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Patricia Reboredo-Rodríguez[1,2], Carmen González-Barreiro[2], Beatriz Cancho-Grande[2], , Tamara Y. Forbes-Hernández[1], Massimiliano Gasparrini[1], Sadia Afrin[1], Danila Cianciosi[1], Alegría Carrasco-Pancorbo[3], Jesús Simal-Gándara[2], Francesca Giampieri[1*], Maurizio Battino[1*]

[1]Dipartimento di Scienze Cliniche Specialistiche ed Odontostomatologiche (DISCO)-Sez. Biochimica, Facoltà di Medicina, Università Politecnica delle Marche, Ancona, Italy.

[2]Nutrition and Bromatology Group, Department of Analytical and Food Chemistry, Faculty of Science, University of Vigo, Ourense Campus, E-32004 Ourense, Spain.

[3]Department of Analytical Chemistry, Faculty of Science, University of Granada, Ave. Fuentenueva s/n, E-18071 Granada, Spain.

Authors' e-mails:

Patricia Reboredo-Rodríguez (preboredo@uvigo.es)
Carmen González-Barreiro (cargb@uvigo.es)
Beatriz Cancho-Grande (bcancho@uvigo.es)
Francesca Giampieri (f.giampieri@univpm.it)
Tamara Y. Forbes-Hernández (tamara.forbe@gmail.com)
Massimiliano Gasparrini (m.gasparrini@univpm.it)
SadiaAfrin (dolla.bihs@gmail.com)
Danila Cianciosi (danila.cianciosi@gmail.com)
Alegría Carrasco-Pancorbo (alegriac@ugr.es)
Jesús Simal-Gándara (jsimal@uvigo.es)
Maurizio Battino (m.a.battino@univpm.it)

***Corresponding author:**

Maurizio Battino (m.a.battino@univpm.it)

Tel.: +39-071-220-4646

Fax: +39-071-220-4123

Abstract

The aim of the present work was to evaluate the phenolic profile of the 'Brava' extra virgin olive oil and assess its potential as a "natural adjuvant" in combination with chemotherapy treatment. The total phenol content of the phenolic extracts was 764 mg gallic acid equivalents/kg and the total antioxidant capacity was 2309, 1881 and 2088 μM trolox equivalents/kg determined by Diphenyl-1-picrylhydrazyl free radical method, Ferric Reducing Antioxidant Power and Trolox Equivalent Antioxidant Capacity assay, respectively. Secoiridoids comprised 83% of the total phenolic compounds. The main secoiridoid from oleuropein was the main isomer of oleuropein aglycone (74 mg/kg). The main secoiridoid from ligstroside was the main isomer of ligstroside aglycone (214 mg/kg). These phenolic extracts showed a significant decrease in cell viability on MCF-7 breast cancer cells in a dose and time dependent manner. 48 h-treatments with different concentrations of the extracts induced intracellular ROS generation and cell death..

Keywords: olive oil; phenolic compounds; cytotoxicity; intracellular reactive oxygen species; apoptosis; breast cancer.

1. Introduction

Breast cancer is the second most common cancer in the world and by far the most frequent cancer among women with an estimated 1.67 million new cancer cases diagnosed in 2012 (25% of all cancers). Moreover, breast cancer ranks as the fifth cause of death from cancer overall (522,000 deaths) (Ferlay et al., 2015). Therefore, new therapeutic strategies and chemotherapeutic candidates need to be urgently explored (Shi et al., 2013; Pan et al., 2017a; Pan et al., 2017b). The current interest is now focused on natural products which are major resources of prospective anti-cancer candidates to treat breast cancer by inducing cytotoxic effects (Bishayee et al., 2011; Zhou et al., 2014).

Olive oil is the main lipid source in the Mediterranean area and one of the most characteristic foods of the Mediterranean diet (Bach-Faig et al., 2011; Martinez-Gonzalez & Martin-Calvo, 2016; Piroddi et al., 2017). The beneficial effects of olive oil are related to its high content in monounsaturated fatty acid (MUFA) oleic acid (Gerber, 1997; Menéndez et al., 2006; Quiles et al., 2006a; Escrich et al., 2007, Bullón et al., 2013), and to the presence of a non saponificable fraction which contains bioactive phenolic compounds (Owen et al., 2000; Sotiroudis et al., 2003; Cicerale et al., 2009, Granados-Principal et al., 2014; Robles-Almazan et al., 2018).

Evidence indicates that phenolic compounds from olive oils can exert chemopreventive effects against different cancers (Acquaviva et al., 2012; Pampaloni et al., 2014). By targeting breast cancer, many studies have been recently published describing their implication on breast cancer cells. It was proved that *Oleuropein*, a heterosidic ester of elenoic acid with hydroxytyrosol, induced apoptosis on MCF-7 cells via a p53-dependent pathway mediated by Bax and Bcl2 genes, suggesting that it may have possible therapeutic potential in breast cancer patients (Hassam et al., 2013). *(+)*-

pinoresinol has anti-tumour effects at low concentrations by promoting cytotoxic, antiproliferative and pro-oxidant activities on MCF-7 and MDA-MB-231 breast cancer cells, independently of their oestrogen receptor status (López-Biedma et al., 2016). *S (−)-Oleocanthal*, a naturally occurring secoiridoid from extra virgin olive oil (EVOO), has attracted considerable attention due to its numerous biological effects against breast cancer (Elnagar et al., 2011). It could be a potential therapeutic option in combination with endocrine treatments in hormone-dependent breast cancer (Ayoub et al., 2017) attenuating cell proliferation, invasiveness and tumor growth in different breast cancer models (Alk et al., 2014). Many of these studies have been performed on MCF-7 cells. In fact, MCF-7 breast cancer cells are a widely used epithelial cancer cell line, derived from breast adenocarcinoma (ATCC, Manassas, VA, USA). According to the four main molecular subtypes, including the presence or absence of hormone (estrogen or progesterone) receptors (HR+/HR-) and excessive levels of human epidermal growth factor receptor 2 (HER2+/HER2-), MCF-7 can be classified as *Luminal A (HR+/HER2-)* (which represent around 74% of breast cancers) (American Cancer Society, 2017).

The first report research on prospecting, collecting and characterizing olive plant material from the main olive-growing area of Galicia (NW Spain) has been recently published (Reboredo-Rodríguez et al., 2018). This work shed some light on 'Mansa' and 'Brava' virgin olive oils by contributing detailed quality and genuineness-related information, a sensory description and their phenolic profile. 'Brava' EVOO was chosen among both oils due to its highest phenolic content, which could exert *a priori* greater beneficial health effects against several common diseases.

In this context, the main objective of this paper is to assess the potential health effects of phenolic extracts of 'Brava' EVOO on MCF-7 breast cancer cell line by evaluating the cytotoxic properties and also the effect on apoptosis and intracellular reactive oxygen

species (ROS) generation. Given the fact that phenolic profile of EVOOs can be very effective in its biological activity, a systematic phenolic characterization of the studied oil was undertaken by HPLC/DAD–ESI/MSn.

2. Materials and Methods

2.1. Olive Oil Sample

Olives were harvested in November 2016 in a growing area located between the municipalities of Ribas do Sil (42° 27′59.8″ N, 7° 17′15.8″W) and Quiroga (42° 29′04.8″ N, 7° 12′33.4″W) in the province of Lugo (NW Spain). All olive fruits were obtained under organic agricultural practices. Monovarietal olive oil was obtained from the 'Brava' cultivar by using an Oliomio 50 processing machine (Mori-Tem, Tavarnelle Val di Pesa, Italy) equipped with a knife crusher, a horizontal malaxator and a two-phase decanter. Malaxation trials were carried out at 20 ºC for 45 min. Once in the laboratory, samples were kept at a constant temperature of 4 °C in amber bottles without headspace until the phenolic compound extraction.

Physico-chemical and sensory properties of this Galician olive oil allowed it to be classified as *Extra Virgin* (Reboredo-Rodríguez et al., 2018) in accordance with Regulation EU 1348/2013 since their quality indices fall within the legally established ranges (European Union Commission, 1991, 2013).

2.2. Extraction of Phenolic Compounds from 'Brava' EVOO

2.2.1. Extraction of Phenolic Compounds from 'Brava' EVOO for LC-MS analysis

Phenolic compounds were extracted in duplicate from the EVOO according to the methodology proposed by Bajoub, Ajal, Fernández-Gutierrez, & Carrasco-Pancorbo (2016). Briefly, phenolic compounds were extracted by shaking the olive oil sample (2 g with the addition of 25 µL of the IS solution (amount of IS which was evaporated under N_2)) with *n*-hexane (1 mL) and acetonitrile (6 mL) for 3 min in a vortex; the mixture was then centrifuged at 3500 rpm for 6 min at room temperature. The acetonitrile fraction was separated and the residue was once again extracted with

acetonitrile (2 mL) and centrifuged under the same conditions. The combined acetonitrile extracts were evaporated under reduced pressure at 37 °C (rotary evaporator, Büchi R-210). The obtained residue was reconstituted in 1 mL of acetonitrile:water (50:50, v/v) and filtered through 0.22 μm membrane (nylon) filter.

2.2.2. Extraction of Phenolic Compounds from 'Brava' EVOO for the spectrophotometric and in vitro analysis

Phenolic fraction was extracted from olive oils following the International Olive Council method (IOC/T.20/Doc N 29), with some modifications. Briefly, 2.0 g of olive oil were weighed in a 15 mL screw-cap tube and 5 mL of MeOH:H_2O (80:20, v/v) was added. The tube was shaken for 1 min. The extraction was performed by an ultrasonic bath for 15 min at room temperature. Finally, the tube was centrifuged at 4800 rpm for 25 min and the MeOH:H_2O phase was collected and was filtered through a 0.45 μm filter with a cellulose acetate syringe filter. For subsequent experimental procedures, eight methanolic extracts were concentrated together and dried first with a rotary evaporator and subsequently with a SpeedVac resulting in approximately 20 mg of dried material. After that, samples were stored in aliquots at -80 °C.

2.3. Determination of Total Phenolic Content (TPC)

TPC was determined by using the Folin–Ciocalteu method, as modified by Slinkard and Singleton, (1977). Briefly, 500 μL of hydromethanolic phenolic extract was mixed with 2.5 mL of Folin–Ciocalteu reagent (10%) and kept 5 min at room temperature. Then, 2 mL of CH_3CO_2Na solution (0.7 M) was added. After 2 h of incubation at room temperature in the dark, a UV-Vis spectrophotometer was used to measure the

absorbance at 760 nm. Gallic acid was used as standard and the results were expressed as milligrams of gallic acid equivalents (GAE) per kg of olive oil (mg GAE/kg).

2.4. Determination of Total Antioxidant Capacity (TAC)

For determination of TAC (Quiles et al., 2006b) of the phenolic extracts, three different methods were employed: Diphenyl-1-picrylhydrazyl (DPPH) free radical method, Ferric Reducing Antioxidant Power (FRAP) and Trolox Equivalent Antioxidant Capacity (TEAC) assays.

DPPH radical assay was performed according to the protocol proposed by Kumaran and Karunakaran, (2007).

FRAP assay was performed according to the protocol proposed by Deighton et al., (2000).

TEAC assay was performed according to the method proposed by Re et al., (1999).

Trolox was used as standard and the results were expressed as μmoles of Trolox equivalents (TE) per kg of olive oil (μM Trolox/kg).

2.5. LC-MS analysis of Phenolic Extracts from 'Brava' EVOO

Phenolic extracts were analyzed using an Agilent 1260-LC system (Agilent Technologies, Waldbronn, Germany) equipped with a diode-array detector (DAD), which was coupled to a Bruker Daltonic Esquire 2000TM ion trap mass spectrometer (Bruker Daltonik, Bremen, Germany) by an electrospray ionization (ESI) interface. The same LC system was also coupled to a micrOTOF-Q II^{TM} mass spectrometer (Bruker Daltonics) by means of an ESI source. Several injections of each extract were made using high resolution-MS to corroborate the identity of the compounds under study. LC-ESI-IT-MS was employed to carry out the quantification experiments in full scan mode.

Chromatographic data acquisition and examination of DAD signals was performed by using ChemStation B.04.03 software (Agilent Technologies). Bruker mass spectrometers were controlled using the software Esquire Control and the resulting files were treated with the software Data Analysis 4.0 (Bruker).

A Zorbax C18 analytical column (4.6 × 150 mm, 1.8 μm particle size) protected by a guard cartridge of the same packing was used. Column oven temperature was set at 25 ºC. The mobile phases were 0.5 % acetic acid in water (A) and acetonitrile (B). A flow rate of 0.8 mL/min was used and an injection volume of 10 μL of the phenolic extracts and/or standards was selected. The established elution gradient was the following: 0 to 10 min, 5-30 % B; 10 to 12 min, 30-33 % B; 12 to17 min, 33-38 % B; 17 to 20 min, 38-50 % B; 20 to 23 min, 50-95 % B. Finally, the B content was decreased to the initial conditions (5 %) in 2 min and the column re-equilibrated for 2.5 min before the subsequent injection.

The Q-TOF MS system operated in negative and positive mode (to increase the information achieved about the olive oil samples) within the range of 50-1200 m/z, at a scan speed of 240 ms. A drying gas (N_2) temperature of 300 ºC and a flow of 9.0 L/min were selected as optimum. The capillary voltage was set at 4500 V and the end plate offset at -500 V. Internal calibration was performed using sodium formate clusters. The ion trap mass analyzer was used in negative ion mode. The capillary voltage was set at +3200 V and the MS detector was programmed to perform scans with the m/z range 50-800. The following parameters of ESI-MS were used: drying gas temperature, 300 °C; drying gas flow, 9 L/min; and nebulizing gas pressure, 30 psi.

Identification of the phenolic compounds present in the analyzed samples was based on the use of pure standards (when available), retention time data, extracted ion

chromatograms, accurate MS signals and comparing the MS/MS spectra with previously published results (Bajoub et al., 2016).

2.6. Cell Culture

MCF-7 breast cancer cell line was kindly provided by Prof. Mirco Fanelli of the Department of Biomolecular Sciences (Università degli Studi di Urbino Carlo Bo, Urbino, Italy) and was grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum as well as 100 IU/mL penicillin and 100 µg/mL streptomycin. Cells were maintained in a CO_2 incubator at 37 °C under a humidified atmosphere (95% air, 5% CO_2).

2.7. Cell Viability: MTT Assay

Cells were seeded into 96-well plates at a density of 5×10^3 cells/well and treated with different concentrations (from 0 to 1 mg/mL) of the dried hydromethanolic phenolic extract for 24, 48, and 72 h. The dried extract was directly dissolved in the cell culture medium containing 1% of dimethyl sulfoxide (DMSO). After incubation, 30 µL of RPMI medium containing 2 mg/mL of MTT were added to each well, and cells were incubated for another 2 h at 37 °C. MTT solution was then discarded, and 100 µL of DMSO were added to each well to dissolve the formazan crystal. The level of colored formazan derivative was analyzed on a microplate reader at a wavelength of 590 nm (Moongkarndi et al., 1991; Studzinski et al; 1995). The viable cells were directly proportional to the formazan production and the percentage of viable ones was calculated as (absorbance of treated cells/absorbance of control cells) x 100.

2.8. Assessment of Intracellular ROS Production by the Tali® Image-Based Cytometer

Determination of intracellular ROS levels was performed using the CellROX®

Oxidative Stress Kit (Invitrogen TM, Life Technologies, Milan, Italy) according to the manufacturer's instructions. Cells were seeded in 6-well plates at a density of 1.5×10^5 cells/well and treated with different concentrations of the dried phenolic extract (0.01, 0.05, 0.10, 0.25, and 0.50 mg/mL) for 48 h. After treatment, cells were trypsinized and centrifuged at 1500 rpm for 10 min at room temperature. Then, the supernatant was discarded and the cellular pellet was re-suspending in 0.5 mL of complete medium. Finally, 1 µL of CellROX® Orange Reagent was added and samples were incubated for 30 min at 37 °C. Cells were centrifuged once again to remove the medium and re-suspended again in 100 µL PBS. Finally, samples were analyzed with the Tali® Image-Based cytometer (Thermo Fisher Scientific, Milan, Italy), and the results were expressed as the percentage of cells with increased ROS levels compared with the control (Alvarez-Suarez et al., 2016; Giampieri et al., 2017).

2.9. Apoptosis Detection using the Taly® Image-Based Cytometer

Apoptosis was measured using the Tali® apoptosis assay kit and propidium iodide (Invitrogen®, Life Technologies, Milan, Italy) as previously reported (Giampieri et al., 2014; Islam et al., 2017). Cells were seeded in 6-well plate at a density of 1.5×10^5 cells/well and treated with different concentrations of the dried phenolic extract (0.01, 0.05, 0.10, 0.25, and 0.50 mg/mL) for 48 h. At the end of the treatment, cells were centrifuged (1500 rpm for 5 min) and resuspended in 100 µL of Annexin V Binding Buffer (ABB). After that, 5 µL of Annexin V Alexa Fluor® 488 were added, mixed again and the solution was incubated in the dark at room temperature for 20 min. Then, cells were centrifuged again at 1000 rpm for 10 min, resuspended in 100 µL of ABB and 1 µL of propidium iodide was added, mixed and incubated in the dark at room temperature for another 5 min. Finally, samples were analyzed using the Tali® Image-

Based cytometer and the percentage of early apoptotic, dead and live cells was determined on the basis of the corresponding fluorescence histogram compared with an untreated control. The annexin-V positive/propidium iodide negative cells were recognized as apoptotic cells by the cytometer software whereas the annexin V - positive/propidium iodide positive cells were identified as dead cells. Similarly, the annexin V-negative/PI negative cells were identified as viable cells. The results were expressed as fold increase compared with the control.

2.10. Statistical Analysis

The results are expressed as the mean values with standard deviations (SD) of three experiments and the statistical analysis was performed using STATISTICA software (Statsoft Inc., Tulsa, OK, USA). The significant differences represented by letters were obtained by a one-way analysis of variance (ANOVA) followed by a Tukey's honestly significant difference (HSD) post hoc test *($p<0.05$)*.

3. Results and Discussion

3.1. Characterization of Phenolic Extracts of 'Brava' EVOO

3.1.1. Spectrophotometric characterization Spectrophotometric characterization was carried out in order to evaluate the TPC and TAC of the phenolic extracts, as well as possible losses after the drying step. The results obtained are shown in **Table 1**. As can be seen, the TPC before drying the phenolic extracts of 'Brava' EVOO was 764 mg GAE/kg. Olive oils can be categorized according to their TPC as "low content" (50-200 mg GAE/kg), "medium content" (200-500 mg GAE/kg) and "high content" (500-1000mg GAE/kg) (Montedoro et al., 1992). Taking into consideration this classification, 'Brava' EVOO can be categorized as "high content". Moreover, the results here obtained were compared with the TPC of the best known varieties worldwide (**Table 1**): Arbequina, Cornicabra and Picual from Spain; Frantoio and Leccino from Italy; Galega from Portugal; Koroneiki from Greece and Chemlal from North Africa. 'Brava' EVOO showed the highest concentration of TPC.

TAC was evaluated by DPPH, FRAP and TEAC assays (**Table 1**). As with the TPC, a comparison with the TAC of the best known varieties worldwide was also performed (**Table 1**). Concerning the DPPH assay, 'Brava' EVOO showed higher values than Leccino, Cornicabra, Arbequina and Picual. Regarding the FRAP assay, also 'Brava' EVOO showed higher values than Picual and Arbequina. When the results of the TEAC assay of the 'Brava' EVOO were compared with other varieties like Picual, Cornicabra, Arbequina, Koroneiki, Chemlal and Leccino, it ranked third after Picual and Cornicabra. This remarkable antioxidant capacity makes phenolic extracts from 'Brava' EVOO good candidates to evaluate its potential as chemotherapeutic agent on MCF-7 cells. In fact, several studies reported that the cytotoxic effect observed in different cell

lines after treatment with different fractions (broccoli extracts, Radošević et al., 2017; or exopolysaccharides from *Grifola frondosa*, Zhang et al., 2016) correlate well with the content of total phenols and antioxidant capacity.

Finally, significant differences ($p<0.05$) were observed among TPC and TAC – determining by DPPH and FRAP assays– before and after drying the phenolic extracts of 'Brava' EVOO.

3.1.2. Phenolic profiling by LC-MS analysis

Characterization of 'Brava' EVOO was also determined by LC-ESI-IT-MS. **Table 2** summarizes the main phenolic compounds identified, including their name, acronym, molecular formula, m/z ESI-IT MS signal considered for their quantification, as well as their retention time. Additionally it includes LC-ESI-Q-TOF MS data (accurate MS signals, mSigma error, molecular formula and fragmentation patterns). A calibration curve of every available pure standard was constructed using different concentrations of the standard mixture solution and plotting the peak areas versus the concentrations, obtaining correlation coefficients (r^2) above 0.999. When a pure standard was not available, the quantification was made using the calibration curve of a similar (or structurally related) compound: hydroxytyrosol (HyT) was used for oleuropein aglycon (Ol Agl) and related compounds; tyrosol (Tyr) was used for ligstroside aglycon (Lig Agl) and related compounds; lignans were quantified in terms of pinoresinol (Pin); luteolin (Lut) was used for diosmetin (Dios); ferulic acid was used for vanillic acid (Van) and vanillin (Val); and finally, oleuropein (Ol) was used for all elenolic acid (EA) -derivatives. The results were expressed in mg per kg of olive oil, as mean±SD (of three different extracts; n = 3).

Table 3 displays the respective single identification and quantification of the phenolic compounds, whereas a comprehensive description of the results is shown below. Phenolic acids, flavonoids, lignans, phenyl alcohols and secoiridoids are the main families identified in the EVOO sample. Additionally, **Figure 1** displays the base peak chromatogram of 'Brava' EVOO extract.

Phenolic acids. Van, *p*-coumaric acid (*p*-Cou) and Val were the phenolic acids identified in 'Brava' EVOO showing concentrations of 0.3, 0.2 and 0.04 mg/kg, respectively.

Flavonoids. Lut, apigenin (Apig) and Dios were present in 'Brava' EVOO with concentrations of 2.1, 0.6 and 0.1 mg/kg, respectively.

Lignans. Syringaresinol (Syr), Pin and acetoxypinoresinol (Ac-Pin) were the phenolic compounds identified in this family. These phenolics seem to be among the most stable ones during the processing and oil storage. Syr and Pin were quantified at concentrations of 0.06 and 0.2 mg/kg, respectively.

Phenyl alcohols. Hyt, Ty, hydroxytyrosol acetate (Hyt-Ac) and oxidized hydroxytyrosol (O-Hyt) were the group of phenyl alcohols often related to the healthy properties attributed to olive oil consumption (Commission Regulation (EU) 432/2012, 2012). Hyt and Tyr were quantified at levels of 8 and 11 mg/kg, respectively.

Secoiridoid derivatives. Oleuropein and ligstroside are the predominant phenolic compounds in *Oleaceae* olive fruits. In fact, the predominant phenols in olive oils are the respective aglycones of oleuropein and ligstroside (which will be discussed below). Both of them were hydrolyzed by endogenous glucosidases during olive oil extraction. Secoiridoids comprised 83 % of the total phenolic compounds. 'Brava' EVOO (618 mg/kg) was compared with two EVOOs obtained from the best known varieties in

Spain: 'Cornicabra' (865 mg/kg) and 'Picual' (506 mg/kg) (Figueiredo-González et al., 2018). ‘Brava’ EVOO ranked second after ‘Cornicabra’ EVOO.

Secoiridoids from oleuropein are Hy-D-Ol-Agl, DOA, 10 Hy-Ol-Agl, OlAgl, 2 isomers of OlAgl and methyl OlAgl. All of them were tentatively quantified using Hyt as reference standard due to the lack of commercial standards. The main secoiridoids from oleuropein in 'Brava' EVOO were DOA (3,4-DHPEA-EDA, 59 mg/kg) and the main isomer of OlAgl (3,4-DHPEA-EA, 74 mg/kg).

Secoiridoids from ligstroside are D-LigAgl and 4 isomers of LigAgl. They were tentatively quantified using Ty as reference standard. They represented 73 % of the total secoiridoid compounds in 'Brava' EVOO. The main secoiridoids from ligstroside were D-LigAgl (*p*-HPEA-EDA or oleocanthal, 23 mg/kg) and the main isomer of LigAgl (*p*-HPEA-EA, 214 mg/kg).

EA- derivatives. Secoiridoids are characterized by the presence of EA in its glucosidic or aglyconic form in their molecular structure. Partial modification of oleuropein and ligstroside secoiridoids during olive oil extraction process resulted in EA derivatives, which were considered non-phenolic (but related) compounds. D-Ald-D EA, Desoxy-EA, Hy-EA and EA were determined in ‘Brava’ EVOO and quantified using oleuropein as reference standard. D-Ald-D EA, Desoxy-EA and EA were present at concentrations of 13, 25 and 68 mg/kg.

3.2. Cytotoxic effects of Phenolic Extracts of ‘Brava’ EVOO on MCF-7 cells

To evaluate the possible cytotoxic effects of the phenolic extracts of ‘Brava’ EVOO on MCF-7 cells, a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay was performed. Cells were treated for 24, 48, and 72 h with different concentrations (0.00, 0.01, 0.05, 0.10, 0.25, 0.50, 0.60, 0.70, 0.75, 0.80, and

1.00 mg/mL) of the phenolic extracts corresponding approximately to 0.00, 6.41, 32.05, 64.10, 160.25, 320.50, 384.60, 448.70, 480.75, 512.80, and 641.00 μg phenolic compounds/mL (considering that the total concentration of phenolic compounds is 641 mg/kg since quinic acid and elenoic acid derivatives were not considered) (**Figure 2**). The IC_{50}− which is defined as the concentration required for 50 % inhibition of cell growth− was calculated for such treatments. As shown in **Figure 2**, the IC_{50} was not reached with treatments at 24 h meanwhile it was around 0.50 mg/mL at 48 h and 72 h. The cytotoxic effects in breast cancer cell lines for some single phenolic compounds present in olive oil have already been studied. (-)-Oleocanthal treatment resulted in a dose-dependent inhibition of MCF-7 cell growth in mitogen-free media with IC_{50} values of 24.07 μM (7.32 μg/mL) (Ayoub et al., 2017). On the other hand, the IC_{50} values for (-)-oleocanthal treatment in HGF-supplemented media were 10.9, 20.1 and 25.4 μM (3.32, 6.12 and 7.73 μg/mL) on MDA-MB-231, MCF-7 and BT-474 breast cancer cells, respectively. However, high IC_{50} values have been described for (-)-oleocanthal treatment in HGF-free media, 16.2, 40.8 and 58.4 μM (4.93, 12.42 and 17.77 μg/mL) on MDA-MB-231, MCF-7 and BT-474 breast cancer cells, respectively (Alk et al., 2014). In all cases, the IC_{50} concentrations were really low suggesting that (-)-oleocanthal health benefits could be observed from concentrations at μM levels. Finally, López-Biedma et al. (2016) observed an interesting statistically significant cytotoxic effect following 0.01 μM (3.58 ng/mL) (+)-pinoresinol treatment on MCF-7 and MDA-MB-231 human breast tumour cells tested, but not on human mammary epithelial cells (MCF-10A).

In the present study, phenolic extracts decreased cell viability in a dose and time dependent manner. The decrease of MCF-7 breast cancer cells viability could be due to the presence of some specific phenolic compounds. *Pin* was detected in the phenolic

extracts at a concentration of 0.2 mg/kg and its anti-tumour effects at low concentrations have been recently reported by López-Biedma et al.(2016). It was demonstrated that *Apig* significantly suppressed the proliferation of both MCF-7 and MCF-7/ADR cells in a dose-dependent manner after 72 h of treatment at 40, 60, 80 and 100 μM (10.80, 18.26, 21.60 and 30,43 μg/mL) (Seo et al., 2017). Also doses from 10-75 mM of *Hty* and *oleuropein* determined statistically significant dose-dependent inhibition of E2-induced MCF-7 cell proliferation and concentrations equal and above 100 mM were toxic (15.42 mg/mL for *Hty* and 54.05 mg/mL for *oleuropein*) (Sirianni et al., 2010). According to our results, it seems that not only the full amount of phenolic compounds could exert beneficial health effects but maybe also the presence of some 'key' phenolics at very low concentrations.

The subsequent experiments were performed with phenolic extracts of 'Brava' EVOO at concentrations of 0.00, 0.01, 0.05, 0.10, 0.25, and 0.50 mg/mL for 48 h corresponding to approximately 100.0, 111.9, 101.1, 95.0, 78.5, and 41.0% of viable cells.

3.3. Intracellular ROS Production by Phenolic Extracts of 'Brava' EVOO on MCF-7 cells

ROS are by-products of the aerobic metabolism, and include superoxide anion, hydrogen peroxide and hydroxyl radicals (Espinosa-Díez et al., 2015). ROS have a dual effect on cancer: on the one hand, higher ROS levels have been found to play a role in tumor initiation but, on the other hand, they perform a chemotherapeutic effect in suppressing cancer growth by promoting apoptosis and cell death (Liou et al., 2010). To determine intracellular ROS production, MCF-7 cells were treated with different concentrations of the phenolic extract (0.00, 0.01, 0.05, 0.10, 0.25, and 0.50 mg/mL) for 48 h and analyzed using the CellROX® Orange assay kit by Tali® Image-based

Cytometer (**Figure 3**). As can be seen, the highest percentage of intracellular ROS production was obtained after treatment with 0.50 mg/mL of the phenolic extract (9.8% fold increase compared to the control), showing significant differences ($p<0.05$) compared to the control and the other lower concentrations. These results suggested that phenolic extracts of 'Brava' EVOO induced ROS generation, which could be considered a chemotherapeutic effect in suppressing cancer growth. Recently, it has been demonstrated that oleocanthal exerts antitumor effects on human liver and colon cancer cells through ROS generation (Cusimano et al., 2017). Other natural phenolic compounds, such as carvacrol present in essential oils, showed cytotoxic, genotoxic, apoptotic, ROS generating, and GSH-reducing effects on human gastric adenocarcinoma (AGS) cells in a dose-dependent manner (Günes-Bayir et al., 2017).

3.4. Apoptosis Rate Regulation by Phenolic Extracts of 'Brava' EVOO on MCF-7 cells

Apoptosis can be defined as a mechanism characterized by a series of different biochemical and morphological changes, which include increase in ROS level, activation of caspases, cell shrinkage, chromatin condensation and nucleosomal degradation (Forbes-Hernández et al., 2014). Apoptosis plays a crucial role in eliminating hyper-proliferating cells from the system (Halder et al., 2008), so induction of apoptosis represents an efficient strategy for cancer chemotherapy in order to evaluate and design new chemotherapeutic agents (Taraphdar et al., 2001). To determine the level of live, dead cells and those in early apoptosis, MCF-7 breast cancer cells were treated with different concentrations of the phenolic extract (0.00, 0.01, 0.05, 0.10, 0.25, and 0.50 mg/mL) for 48 h and analyzed by Tali® Image-based Cytometer. As shown in **Figure 4**, the addition of phenolic extracts of 'Brava' EVOO significantly reduced the level of live cells compared to the control ($p<0.05$) and significantly

induced the level of dead cells compared to the control ($p<0.05$). On the other hand, no significant differences in the level of apoptotic cells compared to the control ($p<0.05$) were found. A possible explanation of these differences could be that the treatments quickly induce apoptosis. If the apoptotic process runs very fast after the treatments in most of cells, a great number of them would be in later apoptotic state at the moment of the analysis and they would be quantified as dead cells. However, other apoptosis-independent cell death mechanisms could be responsible for the increase in cell dead observed after the different treatments, at least in part. Previous studies have demonstrated that (-)-oleocanthal at a concentration of 25 μM induced apoptosis in MDA-MB-231 breast cancer cells (Akl et al., 2014), thereby highlighting their pro-apoptotic effects. Also pentacyclic triterpenes (erythrodiol, uvaol, oleanolic acid, and maslinic acid)−found in the skin of olives− have been evaluated for their pro-apoptotic capacities. The results obtained in this study showed that 100 μM (44.27 μg/mL) of erythrodiol strongly induced apoptosis (increasing the apoptotic rate from 12 % in control untreated cells to 64 %), whereas no effect on apoptosis induction was observed by the rest of the triterpenes (Allouche et al., 2011). In order to elucidate the apoptotic pathway, molecular studies are now underway in our laboratory determining the level of the expression of the main apoptosis-related proteins, such as caspase-3, caspase-8 and caspase-9, cytochrome c, Bax, Bcl-2, p53, and PARP1 (Nikoletopoulou et al., 2013).

4. Conclusions

Local Galician producers traditionally grind together fruits from the ancient autochthonous cultivars 'Brava' and 'Mansa' in different proportions. Up to now, only one paper has reported the spectrophotometic characterization of the monovarietal 'Brava' extra virgin olive oil (EVOO). In the present work, a description of its phenolic profile has been widely assessed. Moreover, phenolic extracts from ‘Brava’ EVOO were tested as a natural alternative chemotherapeutic agent on MCF-7 breast cancer cell line. The results demonstrated that phenolic extracts from ‘Brava’ EVOO induce cell death and increase intracellular ROS production. In order to elucidate the pathways involved in the chemopreventive activity of this “natural alternative chemotherapeutic agent”, molecular studies are now underway in our laboratory.

Acknowledgments:

Patricia Reboredo-Rodríguez acknowledges award of a post-doctoral contract from Xunta de Galicia. The authors are grateful to Miguel Rodríguez González (Aceites Figueiredo) for providing olive samples. The authors wish to thank Monica Glebocki for extensively editing the manuscript. The UVIGO team has received funding for this study from FEDER, Interreg España-Portugal Programme, under the framework of the Projects: ref. 0377_IBERPHENOL_6_E and ref. 0181_NANOEATERS_1_E.

Conflicts of Interest: The authors declare no conflicts of interest.

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Table and Figure Captions:

Table 1. Characterization of the TPC and TAC of 'Brava' and other well-known varieties worldwide.

Table 2. Information for the main phenolic compounds (or related analytes) identified in the studied olive oil by using LC-ESI-Q-TOF MS (accurate MS signals, molecular formula, mSigma error, and fragmentation patterns (indicating the fragments in decreasing order of intensity, without decimal figures to contain the size of the table)).

Table 3. Mean± SD (mg/kg) of the phenolic compounds in the evaluated 'Brava' EVOO.

Figure 1. Base peak chromatogram obtained using the optimum LC/ESI-IT-MS conditions for 'Brava' EVOO. The peak numbers are the same as those used in **Table 2**. Peak numbers: (2) O-Hyt; (3) Hyt; (4) Ty; (5) Van; (6) D-Ald-D EA; (7) *p*-Cou; (8) Val; (9) Desoxy-EA; (11) Hyt-Ac; (12) EA; (13) Hy-D-Ol-Agl; (14) DOA; (15) Syr; (16) Lut; (17) Pin; (20) OlAgl (Is1); (21) D-LigAgl; (22) Apig; (23) Dios; (24) LigAgl (Is1); (25) OlAgl; (26) LigAgl (Is2); (27) OlAgl (Is2); (29) LigAgl; (30) LigAgl (Is3).

Figure 2. Inhibition of cell proliferation by reconstructed phenolic extracts of 'Brava' EVOO (**A**: 24h; **B**: 48h; **C**: 72h) on MCF-7 cell line. After 24 h of cell seeding, MCF-7 were treated with different concentrations of the phenolic extracts (0-1 mg/mL) for 24,

48 and 72 h. Cell viability was measured by using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay. Results were expressed as a percentage (%) of viable cells compared to control cells (containing 1 % DMSO). Data are shown as the mean±SD (n=3).

Figure 3. Reconstructed phenolic extracts of 'Brava' EVOO induce ROS generation on MCF-7 cells. MCF-7 cells were treated with the indicated concentrations of 'Brava' phenolic extracts (0.00, 0.01, 0.05, 0.10, 0.25, and 0.50 mg/mL) for 48 h. Intracellular ROS levels were calculated by using CellROX® Orange assay kit and the Tali® Image-Based Cytometer was used to quantify ROS induction (% of propidium iodide positive) on MCF-7 cells following the treatment previously described. The blue line of the thumbnail histogram indicated the set threshold. Data are shown as the mean±SD of three independent experiments (n=3). Columns with different superscript letters are significantly different from control ($p<0.05$).

Figure 4. Live, dead and early apoptosis levels on MCF-7 cells treated with the indicated concentrations of 'Brava' EVOO reconstructed phenolic extracts (0.00, 0.01, 0.05, 0.10, 0.25, and 0.50 mg/mL) for 48h. Tali® Image-Based Cytometer was used to quantify live, dead and early apoptosis cells following the treatment previously described. The blue line of the thumbnail histogram indicated the set threshold. Data are shown as the mean±SD of three independent experiments (n=3). Columns belonging to the same set of data with different superscript letters are significantly different from control ($p<0.05$).