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Abstract

When compared with other edible vegetable oils, the extra virgin olive oil (EVOO) exhibits excellent nutritional properties due to the presence of biophenolic compounds. Although they constitute only a very small amount of the unsaponifiable fraction of EVOO, biophenols strongly contribute to the sensorial properties of this precious food conferring it, for example, the bitter or pungent taste. Furthermore, it has been found that biophenols possess beneficial effects against many human pathologies such as oxidative stresss, inflammation, cardiovascular diseases, cancer and aging-related illness. In the present work, the biophenolic content of 51 Italian and Spanish EVOOs was qualitatively and quantitatively identified and their antioxidant ability analyzed by oxygen radical absorbance capacity (ORAC) assay. Results indicated that the maximum relationship can be found if the ORAC value is correlated with the concentration of the large family composed by ligstroside and oleuropein derivatives together with their degradation products, hydroxytyrosol and tyrosol. Then, selected biophenolic extracts were tested in NIH-3T3 cell line to verify their ability in the recovery of the oxidative stress revealed by DCFH-DA assay. Results were linearly correlated with the concentration of ligstroside aglycone (aldehyde and hydroxyl form).

Keywords	Biophenols; Olive oils; Oxidative stress; Cell protection

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Dear Editor,

Attached please find the electronic version of the manuscript entitled: "**Bioactive Compounds from Extra Virgin Olive Oils: Correlation Between Phenolic Content and Oxidative Stress Cell Protection"** by G. Presti, V. Guarrasi, E. Gulotta, F. Provenzano, A. Provenzano, S. Giuliano, M. Monfreda, M.R. Mangione, R. Passantino, P.L. San Biagio, D. Giacomazza and myself. The article concerns the study of the content of the antioxidant fractions obtained from 60 extra virgin olive oil samples and its correlation with data obtained by cell culture experiments in which the phenolic extracts were added.

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All authors have seen and approved the submission of the manuscript.

The authors hope that you will consider this paper of interest for submission in the Biophysical Chemistry journal as an original full-length research paper.

Sincerely

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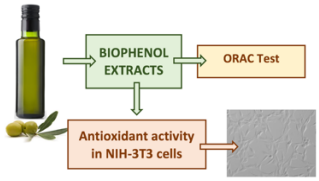
1. Mediterranean Olive Oils as powerful source of biophenols.
2. Complete analysis of the biophenol fraction.
3. Correlation between ORAC value and chemical classes of antioxidant species.
4. Relationship between molecular species and oxidative stress cell protection.



Mediterranean Olive Oils are used as sources of biophenols. Results correlate molecular species and oxidative stress cell protection.

Bioactive Compounds from ExtraVirgin Olive Oils: Correlation Between Phenolic Content and Oxidative Stress Cell Protection

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Abstract

When compared with other edible vegetable oils, the extra virgin olive oil (EVOO) exhibits excellent nutritional properties due to the presence of biophenolic compounds. Although they constitute only a very small amount of the unsaponifiable fraction of EVOO, biophenols strongly contribute to the sensorial properties of this precious food conferring it, for example, the bitter or pungent taste. Furthermore, it has been found that biophenols possess beneficial effects against many human pathologies such as oxidative stresss, inflammation, cardiovascular diseases, cancer and aging-related illness. In the present work, the biophenolic content of 51 Italian and Spanish EVOOs was qualitatively and quantitatively identified and their antioxidant ability analyzed by oxygen radical absorbance capacity (ORAC) assay. Results indicated that the maximum relationship can be found if the ORAC value is correlated with the concentration of the large family composed by ligstroside and oleuropein derivatives together with their degradation products, hydroxytyrosol and tyrosol. Then, selected biophenolic extracts were tested in NIH-3T3 cell line to verify their ability in the recovery of the oxidative stress revealed by DCFH-DA assay. Results were linearly correlated with the concentration of ligstroside aglycone (aldehyde and hydroxyl form).

Keywords: Biophenols; Olive oils; Oxidative stress; Cell protection

1. Introduction

Oxidative stress is one of major cellular features in the onset of many human pathological conditions such as Alzheimer's [1-4] and Parkinson diseases [5-7], renal disease [8,9], diabetes [10], ischemia [11, 12], atherosclerosis [13, 14], pulmonary dysfunction [15], cancer [16-18], and aging [19-20], and it occurs when excessive generation of free radicals produced during the normal cell metabolic processes is unbalanced by the antioxidant defense system. The latter includes endogenous enzymes (e.g. superoxide dismutase, catalase, glutathione reductase, glutathione (GSH) and glutathione peroxidase), metal binding proteins (ferritin, ceruloplasmin, lactoferrin and albumin) and exogenous chemical compounds mainly derived from fruits and vegetables (e.g. citrulline, taurine, creatine, selenium, zinc, A, C and E vitamins, and phenols) [3].

Free radicals, i.e. reactive oxygen species (ROS) and reactive nitrogen species (RNS), are generated by unstable and highly reactive oxygen- and nitrogen-based molecules with unpaired electrons. Generally, oxidative damage to the cellular components results in alteration of the membrane properties such as fluidity and ion transport, enzyme activities and protein cross-linking [21]. Furthermore, damages can extend to nuclear component and cellular organelles [22].

Under physiological conditions, free radicals are normally produced during the cellular metabolism, the main source being the mitochondrion and, in particular, the electron transport chain (ETC) where the continuous movement of high-energy electrons can generate superoxides [3] after interaction with O_2 molecules. In addition, other external factors can increase the levels of free radicals such as UV-light [23, 24], air pollution [25, 26], ionizing radiations [27, 28] and smoking [29, 30].

Due to the unhealthy consequences of oxidative stress and its implications, a huge number of studies have been devoted to the investigations of the therapeutic administration of antioxidants. They are chemical compounds, exclusively present in fruit and vegetables, able to prevent, slowing or terminating the radical formation reactions.

Thus, the interest of the researchers has been devoted to the discovery and valorization of food containing a large variety of antioxidants. Among these, extra virgin olive oil (EVOO) deserves a special place. EVOO is a key component of the traditional Mediterranean diet, which is believed to be associated with a relatively long life in good health.

Extra-virgin olive oil (EVOO) is recognized as one of the best food [31] for its capacity to prevent some diseases, such as cancer [32] and cardiovascular diseases [33], and to reduce their incidence in the western population. Furthermore, numerous studies show that olive oil reduces cholesterol [34, 35], lowers blood pressure [34] and incidence of breast cancer [36] and inhibits platelet aggregation [37]. EVOO is a complex mixture composed for about 98% by fatty acids esterified with glycerol, as mono-, di- and, prevalently, triglycerides, and unsaponifiable substances for the remaining 2%. This unsaponifiable fraction is constituted by squalene (about 50%), sterols, terpenic and aliphatic alcohols, methylsterols, biophenols and other compounds responsible for the oil particular taste and aroma [38].

Biophenols are responsible of the shelf-life of EVOO, because they avoid lipid oxidation through a variety of mechanisms based on radical scavenging, hydrogen atom transfer and metal-chelation [39].

Despite their low concentrations, phenolic compounds have been related with the healthy beneficial effects derived from consuming EVOO [40, 41].

In the present work, the phenolic fraction of 51 Sicilian EVOO samples was examined and the individual components were qualitatively and quantitatively determined. Moreover, the antioxidant efficacy of the 51 phenolic fractions was tested by the oxygen radical absorbance capacity (ORAC) method. Correlations between the conconcentration of the individual bioactive compounds or groups of compounds and ORAC values were studied. The highest correlation value was found with the large group composed by ligstroside and oleuropein derivatives, hydroxytyrosol and tyrosol. On the basis of this results, 9 biophenol extracts were chosen and evaluated for their ability to reduce ROS level induced in a cell model system by DCFH-DA assay.

2. Materials and Methods

2.1 Extravergin Olive Oil samples

32 Italian olive oil samples from Trapani geographical area (Trapani, Sicily) and 19 Spanish olive oil samples were analyzed. The Italian samples were a blend of the *Nocellara del Belice*, *Biancolilla* and *Cerasuola* cultivars while the Spanish ones were monocultivar samples of *Arbequina* and *Picual* olives. All the EVOOs were PDO (Protected Designation of Origin) registered, produced in 2014.

2.2 Chemicals and reagents

Folin-Ciocalteau, sodium carbonate, potassium persulfate ($K_2S_2O_8$), fluorescein, 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), gallic acid, Dulbecco's Modified Eagle's Medium (DMEM), Bovine Calf Serum, Penicillin-Streptomycin Solution (10,000 U/mL and 10,000 µg/mL, respectively), 2',7'-Dichlorofluorescin diacetate (DCFH-DA), Luperox® TBH70X thert-Butyl hydroperoxide solution (TBH) were purchased from Sigma-Aldrich (Milan, Italia). CellTiter 96® Aqueous One Solution Assay (MTS) was purchased from Promega. The water used in all experiments was Millipore MilliQ.

2.3 Biophenol extract preparation

The biophenol extraction was based on the standard method for the determination of biophenols in olive oils of the International Olive Council [42] with slight modifications. 2 g of olive oil were used and 5 mL of methanolic solution (80:20 v/v) were added. The mixture was put into vortex for 2 min and in ultrasonic bath for 25 min, then was centrifuged at 5000 rpm for 25 min at 20°C in a Beckman Avanti 30 Compact centrifuge (Beckman Coulter, Italy). All extracts were stored in the dark at 4° C.

2.4 Folin-Ciocalteu colorimetric assay

After retrieving the polar part, the determination of biophenols was done by using the Folin-Ciocalteu colorimetric assay. This method was performed accordingly to Hrncirik et al. [43] with minor modifications. An aliquot (0.2 mL) of the methanolic phase was diluted with water to a total volume of 5 mL, followed by the addition of 0.5 mL Folin-Ciocalteu reagent. After 3 min, 1mL sodium carbonate solution (20% w/v) was added to the reaction mixture which was finally mixed and diluted with water to 10 mL. The absorbance of the solution was measured after 2 h against a blank sample on a Shimadzu Spectrophotometer at the wavelength of 765 nm. Concentration values were reported by using a standard curve obtained with gallic acid.

2.5 FAST HPLC measurements

The methanol:water (80:20 v/v) extracts were analysed using a THERMO ACCELA HPLC equipped with an UV-vis photodiode array detector and a 5-µL sample loop. An Accucore C18 column (2,1mm i.d. x 100mm with particle size 2,6 µm) was used with detection at 280 nm. The column was maintained at 25 °C, the flow rate was 0.5 mL/min using a mixture of methanol/ acetonitrile (1:1 v/v) (A) and an aqueous solution of phosphoric acid (0.2% v/v)

(B) as mobile phase. The gradient used is shown in Table I below. The recognition of the biophenols and the determination of their concentrations were done through the use of siringic acids and tyrosol as standards. The sample injected volume was 1 μL.

TIME (min)	A(%) (v/v)	B(%) (v/v)	μL/min
0.00	4.0	96.0	500.0
3.00	4.0	96.0	500.0
20.00	50.0	50.0	500.0
23.00	60.0	40.0	500.0
24.00	60.0	40.0	500.0
25.00	100.0	0.0	500.0
28.00	50.0	50.0	500.0
30.00	4.0	96.0	500.0
35.00	4.0	96.0	500.0

Table I - FAST HPLC gradient. Gradient applied during the HPLC experiments for the identification of the phenolic fraction of the oil extracts.

2.6 HPLC-MS measurements

In order to confirm the biophenol content obtained by Fast HPLC measurements, HPLC-MS experiments on a HPLC Thermo, equipped with autosampler and MS/MS detector Quantum Access Thermo triple quadrupole, were done on the same methanol:water (80:20 v/v) extracts described in 2.5 paragraph. An Hypersil Gold column (2.1 mm i.d. x 15 mm with particle size 1.9 μm) was used with detection at 280 nm. The column was maintained at 25 °C, the flow rate was 0.35 mL/min using a mixture of water/acetic acid (97.5:2.5 v/v) (A) and methanol/acetonitrile (1:1 v/v) (B) as mobile phase. The gradient used is shown in Table II, below. The sample injected volume was 10 μL.

The HESI source settings are: 350 °C capillary temperature; 300 °C vaprorization temperature; 35.0 (a.u.) sheath gas pressure and -4000.0 (negative polarity) spray voltage. The main biophenolic molecules identified and confirmed were oleuropein derivatives (*m/z* 319 e 377) and ligstroside derivatives (*m/z* 303 e 361) as reported by Tasioula-Margari and Tsabolatidou [44].

TIME (min)	A(%) (v/v)	B(%) (v/v)	µL/min
0.00	95.0	5.0	350.0
2.00	75.0	25.0	350.0
10.00	65.0	35.0	350.0
22.00	65.0	35.0	350.0
23.00	35.0	65.0	350.0
30.00	0.0	100.0	350.0
35.00	95.0	5.0	350.0
40.00	95.0	5.0	350.0

Table II - HPLC-MS gradient. Gradient applied during the HPLC-MS experiments for the identification and confirmation of the phenolic fraction of the oil extracts previously identified by FAST HPLC measurements.

2.7 Oxygen Radical Absorbance Capacity (ORAC)

The ORAC assay was performed accordingly to Cao et al. [45] and Ninfali et al. [46] and slightly modified. The reaction was carried out in 0.075 M Na-K phosphate buffer (pH 7.0), and the final reaction mixture was 200 µL. 160 µL of 0.04 µM fluorescein and 20 µL of properly diluted biophenolic extract or 20 µL of 100 µM Trolox were placed in a 96 well black microplate. Samples were preincubated for 10 min at 37°C in the dark and the reaction was started with the addition of 20 µL of 40 mM AAPH in each well. The control was the methanol solution (80:20 v/v) properly diluted in 0.075 M Na-K phosphate buffer (pH 7.0). The fluorescence decay was measured every 5 min at 37°C using a Thermo Scientific Fluoroskan Ascent F2 Microplate at 485 nm excitation and 538 nm emission until zero fluorescence was detected. All the reaction mixutures were prepared in triplicate and at least three independent assays were performed for each sample. Because the reaction is driven to completion, one can quantitate the protection by calculating the area under the curve from the experimental sample. Thus, the ORAC value refers to the net area under the curve of fluorescein decay in the presence of biophenolic extract or Trolox, minus the blank area. The activity of the sample was expressed as µmol of Trolox Equivalents (TE) per gram of oil, with the following equation:

$$ORAC\left(\mu mol\ TE\ g^{-1}\right) = k \cdot a \cdot h\left[\frac{\left(S_{sample} - S_{blank}\right)}{\left(S_{Trolox} - S_{blank}\right)}\right] \quad (1)$$

where k is the final dilution of the methanolic extract; a is the ratio between the volume (liters) of the methanolic extract and grams of oil; h is the final concentration of Trolox expressed as μmol/L; and S_{sample}, S_{blank} and S_{Trolox} are the areas under the fluorescence decay curve of fluorescein in the presence of sample, methanol solution properly diluted in the buffer or Trolox, respectively.

2.8 Cell cultures and treatments

NIH-3T3 mouse embryo fibroblast cell line was purchased from Sigma-Aldrich. Cells were grown in DMEM supplemented with 10% Bovine Calf Serum (Sigma-Aldrich) and 1% penicillin-streptomycin (10,000 U/mL and 10,000 μg/mL, respectively) at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

2.9 Cell viability (MTS) assay

NIH-3T3 cells were seeded at a density of 10^4 cells per well in a 96-well plate. 24 hours later, cells were untreated or treated in triplicate with 25, 50 or 100 ng/mL of each biophenol extract selected on the basis of the results obtained in Figure 2. Cells were also treated with aqueous methanol solution at 0.1% (v/v) final concentration (f.c.) and corresponding to the maximum methanol concentration in the cell culture. Cell viability was measured by MTS assay. After 24 hours of treatment, 20 μL of the MTS solution was added to each well, and the incubation was prolonged for 4 hours at 37°C and 5% CO_2. The absorbance was read at 490 nm in the Bio-Rad iMark™ Microplate Reader. Results were expressed as the viability percentage of treated cells, considering untreated cells as the 100% reference.

2.10 Cellular ROS-level (DCFH-DA) assay

Intracellular reactive oxygen species (ROS) level after incubation with biophenol extracts was evaluated by 2', 7'-Dichlorofluorescin diacetate assay accordingly to Wang and Josef [47] with few modifications. In brief, 10^4 cells/well were seeded in 96-well black plate in medium culture lacking phenol red. The next day, cells were untreated or treated for 24 hours with each of the biophenol extracts, corresponding to the groups of samples selected on the basis of the results obtained in Figure 2. After treatment, cells were washed three times with PBS and then treated for 1 hour with DCFH-DA (final concentration, 50 μM in PBS pH 7.0; 0.5% DMSO v/v), washed again three times and then treated with 500 μM TBH for 30 min at 37°C to induce oxidative stress. The fluorescence resulting from oxidation of the nonfluorescent

dichlorofluorescin probe to fluorescent DCF by production of intracellular ROS was determined from measurements at 30 min after TBH addition in the Fluoroskan Ascent FL Thermo Scientific microplate reader (excitation 485nm and emission wavelength 528 nm). The emitted fluorescence is proportional to the ROS level produced within the cell cytosol. Results were expressed as ROS% of TBH-treated control, stated as 100%. All treatments and fluorimetric determination were performed in the dark.

2.11 Statistical analysis

Statistical analysis of the results was performed using the STATSOFT 6.0 program (Vigonza, Padova, Italy). The significant differences ($p \leq 0.05$) were evaluated by variance analysis (ANOVA) and the means separation was conducted using the Tukey post-hoc test.

To study the correlation between the polyphenol compounds (TPC and FC) and the antioxidant activity evaluated by ORAC assays. Pearson's correlation coefficient was applied carried out using SIGMAPLOT 10 software (Systat software Inc., San Jose, CA, USA).

3. Results and Discussions

In the recent decades, antioxidants have captured the interest of the researchers because of the discovery of the damages induced by oxidative stress during the cell functioning. These chemical compounds are available in vegetables and fruits where they are present in very small amounts. Their redox effect is exerted against oxidation of proteins [48, 49], nucleic acids [50] and lipids [48, 49, 51].

EVOO is a key component of the traditional Mediterranean diet and it is believed to be associated with a relatively long life in good health. EVOO is unique among other vegetable oils because of the high levels of valuable biophenolic compounds, to which, together with the high content of unsaturated fatty acids, the healthy benefits of EVOO are attributed. The biophenolic fraction of EVOO consists of a heterogeneous mixture of compounds, each of them influencing the chemical properties and quality of EVOO. In Figure 1 is reported the typical HPLC chromatogram obtained by analyzing one of the 51 biophenol extracts chosen as representative example. The biophenol concentration in olive oils ranges from 50 to 800 mg/kg, with a mean value of 180 mg/kg [52, 53]. The EVOO biophenolic fraction is chemically composed by phenolic acids (caffeic, vanillic, syringic, *p*-coumaric, *o*-coumaric, protocatechuic, sinapic, *p*-hydroxybenzoic and gallic acid), phenolic alcohols (tyrosol and

hydroxytyrosol), flavonoids, lignans and secoiridoids, the last deserving particular attention and being the most abundant compounds in the olive drupes [44].

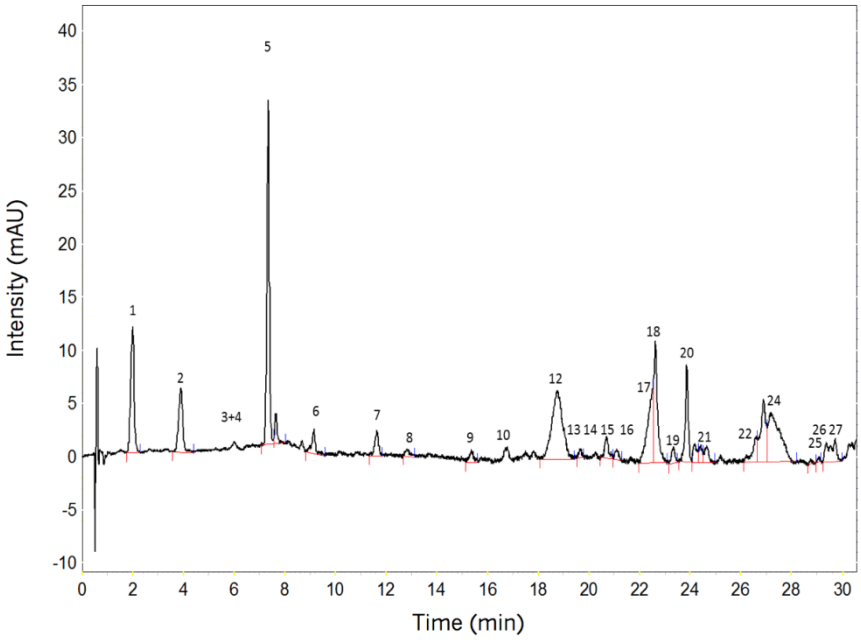

Figure 1 – FAST HPLC profile of biophenolic extract. Typical chromatogram of one of the 51 biophenolic extracts. The numbers in correspondence of the peaks indicate the biophenolic compounds: (1) hydroxytyrosol; (2) tyrosol; (3) vanillic acid; (4) caffeic acid; (5) syringic acid, internal standard; (6) vanillin; (7) *p*-coumaric acid; (8) hydroxytyrosyl acetate; (9) ferulic acid; (10) *o*-coumaric acid; (11) decarboxymethyl oleuropein aglycone, oxidized dialdehyde form; (12) decarboxymethyl oleuropein aglycone, dialdehyde form; (13) oleuropein; (14) oleuropein aglycone, dialdehyde form; (15) tyrosyl acetate; (16) decarboxymethyl ligstroside aglycone, oxidized dialdehyde form; (17) decarboxymethyl ligstroside aglycone, dialdehyde form; (18) pinoresinol; (19) cinnamic acid; (20) ligstroside aglycone, dialdehyde form; (21) oleuropein aglycone, oxidized aldehyde and hydroxylic form; (22) luteolin; (23) oleuropein aglycone, aldehyde and hydroxylic form; (24) ligstroside aglycone, oxidized aldehyde and hydroxylic form; (25) apigenin; (26) methyl luteolin; (27) ligstroside aglycone, aldehyde and hydroxylic form.

More in detail, hydroxytyrosol (3,4-dihydroxyphenylethanol or 3,4-DHPEA) and tyrosol (p-hydroxyphenylethanol or p-HPEA) are abundantly and exclusively present in olives, olive leaves and olive oil as free compounds or bonded to elenolic acid (EA) or its dialdehidic form (EDA) giving rise to the following secoiridoid derivatives: 3,4-DHPEA-EA (oleuropein

aglycon), *p*-HPEA-EA (ligstroside aglycon), 3,4-DHPEA-EDA, oleuropein and *p*-HPEA-EDA (oleocanthal). [53] The latter has been found responsible of the bitter and pungent taste of the olive oil [54] with an antinflammatory activity mimicking the ibuprofen effect [55, 56]. The presence or absence of additional groups, such as aldehydes, carbonyl, methyl as well as the transition from closed to open forms differentiates the chemical details of these molecules [44].

The antioxidant ability of the total biophenolic content of the 51 samples was evaluated with the ORAC assay. The test successfully performed to determine the antioxidant capacity of fruit juices, beverages and olive oils [45, 46, 57, 58] is a good quantitative indicator of the antioxidant power of a pure compound or mixture of compounds. It has also been used to reveal the increase of the plasma antioxidants in humans after intake of antioxidant-rich foods [59, 60]. In the ORAC assay, the time-dependent fluorescence decay of the fluorescein induced by the prescence of the radical initiator (AAPH), is counteracted by the antioxidant activity of the samples or by the Trolox, used as reference compound. Thus, the power of the antioxidant molecules is observed as the maintenance of the fluorescence signal. Data are expressed as equivalents of Trolox used as reference.

In table III are reported the concentrations of the most representative biophenols found in the 51 samples, as obtained by FAST HPLC and HLPC-MS analyses. In the last three columns are reported the total biophenolic fraction measured by HPLC technique (obtained as the sum of each identified component) and Folin-Ciocalteu (F-C) method and the ORAC value, respectively. It can be observed the good agreement between the total biophenol content determined by HPLC and F-C test.

EVOO Sample	HT	TY	VA	Van	FA	TY ac	PN	LU	AP	mLU	Ligst. der.	Oleur. der.	HPLC	F-C	ORAC value
# 1-I	7.1	5.5	0.0	2.7	32.3	0.0	3.0	9.1	0.0	5.0	13.0	34.8	114.7	106.9	4.8
# 2-I	23.3	18.6	0.0	5.9	2.2	5.2	15.6	17.1	1.1	2.9	59.5	79.0	233.8	259.2	9.2
# 3-I	25.6	13.9	0.0	3.5	2.0	3.3	9.6	19.1	3.3	7.8	61.2	112.0	263.8	303.7	10.6
# 4-I	21.5	14.4	0.0	5.1	4.5	0.0	3.8	11.8	0.9	9.5	43.2	69.1	183.8	214.1	8.1
# 5-I	4.3	3.9	0.0	0.0	4.7	0.0	3.1	6.1	0.0	4.4	15.6	21.3	63.5	68.0	3.2
# 6-I	11.5	8.6	0.0	4.4	2.4	0.0	20.9	7.5	0.0	8.6	39.8	55.7	159.4	162.2	6.8
# 7-I	12.8	11.4	1.8	4.3	0.0	5.7	40.8	6.8	5.8	3.4	47.5	71.4	214.3	244.0	9.1
# 8-S	15.3	12.5	0.0	8.5	0.0	5.9	53.5	6.6	7.4	2.2	70.0	124.0	306.0	287.6	9.8
# 9-S	16.4	12.7	1.4	8.2	0.0	5.8	54.9	7.9	6.5	1.6	71.0	91.0	277.6	316.3	9.5
# 10-I	6.4	7.0	0.0	4.0	0.0	0.9	6.9	11.2	3.5	6.0	20.8	55.6	122.3	172.3	6.9
# 11-S	14.7	15.2	1.9	10.1	7.2	0.0	25.9	1.8	2.6	6.0	81.1	65.4	231.9	236.2	8.6
# 12-S	13.1	10.6	0.0	5.7	4.6	4.5	7.7	10.9	7.4	2.9	50.6	104.6	222.6	313.3	10.3
# 13-S	52.1	34.4	2.8	5.5	8.0	3.3	9.3	10.3	4.6	3.5	29.8	58.0	221.4	201.8	7.4
# 14-I	6.7	3.3	0.0	0.0	4.0	4.1	10.5	8.9	6.6	4.1	40.2	72.1	160.5	204.7	7.6
# 15-S	72.5	44.8	0.0	30.7	0.0	0.0	10.0	9.5	2.5	5.0	35.1	39.6	249.8	230.8	8.5
# 16-I	10.2	3.8	0.0	1.7	3.2	3.9	10.5	4.4	2.3	4.3	38.9	63.4	146.5	190.0	6.5
# 17-I	37.1	20.5	0.0	5.3	6.1	2.2	8.3	11.2	1.9	4.2	50.8	64.7	212.3	215.7	7.2
# 18-I	10.6	9.1	2.3	6.7	7.8	0.0	25.7	6.0	2.5	2.3	38.6	80.9	192.4	210.6	5.4

# 19-I	21.7	15.6	0.0	5.2	12.0	0.0	10.0	11.5	4.0	2.5	37.5	64.9	184.9	215.0	5.9
# 20-S	24.3	13.7	0.0	3.0	3.9	0.0	14.7	3.7	2.3	1.4	39.8	67.9	174.6	213.3	6.1
# 21-I	15.7	9.5	0.0	9.3	6.3	0.0	19.0	17.9	2.9	2.7	37.1	79.3	199.7	244.8	5.4
# 22-I	15.0	9.7	0.0	4.4	7.6	1.1	5.0	7.5	2.3	2.3	40.1	48.9	143.8	172.9	5.2
# 23-I	38.4	24.8	0.0	3.1	3.1	2.2	21.6	9.1	3.1	3.0	31.5	51.6	191.6	195.8	7.7
# 24-I	51.8	39.8	0.0	3.3	6.3	0.0	21.1	4.3	1.6	2.6	21.8	31.0	183.4	171.7	6.8
# 25-S	13.9	9.3	2.1	5.3	9.4	4.5	32.3	4.9	2.0	2.9	57.0	78.8	222.3	232.6	8.1
# 26-S	15.8	8.8	0.0	1.2	3.5	3.6	21.4	9.9	5.0	7.6	54.8	90.7	222.3	253.6	10.6
# 27-I	8.6	5.4	0.8	3.5	0.0	2.4	28.7	10.0	2.5	6.5	32.4	89.5	190.3	219.1	8.4
# 28-S	29.9	9.5	1.4	9.8	4.3	2.2	27.9	4.1	4.8	9.9	54.1	87.7	245.5	265.4	8.8
# 29-S	22.0	14.3	1.4	3.5	5.0	2.9	39.1	5.8	3.7	6.0	78.2	96.4	281.4	290.9	10.1
# 30-I	10.6	7.4	0.9	3.0	2.4	3.2	16.3	10.8	3.3	4.8	42.1	78.5	183.3	230.1	7.1
# 31-I	7.1	6.6	0.0	0.0	3.2	2.1	9.5	9.4	2.7	2.1	32.8	63.9	139.5	163.8	6.5
# 32-I	14.8	6.2	0.6	1.8	1.8	2.2	20.3	4.7	3.5	5.3	32.9	72.4	166.6	184.3	8.0
# 33-I	4.7	2.8	1.1	4.5	5.8	0.0	37.0	1.0	3.1	2.5	41.3	75.9	179.7	162.9	6.4
# 34-I	13.3	17.5	1.2	1.7	7.2	0.0	23.6	6.4	3.3	2.3	49.5	52.6	178.7	182.3	6.9
# 35-S	17.2	13.8	1.8	3.8	3.6	4.1	25.1	7.9	5.9	7.6	61.5	81.3	233.5	259.4	8.7
# 36-I	28.6	19.4	0.0	4.3	6.1	0.0	24.1	11.3	6.0	7.4	50.2	60.5	217.9	221.9	8.1
# 37-S	9.0	6.1	0.0	1.7	2.3	0.0	11.9	5.2	9.2	7.7	32.9	75.0	161.0	203.4	6.2
# 38-I	15.6	12.9	0.0	2.8	2.5	1.2	15.8	3.7	3.9	4.5	32.8	59.7	155.3	197.5	5.4
# 39-I	12.2	8.2	0.0	1.6	2.9	0.0	5.7	5.4	6.9	7.8	32.6	62.6	145.7	212.1	5.9
# 40-S	13.5	9.1	2.2	2.8	7.7	0.0	17.8	6.8	4.4	8.7	45.6	95.5	214.1	248.9	7.1
# 41-S	6.0	4.5	0.0	2.3	4.1	0.0	13.8	3.5	7.0	3.4	33.7	60.6	138.8	171.1	5.8
# 42-I	8.3	5.8	0.0	1.5	2.9	0.0	13.8	1.8	7.2	4.0	29.2	40.6	115.0	119.4	4.0
# 43-S	18.5	13.1	1.4	2.8	1.4	0.0	17.0	4.8	2.4	5.1	59.7	76.9	203.2	274.5	7.7
# 44-I	14.9	9.8	2.7	5.0	9.4	0.0	28.8	4.2	6.0	14.0	55.1	72.9	223.0	225.3	5.6
# 45-S	22.2	15.8	0.0	0.0	5.6	0.0	27.0	3.8	19.4	12.3	45.0	60.9	212.1	223.7	6.9
# 46-S	17.0	10.0	0.0	2.2	3.0	0.0	12.2	6.0	4.6	7.3	42.1	100.1	204.5	295.0	9.0
# 47-I	8.3	6.3	0.0	3.9	0.0	0.0	2.2	6.0	7.4	6.5	24.8	70.4	135.8	162.8	5.5
# 48-I	8.6	5.6	0.0	1.9	5.3	1.4	14.6	5.9	3.4	3.6	35.9	72.3	158.6	188.5	5.7
# 49-I	9.2	5.4	0.0	1.5	1.8	2.0	13.9	3.4	4.3	12.2	38.9	59.4	152.1	190.8	4.7
# 50-I	10.7	8.5	0.0	2.5	2.3	0.0	10.4	4.8	7.2	4.9	35.3	65.9	152.7	195.6	4.8
# 51-S	1.1	1.1	0.0	0.0	0.0	0.0	2.5	0.0	0.0	0.0	0.0	1.2	5.8	25.9	0.2

Table II – Composition of the EVOO biophenol extracts. Concentration of biophenols measured in the 51 EVOO samples expressed as mg/Kg of EVOO. S or I after the identification number of the sample symbolize the Italian (I) or Spanish (S) origin of the sample.
HT = Hydroxytyrosol; TY = Tyrosol; VA = Vanillic acid; Van = Vanillin; FA = Ferulic acid; TY ac = Tyrosyl acetate; PN = Pinoresinol; LU = Luteolin; AP = Apigenin; mLU = metil-luteolin; Ligstr. der. = Total concentration of the ligostride derivatives; Oleur. der. = Total concentration of the oleuropein derivatives; HPLC = Biophenol total concentrations measured by HPLC technique and espressed as mg/Kg of EVOO; F-C = Biophenol total concentrations measured by Folin-Ciocalteu method and espressed as mg/Kg of EVOO; ORAC value = Oxygen Radical Absorbance Capacity values expressed as μmol TE/g.
Chemical species whose concentration in all samples is ≤ 1.0 mg/Kg are omitted to simplify the reading of the table.

Many attempts were done to find a good correlation among the diverse parameters shown in Table II and, in particular, to analyze how the differences recorded by the ORAC values can be explained on the basis of the concentrations of the biophenols, or, in particular, wich molecule or family of molecules were responsible for the ORAC variability.

x variable concentration	r^2
Total biophenol measured FAST HPLC	0.72519
Hydroxytyrosol	0.12189
Tyrosol	0.10631
Decarboxy-methyl oleuropein aglycone (dialdehyde form)	0.46425
Decarboxy-methyl ligstroside aglycone (dialdehyd and oxidized form)	0.39179
Decarboxy-methyl ligstroside aglycone (dialdehyde form)	0.40527
Oleuropein derivatives	0.54625
Ligstroside derivatives	0.54279
Ligstroside derivatives + tyrosol	0.58205
Oleuropein derivatives + hydroxytyrosol	0.71306
(Ligstroside + oleuropein) derivatives + + tyrosol + hydroxytyrosol	0.76138

Table III – Fit correlation. Pearson's correlation coefficient values, r^2, obtained by plotting the ORAC data vs. the concentration of the molecules or molecular families reported in table.

As indicated in table III, the higher coefficient of determination, r^2, was obtained by plotting the ORAC value against the concentration of the molecular family composed by ligstroside and oleuropein derivatives together with the concentration of hydroxytyrosol and tyrosol (Figure 2).

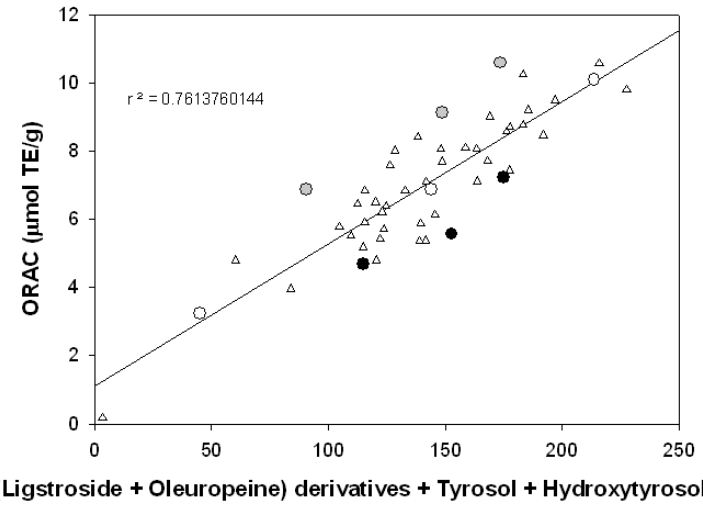

Figure 2 – Correlation curve. Fit indicaticating the correlation between the ORAC data and the total concentration of the biophenols corresponding to the ligstroside and oleuropein derivatives, plus hydroxytyrosol and tyrosol for the 51 samples examined (empty triangles). The circles indicate the biophenol extracts selected for further investigations and placed above (samples 7-I, 10-I, and 26-S; gray circles), below (samples 17-I, 44-I, and 49-I; black circles) and on the fit line (samples 5-I, 29-S, and 45-S; white circles).

It is nothworthy that both hydroxytyrosol and tyrosol are strictly linked to oleuropein and ligstroside, respectively, being their hydrolysis products and this common chemical pathway is the rational of their presence inside the group. Due to these results, further experiments were perfomed on 9 biophenol extracts chosen on the basis of their position in the plot of Figure 2 and accounting for the maximum sample variability. In particular, 3 samples placed above (samples 7-I, 10-I, and 26-S in Table II; gray circles), below (samples 17-I, 44-I, and 49-I in Table II; black circles) and on the fit line (samples 5-I, 29-S, and 45-S in Table II; white circles) were selected.

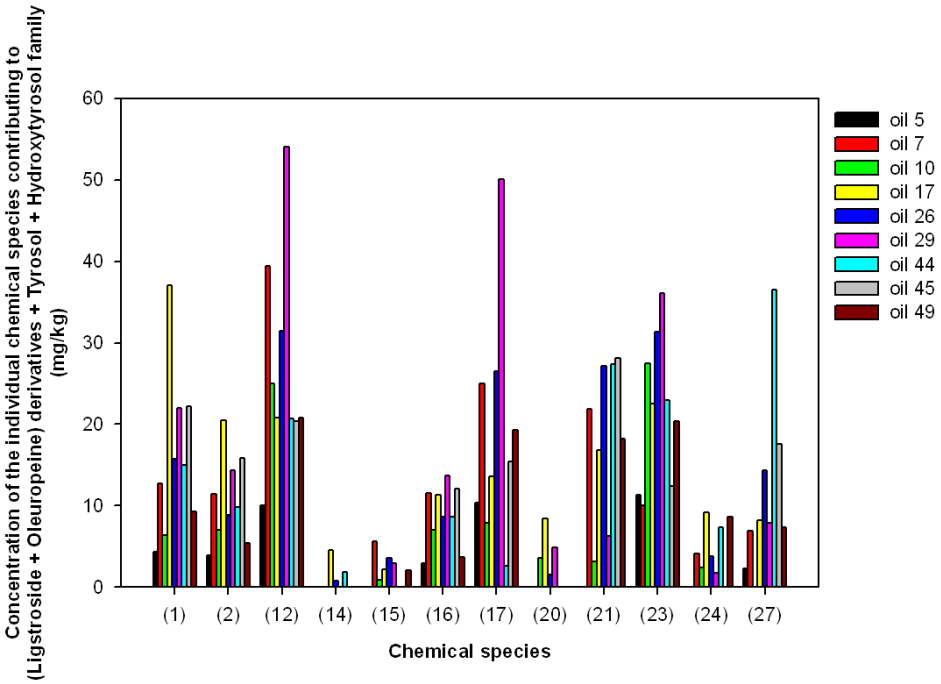

Figure 3 – Concentration of the chemical species represented in the ligstroside and oleuropein derivatives, hydroxytyrosol and tyrosol group. The numbering of the x axis refers to the peaks in Figure 1. (1) hydroxytyrosol; (2) tyrosol; (12) decarboxy-methyl oleuropein aglycone (dialdehyde form); (14) oleuropein aglycone (dialdehyde form); (15) tyrosyl acetate; (16) decarboxy-methyl ligstroside aglycone (dialdehyde and oxidized form); (17) decarboxy-methyl ligstroside aglycone (dialdehyde form); (20) ligstroside aglycone, dialdehyde form; (21) oleuropein aglycone, oxidized aldehyde and hydroxyl form; (23) oleuropein aglycone (aldehyde and hydroxyl form); (24) ligstroside aglycone (oxidized aldehyde and hydroxyl form); (27) ligstroside aglycone (aldehyde and hydroxyl form).

In the hystograms of Figure 3 are reported the concentrations of the single chemical species contributing to the large group composed by ligstroside and oleuropein derivatives, hydroxytyrosol and tyrosol for the 9 biophenol extracts selected in Figure 2.

The great concentration variability of the chemical compounds contributing to the ligstroside and oleuropein derivatives, hydroxytyrosol and tyrosol family in the different samples analyzed is evident from the analysis of Figure 3.

The synthesis of the biophenolic molecules takes place after the tissue disruption during the oil productions [61] but it continues during the malaxation of the olive paste [62] producing further remarkable changes in the chemical composition of oil due to catalytic action of the enzymes released during the olive grinding. Furthermore, the phenolic content of EVOO is also influenced by the cultivar, the geographical zone of cultivation, the ripening of the drupe, the techniques used for oil extraction, storage and preservation methods [63]. Both oleouropein and ligstroside derivatives belong to the secoiridoid class of biophenols, molecules exclusively present in the plants belonging to the *Olearaceae* group including *Olea europaea* [64]. In particular, the aglycone forms are obtained by enzimatic action of the β-glucosidase during the olive ripening, crusching and malaxation [52, 64]. Because of their amphiphilic chemical nature, during EVOO production these substances migrate towards oil and mill waste water where they are mainly present [64].

The antioxidant ability of biophenol extracts belonging to the three groups selected in Figure 2 was analyzed in NIH-3T3 mouse normal fibroblast model using the same concentration for each individual extract. TBH was used to induce oxidative stress and the consequent ROS production was detected by DCFH-DA assay in treated and untreated cells. Before investigating the potential protective role of the 9 biophenol extracts against ROS-induced cell damage, a control experiment by using MTS assay was done to assess their cytotoxicity on NIH-3T3 cells and to avoid false positive results. The percentage of cell viability after 24-hour treatment with the three different concentrations (25, 50 or 100 ng/mL) of each biophenol extract is reported in Figure 4. The methanol concentration in medium (0.1 % v/v) had no toxic effect on cells, as shown in Figure 4 A. Untreated cells were indicated as 100% viability (dashed line in Figure 4A, B and C). All biophenol extracts showed a dose dependent effect on the viability of the NIH-3T3 cells and in most of the samples a weak cytotoxic effect was detected at the concentration of 100 ng/mL (Figure 4A, B and C). Therefore, the highest non cytotoxic concentration (50 ng/mL) was chosen for oxidative stress experiments.

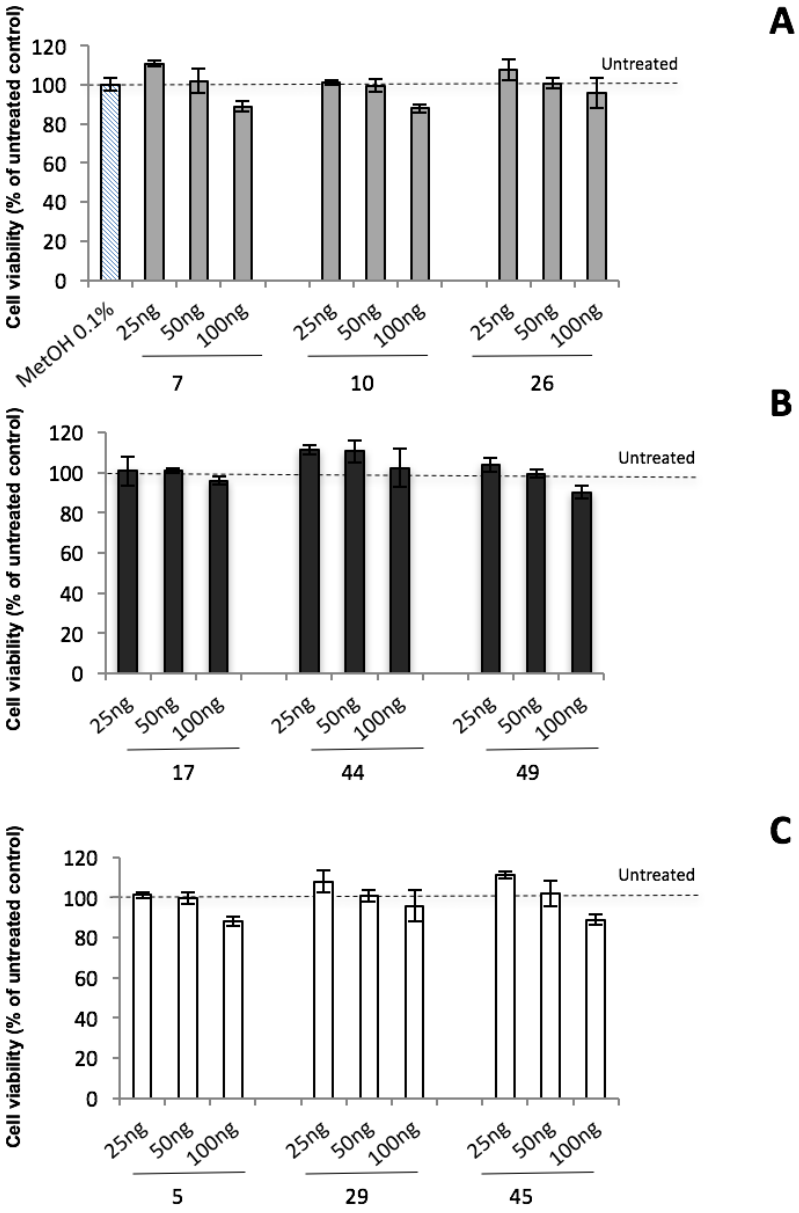

Figure 4 - Effects of EVOO biophenol extracts on cell viability in NIH-3T3 cells. The cells were treated for 24 h with 100, 50 or 25 ng/mL of the biophenol extracts corresponding to the points placed above the fit line (A) (samples 7-I, 10-I, and 26-S), below the fit line (B) (sample 17-I, 44-I, and 49-I) and on the fit line (C) (samples 5-I, 29-S, and 45-S) shown in Figure 2. Cells were also treated with methanol solution at 0.1% f.c. (v/v), corresponding to the concentration of methanol present in the sample containing the lowest concentration of biophenols in the extract. Cell viability was evaluated using MTS assay and expressed as the ratio between treated and untreated (control) cells percent (dashed line, 100% cell viability). Results are the means of three independent experiments with standard errors of the mean as indicated.

To test the antioxidant efficiency of the 9 selected samples, NIH-3T3 cells were preincubated for 24 h with the same concentration (50 ng/mL) of biophenol extract and, then, exposed to TBH, as described in the Experimental section. All samples decreased the TBH-induced ROS production in NIH-3T3 cells by at least 20% with the exception of sample 10-I belonging to the above fit line group and allowing a ROS reduction of about 18%. On the contrary, sample 44-I, belonging to the below fit line group, showed an intense antioxidant activity and caused a 41% ROS level reduction (Figure 5). The low concentrations used in this work are coherent with literature data. Fabiani et al. [65] have reported that very low concentration of olive oil phenol compounds, both purified and in a complex crude extract, protect DNA from oxidative damage in peripheral blood mononuclear cells (PBMC).

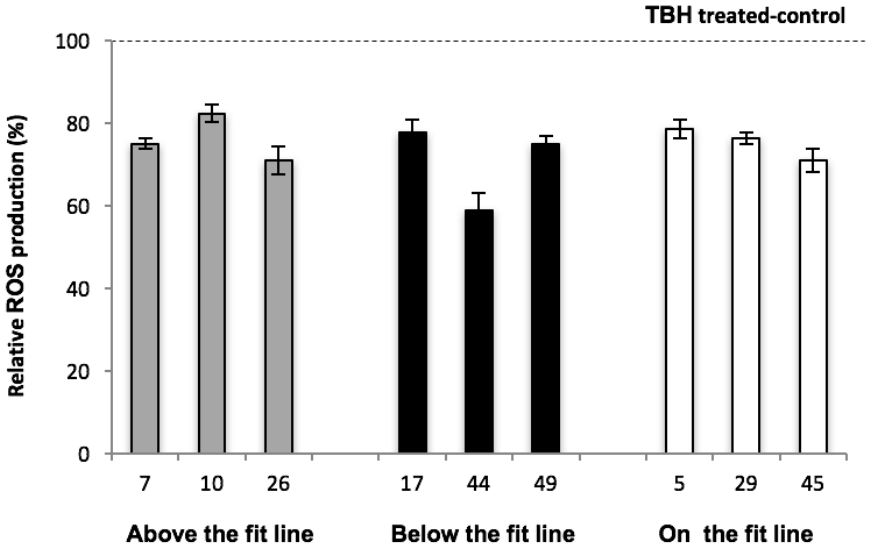

Figure 5 - TBH-induced intracellular ROS-level inhibition in NIH-3T3 cells by EVOO biophenol extracts. Cells were pre-treated for 24 h with 50 ng/mL of the biophenol extracts, selected on the basis of the results obtained in Figure 2, then the TBH was added. The ROS production, after 30 min TBH-treatment, was expressed for each sample as relative percentage, considering as 100% of the fluorescence signal the TBH treated-sample. Each datum represents the mean of the ROS% calculated from at least three independent experiments, with standard errors of the mean as indicated.

Although there is a good overall correspondence between the antioxidant ability of samples tested and their ORAC values, at a first glance, some samples seem to fail this rule. Indeed, the sample 44-I showed a low ORAC value (5.6 μmol TE/g) even though its biophenol content, measured both by FAST HPLC (223.0 mg/kg) and F-C (225.3 mg/kg) was relatively high if

compared with other samples. Nevertheless, it had the highest ability to counteract the oxidative stress induced by the presence of TBH in cell culture. These results suggested that the antioxidant ability strongly depends on the type and composition of molecular species present in the mixture [66] and, due to the high complexity of biological systems, antioxidants may operate via multiple mechanisms.

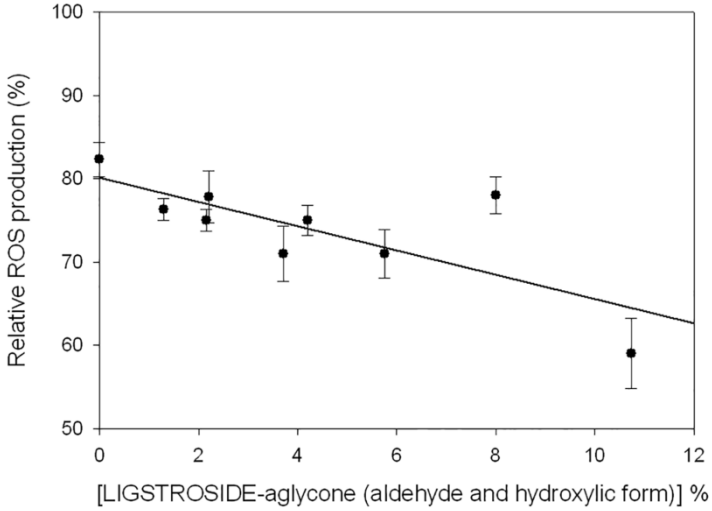

Figure 6 – The decrease of the ROS production linearly depends on ligstroside aglycone (aldehyde and hydroxyl form) concentration. Relative ROS production reported as a function of the ligstroside aglycone (aldehyde and hydroxyl form) expressed as a percentage of the total biophenol content in each of the 9 biophenol extracts selected in Figure 2.

A relationship was found between cellular response and biophenol treatment. Data reported in Figure 6 indicated that a linear dependence can be found when NIH-3T3 cells were treated with total biophenol extracts. The ROS-induced production due to the presence of TBH was correlated with the concentration of the molecular species contributing to the ligstroside and oleuropein derivatives together with the concentration of hydroxytyrosol and tyrosol group. Results indicated that a linear dependence can be found if the TBH effect is plotted agains the concentration of ligstroside aglycone (aldehyde and hydroxil form). The ligstroside aglycone (p-HPEA-EA) and its derivatives belong to phenolic secoiridoid group. Secoiridoids were found, for the first time, in VOO by Montedoro et al. [67, 68] who also assigned their chemical structures in 1993 [69], later confirmed by other authors [70, 71]. Over the last decade, many *in vitro* studies have demonstrated that especially one ligstroside aglycone derivative, namely the oleocanthal (p-HPEA-EDA), possesses strong anti-inflammatory and neuroprotective properties [55, 72-75]. Recently, two studies have also demonstrated anti-cancer activity of

oleocanthal in *in vivo* human tumor mouse xenograft models [76, 77]. On the contrary, few literature data were found on biological activity of ligstroside aglycone [78-80].

Polyphenols modulate gene expression and activity of a variety of different signaling proteins depending on their concentration, the cell system, type or stage of cell damage. It would be very interesting to investigate the cell antioxidant effect of purified ligstroside aglycone (aldehyde and hydroxylic form), both individual or in combination with other polyphenols, in our cell system (NHI-3T3) and in other cell types. This future study may contribute further to already broad range of knowledge on health effects of olive oil biophenols.

Conclusions

Data presented here suggested that the cellular response to the addition of mixture of biophenols, can be considered as the sum of two different contributions: the first one due to the presence of the total polyphenol amount and accounting for about 20% of the cell ROS reduction and the second one exclusively due to the presence of ligstroside aglycone (aldehyde and hydroxylic form) and linearly dependent on its concentration in the total extract.

Authors contribution

G.P., S.G., M.M. and E.G. performed and analyzed HPLC and HPLC-MS measurements and designed research. V.G., M.R.M. and R.P. designed research. F.P. and A.P. performed and analyzed ORAC measurements, F-C tests and biological experiments. P.L.S.B., M.A.C and D.G. wrote the paper and approved the final version of the manuscript.

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Conflict of interest statement

The Authors declare the absence of any conflict of interest.

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