. The column used was a Discovery® C-18 (150 mm × 2.1 mm × 5 μm) supplied by Supelco (Agilent Technologies, Waldbronn, Germany). The chromatographic separation was carried out at room temperature with a gradient elution program at a flow rate of 0.2 mL min^{-1}. The mobile phases consisted in water/acetonitrile (90:10, v/v) (A) and acetonitrile (B), both with 0.1% of formic acid. The following multistep linear gradient was applied: 0 min, 0%B; 3 min, 5%B; 18 min, 20%B; 19 min, 24%B; 24 min, 25%B; 30 min, 26%B; 31 min, 30%B; 61 min, 60%B; 67 min, 100%B. The injection volume in the HPLC system was 25 μL and the UV/vis detection was performed at 280 nm. Before HPLC injection, extracts were dissolved in water (HPLC grade) and then filtered through a 0.2 μm nylon syringe filter (VWR International, Carnaxide, Portugal).

The HPLC system was coupled to a Micromass spectrometer (Manchester, UK), operating in negative mode, equipped with an electrospray ionization source and a triple quadrupole (QqQ-MS) analyzer. The cone and capillary voltages were set at −30.0 V and −2.6 kV, respectively. The source temperature was 143 °C and the desolvation temperature was 350 °C. MS/MS spectra were obtained using argon as collision gas with the collision energy set between 20 and 25 V. The detection was carried out considering a mass range of 50–1000 m/z, with scan duration of 0.5 s. The data acquisition was done by using the MassLynx® data system (Waters, Milford, MA, USA). Phenolic compounds were identified by comparing their mass spectra with data from literature.

HPLC–MS^n analyses were carried out in a Thermo Scientific liquid chromatograph (San Jose, CA, USA) equipped with an injector of 10 μL loop, a quaternary pumping system and a diode-array detector (210–450 nm). The column used was a Discovery® C-18

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(150 mm × 2.1 mm × 5 μm) supplied by Supelco (Agilent Technologies, Waldbronn, Germany). The chromatographic separation was carried out at room temperature with a gradient elution program at a flow rate of 0.2 mL min^{-1}. The mobile phases consisted in water/acetonitrile (90:10, v/v) (A) and acetonitrile (B), both with 0.1% of formic acid. The following linear gradient was applied: 0 min, 0%B; 3 min, 0%B; 10 min, 10%B; 30 min, 20%B; 35 min, 25%B; 50 min, 50%B; 60 min, 0%B. The injection volume in the HPLC system was 25 μL and the chromatographic runs were performed at 280 nm. Samples were prepared for injection as described above.

The HPLC system was coupled to a LCQ Fleet spectrometer (San Jose, CA, USA), operating in negative mode, equipped with an electrospray ionization source and a Linear Ion Trap (IT-MS) analyzer. Optimal ESI conditions were as follows: nitrogen sheath gas, 40 abs; spray voltage, 5 kV; capillary temperature, 300 °C; capillary voltage, −28 V and tube lens voltage, −115 V. MS^n experiments were performed on mass-selected precursor ions using standard isolation and excitation configurations. The collision energy used was in the range of 30–45 (arbitrary units). Data acquisition was carried out with the Xcalibur data system (Thermo Scientific).

2.6. Quantification of hydroxytyrosol by HPLC/UV analysis

HPLC/UV analyses were carried out in a Merck-Hitachi HPLC system (Tokyo, Japan) that included an injector with a 10 μL loop, quartenary pump and UV/vis detector. Separations were achieved on a Sherisorb ODS-2 C18 column (250 mm × 4.6 mm × 5 μm) proceeded by a Sherisorb ODS-2 C18 pre-column (30 mm × 4.6 mm × 5 μm), both supplied by Waters (Milford, MA, USA). The mobile solvents consisted in water/acetic acid (95:5, v/v) (A) and methanol (B). The chromatographic separation was carried out at room temperature with a gradient elution program at a flow rate of 1 mL min^{-1}. The solvent elution was performed in gradient mode according to literature (Jerman et al., 2010). Samples were filtered using 0.2 μm nylon syringe filter (VWR International, Carnaxide, Portugal). The injection volume in the HPLC system was 25 μL and the UV/vis detection was performed at 280 nm.

Phenolic compounds identified by MS were quantified from the calibration curve (5–400 μg/mL) prepared with an authentic standard HT ($y = 10\,133x - 1872$ where x and y represent HT concentration (μg/mL) and peak area, respectively; $r^2 = 0.9998$). Standard solutions were prepared by diluting adequate volumes of a stock solution (1000 μg/mL) with HPLC grade water. These solutions were filtered prior to HPLC analysis as described above.

2.7. Evaluation of antioxidant activity

2.7.1. Reducing power

The reducing power was determined according to literature (Oyaizu, 1986). Samples (2.0 mL) containing different extract concentrations (1–367 μg/mL) in distilled water were mixed with 2.0 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 2.0 mL of 1% (w/v) potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. To stop the reaction, 2.0 mL of 10% (w/v) trichloroacetic acid solution was added and the mixture was shaken and centrifuged at 1000 rpm for 8 min (Hettich Universal 16A centrifuge, Tuttlingen, Germany). The upper layer (4.0 mL) was added to 4.0 mL of distilled water; plus 0.8 mL of 0.1% (w/v) ferric chloride (III) solution; the mixtures were well vortex before reading the absorbance of Prussian blue at 700 nm. An increase in absorbance indicated higher reducing power. Concentration providing 0.500 of absorbance (EC_{50}) was calculated from the absorbance graph plotted at 700 nm against concentration. HT and BHA (0.1–10.0 μg/mL) were used as reference compounds.

2.7.2. DPPH radical scavenging effect

DPPH scavenging capacity was assessed according to literature (Hatano et al., 1988). Samples (0.3 mL) containing several extract concentrations (1–734 μg/mL) in distilled water were mixed with 2.7 mL of a 6×10^{-5} M DPPH methanolic solution. The mixture was vortexed and left to stand in the dark for 1 h. The absorbance of DPPH free radicals was measured at 517 nm against a blank constituted by 0.3 mL of distilled water and 2.7 mL of methanol. DPPH scavenging effect was calculated as percentage of DPPH discoloration using the equation: % scavenging effect = $[(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where A_S is the sample absorbance and A_{DPPH} is the DPPH solution absorbance. The concentration providing 50% DPPH neutralization effect (EC_{50}) was calculated from the graph of scavenging effect percentage against the concentration logarithm. HT (0.10–6.00 μg/mL) and BHA (0.25–100.00 μg/mL) were used as reference compounds.

2.8. Evaluation of breast cancer antiproliferative activity of OP and DOR extracts, HT and 5-FU

2.8.1. Cell culture

The human breast cancer cell line MDA-MB-231, an estrogen receptor-negative cell line derived from a metastatic carcinoma, was obtained from American Type Cell Culture (ATCC, Manassas, VA, USA). The cells were grown in DMEM supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) penicillin–streptomycin mixture. The cell culture was maintained at 37 °C in a 5% CO_2 humidified atmosphere (C150, Binder GmbH, Tuttlingen, Germany). Before confluence, cells were trypsinized with trypsin (0.5 g/L)/EDTA (0.2 g/L) solution and suspended in fresh growth medium before platting.

2.8.2. Viability assay

MDA-MB-231 cells were seeded, at 2×10^5 cells/mL, in 12-well cluster plates (Nunc, Roskilde, Denmark). The next day, medium was changed and further replaced by fresh DMEM supplemented with 10% FBS and different concentrations of: OP water extract (OPW) (1000–20 000 μg/mL); DOR water extract (DORW) (100–6000 μg/mL), HT (162–2595 μM) and 5-FU (1–10 000 μM). The HT and DORW were also studied concurrently with the 5-FU. Briefly, cells were incubated, during 48 h, with the IC_{50} value of 5-FU, in combination with either HT or DORW extract IC_{50} concentrations. Control cells were kept with vehicle alone (ethanol or DMSO) each assay was performed in three replications. After 48 h incubation period, the medium was discarded and cells were washed with phosphate buffer solution. The number of viable cells was counted after trypsinization in the hemocytometer (Neubauer Improved; 0.0025 mm^2, Labor Optic, Bad Hamburg, Germany) using two separate measurements per well. The concentration providing 50% cell growth inhibition (IC_{50}) was determined from cell viability graph, compared to control, against the concentration logarithm.

2.9. Statistical analysis

All experimental results were performed at least in triplicate ($n = 3$) and the data are expressed as means ± standard deviation. The IC_{50} comparative analysis of antiproliferative effect was done using the MIXED procedure of SAS (SAS Institute, Inc., Cary, NC, USA), considering extracts and wells, as fixed and random effects, respectively; the determinations within each well as repeated measures. The remaining data were analyzed using GLM procedure of SAS considering the extract as fixed effect. Where differences existed, the source of the differences at a $P < 0.05$ of significance

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Table 1
Total phenolic content expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g DW) in OP and DOR extracts and biomass, using water (OPW and DORW) and methanol/water (OPM and DORM).*

Extract	Extraction yield (%)	Total phenolic content (mg GAE/g DW)	
		Extract	Biomass
OPW	16.09	45.81 ± 1.42 c	7.37 ± 0.23 c
OPM	19.43	33.36 ± 0.46 d	6.48 ± 0.09 d
DORW	28.06	78.20 ± 1.61 a	21.94 ± 0.45 a
DORM	18.97	73.38 ± 0.90 b	13.92 ± 0.17 b

* Each value is expressed as mean ± standard deviation ($n = 3$). In each column different letters mean significant differences ($P < 0.05$) between different samples (Tuckey's test).

level was identified by all pairwise multiple comparison procedure. The Tuckey's test was used for pairwise comparisons.

3. Results and discussion

3.1. Extraction yields and total phenolic content (TPC)

DOR extraction yield was higher using water than using methanol/water (Table 1), in contrast with OP, which presented lower extraction yield percentages, regardless of using water or methanol/water solutions.

Concerning total phenolic contents, it can be observed (Table 1) that DOR extracts presented TPC 1.7 and 2.2 times higher (for water and methanol/water, respectively) than those obtained for the corresponding OP extracts, or 2.9 and 2.1 times higher if expressed on a starting biomass basis. This increment in extraction yields and particularly in the TPC of DOR extracts results certainly from the partial degradation of high molecular weight components during high temperature drying of OP, leading to the formation of higher amounts of soluble phenolic compounds (Fernández-Bolaños et al., 2002).

Finally, water was the most efficient solvent in terms of TPC for both OP and DOR (Table 1). In general, DOR water extracts will be the preferred subtracts in terms of gravimetric yield and TPC values.

3.2. Identification of phenolic compounds

HPLC chromatograms of OPW and DORW extracts recorded at 280 nm, are illustrated in Fig. 1A and 1B. Seven phenolic compounds were identified in DOR extracts, while only HT could be identified in OP extracts by the interpretation of their main product ions obtained by HPLC–MS/MS and HPLC–MS^n. Table 2 summarizes the retention time, the $[M-H]^-$ ion and the main product ions obtained by MS/MS and MS^n. Compounds (Fig. 2) were identified by comparing their elution order and fragmentation profiles with the literature as indicated in Table 2.

3.2.1. Simple phenols and derivatives

Compound **1** was identified as HT-1-glucoside based on the $[M-H]^-$ ion at m/z 315 and the product ion at m/z 153 (assigned to hydroxytyrosol ion) (De Nino et al., 1999). In addition, MS^3 spectrum of the ion at m/z 153 presented a product ion at m/z 123, also common to HT fragmentation profile, which may correspond to the loss of $-OCH_2$. Thus, the product ion at m/z 123 present in the MS/MS spectrum of this compound may result from the loss of a glucose–O–CH_2 fragment, indicating that the glucose unit is linked to the aliphatic hydroxyl of the HT molecule. This compound was previously identified in other OP methanol extracts (Cardoso et al., 2005).

Compound **2** was identified as HT by comparing its fragmentation profile and retention time with a reference compound ran under the same experimental conditions, and with published

Table 2
Phenolic compounds identified in OP and DOR extracts and the corresponding MS/MS and MS^n fragmentation profiles.[a]

Comp. no.	RT (min)	Compound name	$[M-H]^-$ (m/z)	Product ions (m/z)			Identif[b]	Presence in olive oil by-products[b]
				QqQ-MS/MS	IT-MS^n			
					MS^2	MS^3		
1	5.53	Hydroxytyrosol-1-glucoside	315	315, 217, 153, 135, 123, 119, 97, 89	**153**, 135, 123	123	(1)	OP (5)
2	6.34	Hydroxytyrosol	153	153, 123, 109	123, 85		(2)	OP (6, 7, 8), DOR (10, 11)
3	9.28	Tyrosol	137	137, 108, 93, 81, 53	119, 109, 91, 89, 67		(2)	OP (6, 7, 8), DOR (10, 11)
4	12.58	Oleuropein aglycone isomer	377	377, 197, 153, 119, 89	333, 309, **241**, 197, 153, 135	197	(2, 3)	OP (3, 5), DOR (11)
5	19.86	Verbascoside	623	623, 569, 461, 298, 161, 132	555, **461**, 443, 315	315, 297, 161, 135	(4)	OP (3, 6, 12), DOR (11)
6	23.17	Oleuropein	539	539, 377, 368, 345, 307, 275, 241, 223, 197, 191, 179, 119, 89, 57	**377**, 307, 275, 223	307, 275, 153, 149	(3, 4)	OP (3, 6, 12), DOR (11)
7	27.43	De(carboxymethyl)oleuropein aglycone isomer in aldehyde form	319	319, 183, 139, 111, 95	319, 273, 251, **183**, 139, 95	165, 139, 95	(2)	OP (3)

[a] m/z in bold was subjected to MS^n analysis.
[b] (1) De Nino et al. (1999), (2) De La Torre-Carbot et al. (2005), (3) Obied et al. (2007a), (4) Ryan et al. (1999), (5) Cardoso et al. (2005), (6) Romero et al. (2002), (7) Nastri et al. (2006), (8) Lesage-Meessen et al. (2001), (9) Obied et al. (2007b), (10) Aranda et al. (2007), (11) Sampedro et al. (2004), and (12) Obied et al. (2008).

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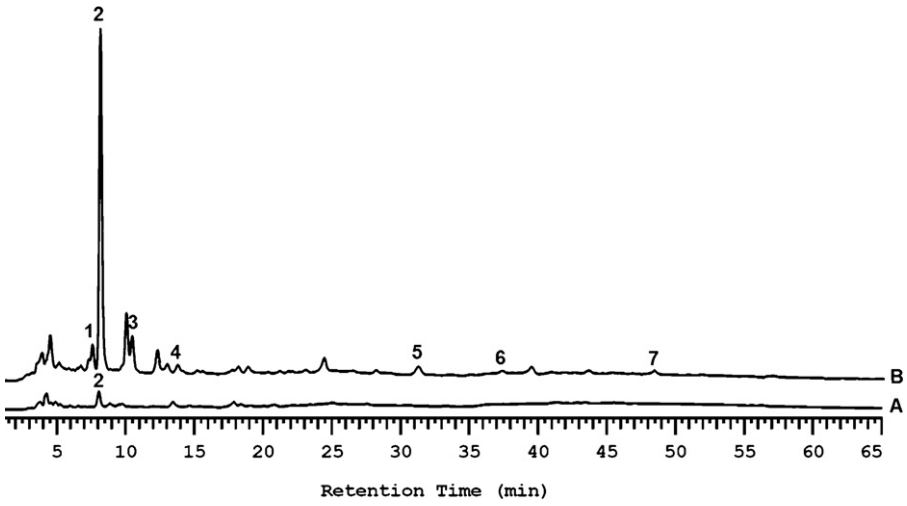

Fig. 1. HPLC chromatograms of (A) OPW and (B) DORW extracts at 280 nm. Peaks identification (numbering refers to compounds identified in Tables 2 and 3).

fragmentation profiles (De La Torre-Carbot et al., 2005). Compound **3** was identified as tyrosol by comparing the MS/MS product ions with published fragmentation profiles (De La Torre-Carbot et al., 2005). Both compounds were already identified in OP samples from different origins (Lesage-Meessen et al., 2001; Nastri et al., 2006; Obied et al., 2007a,b; Romero et al., 2002). HT and tyrosol were also found in DOR samples (Aranda et al., 2007; Sampedro et al., 2004).

3.2.2. Cinnamic acids

Compound **5** was identified as verbascoside based on the $[M-H]^-$ ion at *m*/*z* 623, as well as the corresponding MS^2 product ions at *m*/*z* 461 (resulting from the loss of a caffeic acid moiety) and *m*/*z* 161 (corresponding to an anionic ketene derivative of caffeic acid) (Ryan et al., 1999). Moreover, the MS^3 spectrum of the ion at *m*/*z* 461 showed a product ion at *m*/*z* 315 due to the loss of

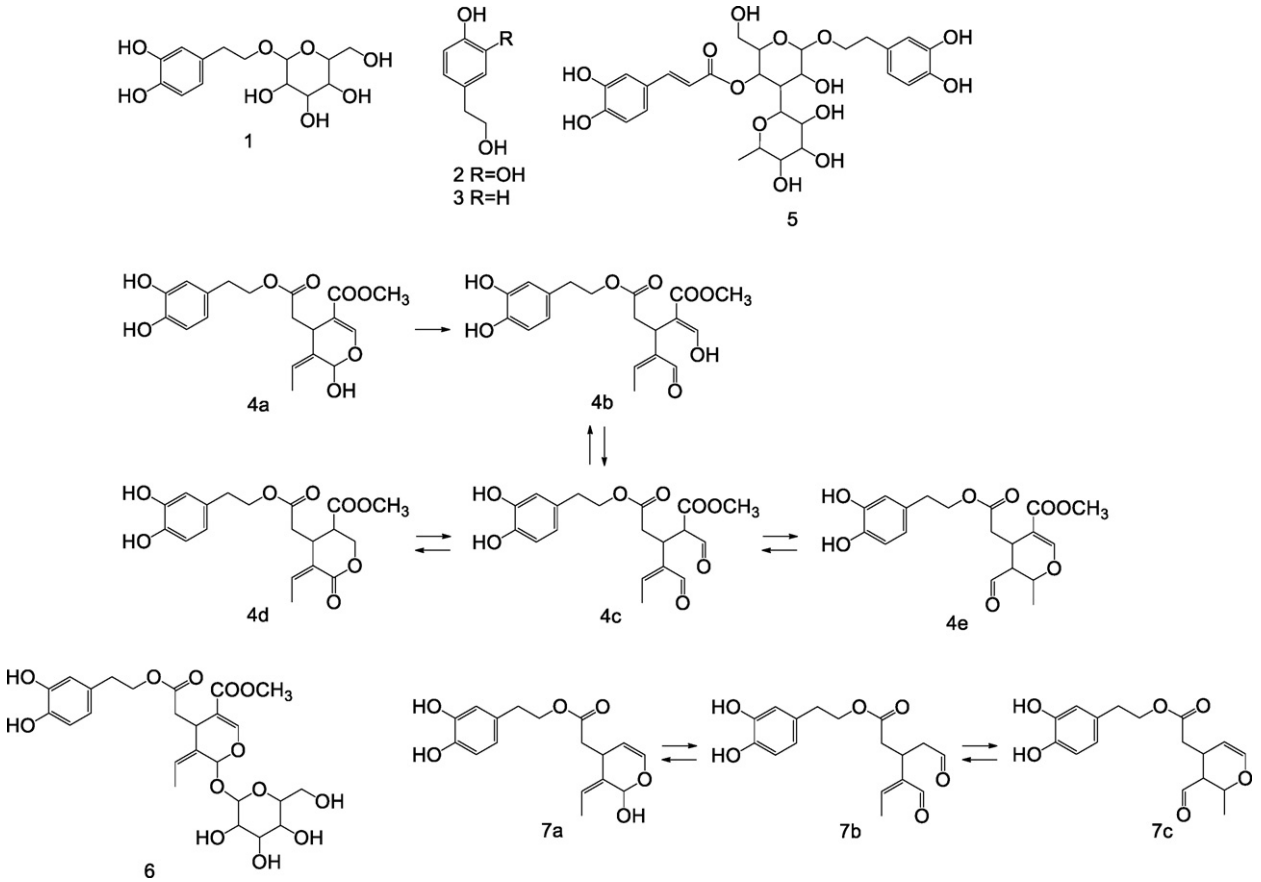

Fig. 2. Chemical structures of the phenolic compounds identified in OP and DOR extracts. **1**: hydroxytyrosol-1-glucoside; **2**: hydroxytyrosol; **3**: tyrosol; **4a**: oleuropein aglycone (3,4-DHPEA linked to elenolic acid); **4b**: acyclic enol form of oleuropeindial; **4c**: 3,4-DHPEA-EDA (3,4-DHPEA-elenolic acid dialdehyde) or acyclic dialdehyde form of oleuropeindial; **4d**: lactone product of Cannizzaro reaction; **4e**: 3,4-DHPEA-elenolic acid aldehyde (3,4-DHPEA-EA) (scheme adapted from Obied et al., 2007a); **5**: verbascoside; **6**: oleuropein; **7a**: de(carboxymethyl)oleuropein aglycone; **7b**: 3,4-DHPEA-deacetoxyelenolic acid dialdehyde (3,4-DHPEA-DEDA); **7c**: de(carboxymethyl)oleuropein aglycone aldehidic.

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a rhamnose unit. Verbascoside was identified in OP from different origins (Obied et al., 2007a, 2008; Romero et al., 2002), as well as in DOR samples (Sampedro et al., 2004).

3.2.3. Secoiridoids derivatives

Compound **6** was identified as oleuropein by comparing the MS^2 product ions with published data (Obied et al., 2007a; Ryan et al., 1999), namely the $[M-H]^-$ ion at *m/z* 539 and the product ion at *m/z* 377 (resulting from the loss of a glucose unit). In addition, the MS^3 spectrum of the ion at *m/z* 377 showed the product ion at *m/z* 153 which corresponds to the hydroxytyrosol moiety as discussed above. Oleuropein was found in OP (Obied et al., 2007a, 2008; Romero et al., 2002), as well as in DOR samples (Sampedro et al., 2004).

Compound **4** was assigned to an oleuropein aglycone isomer (Fig. 2), based on the fact that it shows several mass fragmentation product ions common to that compound, namely the ion at *m/z* 153 (hydroxytyrosol moiety) resulting from the fragmentation of $[M-H]^-$ ion at *m/z* 377 (De La Torre-Carbot et al., 2005; Obied et al., 2007a). Additionally, the MS^2 spectrum also showed the product ion at *m/z* 241 which corresponds to the elenolic acid ion. Furthermore, its MS^3 spectrum showed a product ion at *m/z* 197, which may result from the loss of a carboxyl moiety (—COO). However, this chromatographic peak may not be unambiguously assigned to any of the oleuropein aglycone isomers indicated in Fig. 2 (chemical structures **4a–e**), including its aldehyde forms (**4b–d**) which were reported to elute after oleuropein (**6**) (Fig. 2) (Obied et al., 2007a,b).

Compound **7** was assigned to the de(carboxymethyl)oleuropein aglycone isomer (Fig. 2 (**7a–c**)), based on the $[M-H]^-$ ion at *m/z* 319 and the corresponding MS^2 intense product ion at *m/z* 183 which is assigned to the de(carboxymethyl)elenolic acid derivative ion (De La Torre-Carbot et al., 2005). Besides, the MS^3 spectrum of the ion at *m/z* 183 showed a product ion at *m/z* 139 which may correspond to the loss of a COO group. In addition, MS^2 spectrum of compound **7** showed a product ion at *m/z* 111 which might be caused by the loss of CO and COO of the elenolic derivative fragment (*m/z* 183) in aldehyde forms: 3,4-DHPEA-deacetoxyelenolic acid dialdehyde (3,4-DHPEA-DEDA, **7b**) or de(carboxymethyl)oleuropein aglycone aldehidic (**7c**) (Fig. 2) (De La Torre-Carbot et al., 2005). However, the MS data did not allow to differentiate between the aldehyde forms of the de(carboxymethyl)oleuropein aglycone isomers.

During olive fruit maturation and olive oil extraction, oleuropein can be hydrolyzed by endogenous β-glucosidases, resulting in oleuropein cyclic aglycone (**4a**) which can undergoes ring opening and further transformations (**4b–4e**) (Fig. 2). These oleuropein aglycone structures have been detected in OP samples (Cardoso et al., 2005; Obied et al., 2007a). Until now, only oleuropein cyclic aglycone (**4a**) was identified in DOR samples (Aranda et al., 2007). Regarding to de(carboxymethyl)oleuropein aglycone isomers, only 3,4-DHPEA-DEDA (**7b**) was identified in OP samples (Obied et al., 2007a). However, the oxidation product of 3,4-DHPEA-DEDA (MW 336) was identified in DOR samples (Aranda et al., 2007). In the present study, we report for the first time the presence of a de(carboxymethyl)oleuropein aglycone isomer (MW 320) in aldehyde form (**7b** or **7c**, Fig. 2) in DOR extracts composition.

Since DORW extract presented the highest TPC (Table 1), quantification of the identified phenolic compounds was carried out. According to the data presented in Table 3, HT was the main phenolic compound of DORW, followed by verbascoside, but in much lower amounts (3.6-fold).

Given the known biological activity of HT, its content was determined in OP and DOR using water and methanol/water as extraction solvents (Table 4), in order to find the most adequate extraction system. There was no significant difference between OP water and methanol/water extracts, neither between DOR water and methanol/water ($P < 0.05$). Regardless the extraction solvent, the overall HT content was significantly higher in DOR than in OP, ranging from 4.46 to 7.07 mg/g DW (Table 4).

Table 3
Phenolic compounds content (milligrams per gram of dry weight (DW)) in DOR extract and biomass using water.*

Comp. no.	Phenolic compound	Content (mg/g DW)	
		Extract	Biomass
1	Hydroxytyrosol-1-glucoside	0.63	0.17
2	Hydroxytyrosol	25.21	7.07
3	Tyrosol	1.30	0.35
4	Oleuropein aglycone isomer	3.10	0.84
5	Verbascoside	7.02	1.90
6	Oleuropein	1.74	0.47
7	De(carboxymethyl)oleuropein aglycone isomer in aldehyde form	tr	tr

* Each value is expressed as the mean of three aliquots analyzed in triplicate (standard deviation < 5%). *Abbreviation*: tr: trace.

The higher HT content in DOR may be caused by the degradation of HT-containing high molecular weight compounds (Fernández-Bolaños et al., 2002; Obied et al., 2008) during the high-temperature drying process of OP before oil extraction. The HT content of the analyzed OP was low, but within previously published ranges (0.3–10 mg/g DW) (Lesage-Meessen et al., 2001; Obied et al., 2008; Perez-Serradilla et al., 2008). These results show that the high temperature treatment of OP before oil extraction can promote HT release, in agreement with previously reported studies (Fernández-Bolaños et al., 2002).

3.3. Antioxidant activity

Antioxidant activity of phenolic extracts was evaluated using two assays: reducing power and DPPH scavenging effect (Table 5).

DOR extracts presented a stronger reducing power than OP ones (5- and 2-fold for methanol/water and water extracts, respectively). OPW was more efficient than the OP methanol/water (OPM) extract, while the inverse relation was observed in DOR extracts. However, OP and DOR extracts were significantly less effective than HT and BHA ($P < 0.05$). When EC_{50} values were expressed in terms of TPC (μg GAE/mL) instead of the extract concentration, the reducing power of DOR extracts was higher than the synthetic BHA ($P < 0.05$) (Table 5). Thus, phenolic compounds present in DOR extracts might be more efficient as reducing agents compared to BHA.

DOR extracts were also more active to scavenge DPPH free radicals than OP ones (4- and 3-fold for methanol/water and water extracts, respectively) (Table 5). In addition, water extracts were more active than the methanol/water ones, being both olive oil by-products extracts significantly less active than HT and BHA ($P < 0.05$). When EC_{50} values were expressed in terms of TPC, the scavenging effect of DOR extracts was still less potent, although in the same range of pure standards (Table 5).

Table 4
HT content (milligrams per gram of dry weight (DW)) in OP and DOR extracts and biomass using water (OPW and DORW) and methanol/water (OPM and DORM).*

Extract	HT (mg/g dry weight)	
	Extract	Biomass
OPW	2.02 ± 0.04 b	0.32 ± 0.01 c
OPM	2.06 ± 0.03 b	0.40 ± 0.00 c
DORW	25.21 ± 0.99 a	7.07 ± 0.28 a
DORM	23.53 ± 1.10 a	4.46 ± 0.21 b

* Each value is expressed as mean ± standard deviation ($n = 3$). In each column different letters mean significant differences ($P < 0.05$) between different samples (Tuckey's test).

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Table 5
Reducing power and DPPH scavenging effect EC_{50} values of OP and DOR extracts, HT and BHA.*

Sample	Reducing power EC_{50}			Scavenging effect EC_{50}		
	μg/mL	μg GAE/mL	μM	μg/mL	μg GAE/mL	μM
OPW	108.72 ± 1.81 b	4.95 ± 0.08 a	–	73.11 ± 0.95 b	3.11 ± 0.04 b	–
OPM	174.55 ± 4.21 a	4.76 ± 0.12 a	–	119.33 ± 2.52 a	3.25 ± 0.07 a	–
DORW	57.15 ± 1.04 c	3.11 ± 0.06 c	–	24.27 ± 0.36 d	1.90 ± 0.03 c	–
DORM	38.68 ± 0.38 d	3.04 ± 0.03 c	–	29.56 ± 0.17 c	1.94 ± 0.01 c	–
HT	1.50 ± 0.11 e	1.50 ± 0.11 d	9.74 ± 0.72 b	1.22 ± 0.05 e	1.21 ± 0.05 d	7.89 ± 0.31 a
BHA	4.34 ± 0.11 e	4.34 ± 0.11 b	24.09 ± 0.63 a	1.13 ± 0.01 e	1.13 ± 0.01 d	6.27 ± 0.06 b

* Each value is expressed as mean ± standard deviation ($n = 3$). In each column different letters mean significant differences ($P < 0.05$) between different samples (Tuckey's test).

So, in general, DOR extracts presented the highest antioxidant potential within the olive oil by-products extracts, which may be related to TPC. Indeed, the reducing power and the DPPH scavenging effect are both inversely correlated with TPC on extract basis, with $R^2 = -0.95$ ($P < 0.05$) and $R^2 = -0.98$ ($P < 0.05$). The antioxidant activity can also be related with HT concentration in DOR extracts, which is about 12 times higher when compared to OP extracts. The reducing power and the radical scavenging antioxidant activities are also inversely correlated with HT concentration on extract basis, with $R^2 = -0.88$ ($P < 0.05$) and $R^2 = -0.90$ ($P < 0.05$), respectively. To the best of our knowledge, DOR antioxidant potential has never been reported in the literature. Our results underlie the high potential of valorization of olive by-products phenolic rich extracts for example as alternative to synthetic food antioxidants.

3.4. Breast cancer antiproliferative activity

The highest TPC and HT content, as well as the strongest antioxidant activity of DORW extract prompted us to investigate its antiproliferative potential, comparatively to OPW extract, on cultured human breast cancer cells (MDA-MB-231) vs control cells. The cell growth inhibition was also assayed with increasing concentrations of a well-known cytostatic agent, named 5-fluorouracil (5-FU). Similarly to the antioxidant activity, DORW extract was significantly more effective than OPW extract to reduce viable human breast cancer cells (Fig. 3A and B). DORW extract underlies a higher antiproliferative potential, being more effective than OPW extract (7-fold) (Table 6). HT (Fig. 3C) and 5-FU (Fig. 3D) inhibited more extensively MDA-MB-231 cell growth, when compared to olive oil by-products derived extracts (Table 6). Moreover, the

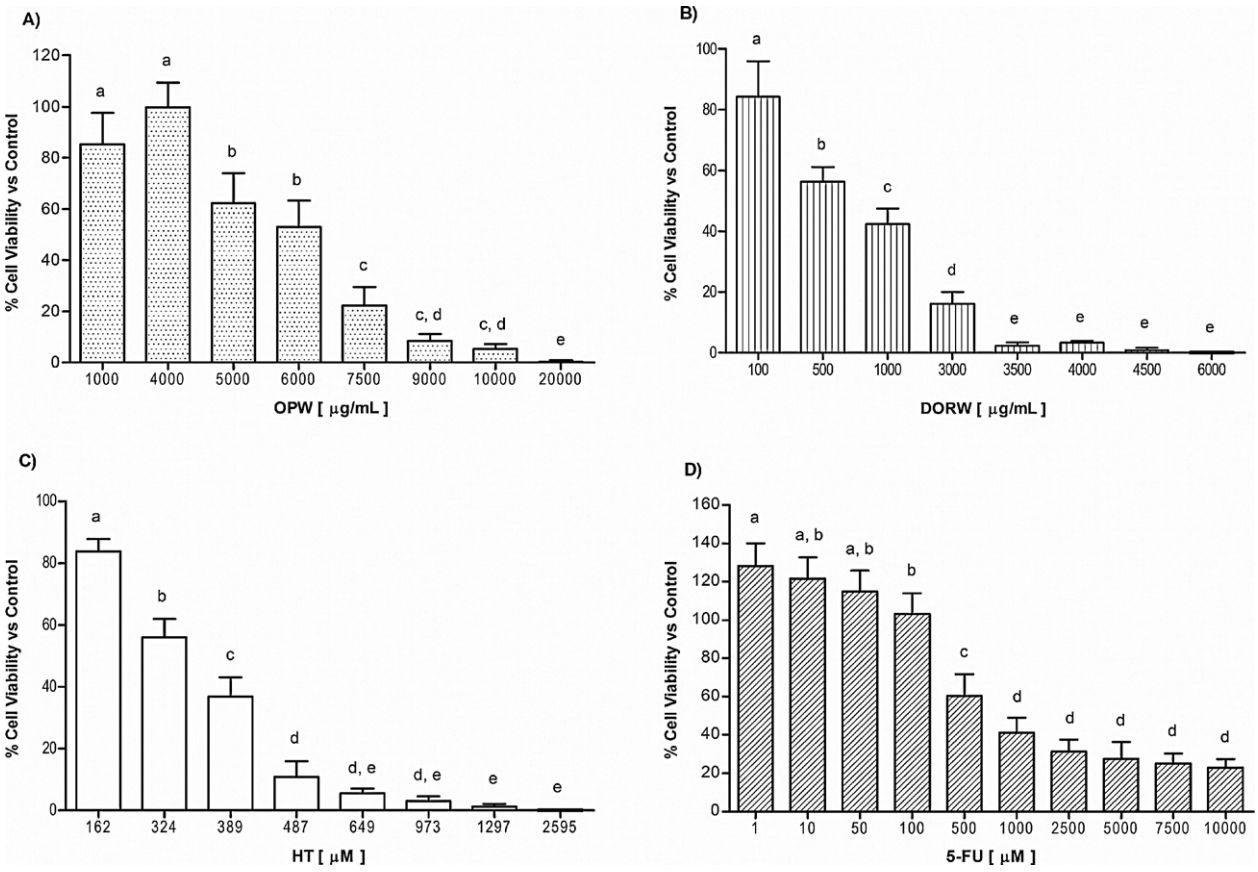

Fig. 3. Effect of different concentrations of (A) OPW, (B) DORW, (C) HT and (D) 5-FU on the proliferation of MDA-MB-231 breast cancer cells. Each value is expressed as mean ± standard deviation ($n = 3$). Different letters in columns of each figure mean significant differences ($P < 0.05$) between different concentrations (Tuckey's test).

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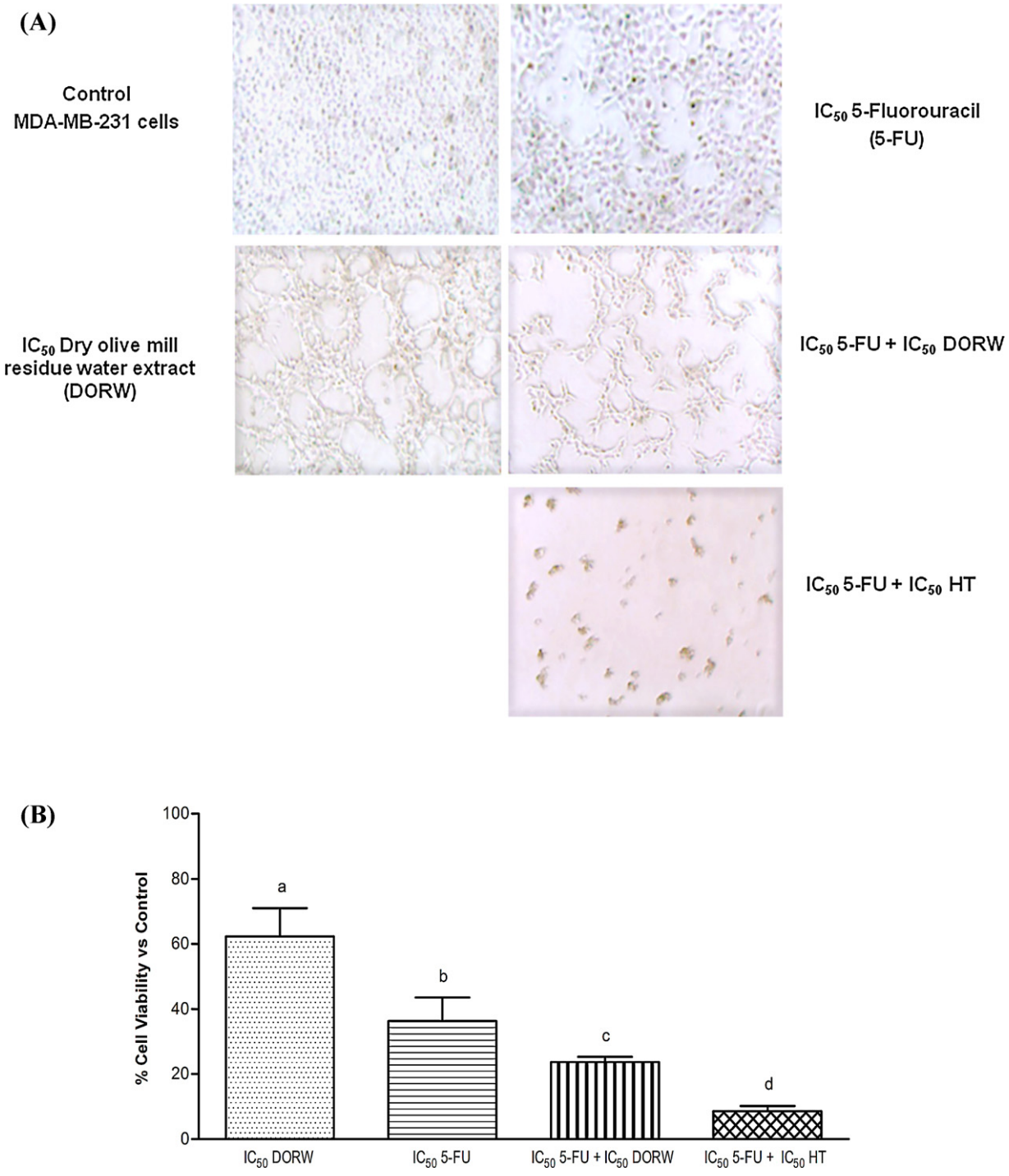

Fig. 4. Antiproliferative activity of the cytostatic agent 5-FU supplemented with DORW extract or HT on MDA-MB-231 cells: (A) morphological aspect and (B) percentage of cell viability after 48 h of incubation. Each value is expressed as mean ± standard deviation ($n = 3$). Different letters in columns of each figure mean significant differences ($P < 0.05$) between different concentrations (Tuckey's test).

antiproliferative activity of HT (IC_{50} value of 367.43 ± 21.10 μM) was about 1.75-fold stronger than 5-FU (IC_{50} value of 643.75 ± 65.83 μM) (Table 6). In fact, a cell viability of 18% (vs control) was even detected at the highest concentration of 5-FU (10 000 μM) (Fig. 3D). When IC_{50} values of extracts were expressed in terms of TPC, there was no statistical difference between the antiproliferative activities of DORW extract and pure standard HT ($P < 0.05$) (Table 6). According to the results, olive phenolic compounds present in DORW extract might be antiproliferative agents as active as HT, on MDA-MB-231 cell line *in vitro* model. At the present knowledge, this is the first study assessing the antiproliferative potential of OP and DOR extracts against the MDA-MB-231 human breast cancer cells.

Given the stronger antiproliferative activity of HT, and even of the phenolic compounds present in DORW extract, comparatively to 5-FU, we further access the MDA-MB-231 cell growth inhibition effect of 5-FU incubated simultaneously with DORW extract or HT. According to Fig. 4A, significant changes were observed in

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Table 6
Antiproliferative effect (IC_{50}) of OPW and DORW extracts, HT and 5-FU against MDA-MB-231 breast cancer cell line.*

Sample	Antiproliferative effect IC_{50}		
	μM	μg/mL	μg GAE/mL
OPW	–	6124.25 ± 489.75 a	280.53 ± 22.43 a
DORW	–	856.98 ± 192.07 b	67.02 ± 15.02 b,c
HT	367.43 ± 21.10 b	56.61 ± 2.85 c	56.61 ± 2.85 c
5-FU	643.75 ± 65.83 a	83.74 ± 8.56 c	–

* Each value is expressed as mean ± standard deviation ($n = 3$). In each column different letters mean significant differences ($P < 0.05$) between different samples (Tuckey's test).

the morphological aspect and cell density of MDA-MB-231 cells treated with 5-FU and DORW extract at the respective IC_{50} concentrations, comparatively to control cells. Interestingly, the 5-FU plus DORW mixture induced the inhibition of cell growth, in a more extensively way, than cells incubated separately with 5-FU or DORW extract (Fig. 4A). Nevertheless, the 5-FU plus HT mixture revealed to be even more active than 5-FU plus DORW mixture (Fig. 4A). According to Fig. 4B, the 5-FU plus HT mixture showed the strongest antiproliferative activity (8.6% of cell viability), which indicates that HT presence can highly potentiate the cytotoxic effect of 5-FU. On the other hand, cell growth inhibition by 5-FU plus DORW mixture was not much lower than 5-FU plus HT, regarding the HT contents in both mixtures (40% (w/w) and 2% (w/w), respectively). In this way, other phenolic compounds present in DORW extract, besides HT, might present synergistic effect with 5-FU, potentiating its cytostatic effect.

4. Concluding remarks

Although the high polyphenol content of olive mill residues has been widely described as a drawback on further valorization of derived waste streams, the extraction of valuable compounds, including phenolic compounds, might represent an interesting valorization route for these olive mill residues, leading to high added value products. The results described in this work demonstrate the potential biological applications of hydroxytyrosol-rich phenolic extracts, derived from dry olive mill residue, for food-nutraceutical applications. The antiproliferative activity of DOR derived extracts, against the breast cancer cell line used (MDA-MB-231) was demonstrated for the first time. Indeed, DORW extract showed to potentiate the breast cancer cell growth inhibition of 5-FU, a well-known cytostatic agent. These related beneficial health promoting effects of olive mill residues seem to be correlated to phenolic compounds, in particular to HT content presented by DOR. These bioactive compounds can be easily extracted with water, being an advantage to further applications in food and pharmaceutical industry. Finally, the fact that DOR extracts show higher bioactivity than OP counterparts allows to entirely preserve the use of this residue to produce OP oil, while obtaining the most valuable extracts from the final residue (DOR), opening new perspectives for the valorization of this agro-industry residue, by extracting high valuable compounds.

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