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# Olive oil with high polyphenolic content induces both beneficial and harmful alterations on rat redox status depending on the tissue

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## ABSTRACT

Olive oil (OO) possesses a predominant role in the diet of Mediterranean countries. According to a health claim approved by the European Food Safety Authority, OO protects against oxidative stress-induced lipid peroxidation in human blood, when it contains at least 5 mg of hydroxytyrosol and its derivatives per 20 g. However, studies regarding the effects of a total OO biophenols on redox status *in vivo* are scarce and either observational and do not provide a holistic picture of their action in tissues. Following a series of *in vitro* screening tests an OO containing biophenols at 800 mg/kg of OO was administered for 14 days to male Wistar rats at a dose corresponding to 20 g OO/per day to humans. Our results showed that OO reinforced the antioxidant profile of blood, brain, muscle and small intestine, it induced oxidative stress in spleen, pancreas, liver and heart, whereas no distinct effects were observed in lung, colon and kidney. The seemingly negative effects of OO follow the recently formulated idea in toxicology, namely the real life exposure scenario. This study reports that OO, although considered a nutritional source rich in antioxidants, it exerts a tissues specific action when administered *in vivo*.

## 1. Introduction

The term “Mediterranean Diet” (MD) was introduced for the first time by Ancel Keys in the early 1960s in order to signify an exceptional dietary practice followed by the countries around the Mediterranean basin [1]. MD is characterized by the next three traits: a) high consumption of fruits, nuts, vegetables, legumes, fish, whole grain cereals, b) low consumption of red and processed meat, and c) moderate or low consumption of dairy products, eggs and red wine. Increasing scientific evidence coming out from meta-analyses reveals that the adoption of MD is highly correlated with reduced mortality and morbidity, lower prevalence of cardiovascular intricacies (e.g., coronary issues, stroke and hypertension), neurodegenerative diseases, cancer and metabolic

diseases (e.g., type 2 diabetes mellitus or metabolic syndrome) [2–5].

Olive oil (OO) is indisputably the most important element of MD and is used as the main culinary fat [1,4,6]. Indeed, the health-promoting properties of MD are mainly attributed to Extra Virgin OO (EVOO) consumption due to its high monounsaturated fatty acid (MUFA) composition, specifically oleic acid that accounts for up to 80 % of its total lipid composition [7], followed by palmitic acid and linoleic acid [8]. Chemically, MUFA possess only one double bond, which makes EVOO less susceptible to detrimental oxidative modifications and also contributes to its antioxidant properties, high stability and a long life compared to polyunsaturated fatty acid (PUFA)-enriched oils [9]. Apart from MUFA, approximately 2–5 % of total EVOO composition is made up by highly bioactive compounds in the

**Abbreviations:** OO, olive oil; HT, hydroxytyrosol; OLEA, oleacein; OLEO, oleocanthal; T, tyrosol; GSH, glutathione; TBARS, thiobarbituric acid reactive substances; CARB, protein carbonyls; TAC, total antioxidant capacity; CAT, catalase

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unsaponifiable fraction (e.g., squalene, sitosterols, triterpenes, pigments), by soluble or hydrophilic compounds (such as  $\alpha$ -tocopherol, sterols, carotenoids) and most importantly by phenolic compounds and especially hydroxytyrosol (HT), oleuropein and ligstroside aglycons, oleacein (OLEA), oleocanthal (OLEO), tyrosol (T), caffeic acid, ligstroside, vanillic acid and hydroxytyrosol esters with potent antioxidant action [5].

The aforementioned substances present in OO offer protection against the excessive generation of reactive species and oxidative stress, which has been defined as an imbalance between the oxidant and antioxidant systems in favor of the former, leading to alterations on redox signaling and control and/or molecular damage [10]. The oxidants are reactive species or free radicals that possess one or more unpaired electrons and are potentially hazardous for lipids, DNA and proteins. Thus, these highly oxidant compounds have been linked to the development of several diseases, such as cardiovascular and neurodegenerative pathologies, and cancer [11]. The antioxidant arsenal of the organisms consists of enzymatic antioxidants, the most important being superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) and non-enzymatic metabolites, such as vitamins C, E and reduced glutathione (GSH) [12]. Additionally, some non-enzymatic antioxidants, namely carotenoids and biophenolic compounds are not intrinsically generated but they are obtained by humans through diet and are probably key factors in the protection of blood and tissues against oxidative stress [12].

The favorable character of OO consumption is nowadays widely recognized. To this end, the European Food Safety Authority (EFSA) [Commission Regulation (EU) 432/2012] [13] has endorsed two relevant health claims. According to them, it is recommended that OO should be used to supersede saturated fats in order to maintain normal blood cholesterol levels. Furthermore, EFSA proposes that OO protects blood lipids from oxidative stress obviously due to the polyphenols that are present in a usual human daily intake of 20 g of OO. This claim is only referred to OO containing 5 mg of HT and its derivatives (e.g., oleuropein complex and T) per 20 g of OO.

Polyphenols (the term "biophenolic compounds" is also in power), although known as strong antioxidants *in vitro*, they can also act as harmful, pro-oxidant agents *in vivo* [14]. Therefore, the impact of diets rich in biophenols on human health is still a matter of debate [15]. For instance, biophenols may help to the preservation of the gastrointestinal tract integrity that is in jeopardy by the harmful action of reactive species formed within the stomach and intestines from the constituents of diet (14). Nevertheless, consumption of high quantities of biophenols through diet or food supplements is not yet encouraged [15]. It is worth to be mentioned that many studies have failed to prove that antioxidant administration protects against detrimental effects of reactive species [15–19]. Indeed, high amounts of  $\alpha$ -tocopherol are ineffective on diminishing levels of lipid peroxidation in humans [20,21]. Moreover, Vissers et al. [22] pointed out that OO consumption by human volunteers minimized the tendency of low-density lipoproteins (LDL) oxidation, but also oil sans antioxidants had the same effect.

The absence of clinical results regarding the use of antioxidants as putative beneficial agents for human health is the most important obstacle. It has been reported that antioxidants may disrupt cell homeostasis related through the disturbance of redox status and the signaling cascades governed by reactive species [23]. This merits the adoption of a systematic and holistic strategy for the administration of antioxidants depending on the nature and type of the targeted disease [24]. Antioxidants have been reported to be beneficial as a treatment strategy for cancer, but at the same time they have been incriminated with deleterious effects (i.e., cancer cell progression) [25]. For instance, N-acetylcysteine (NAC), a precursor molecule of GSH, and vitamin E lead to the increase of the GSH levels, promote tumor progression by reducing ROS and in turn reduce p53 expression levels (ROS-p53 axis). Moreover, the supplementation of the same antioxidants in patients with type 2 diabetes had little or no effect on the increased blood

glucose levels and other oxidative stress biomarkers, such as GSH, GSH/GSSG ratio, TBARS and urine F2a isoprostanes. Conversely, it has been suggested that high doses of NAC in fact could be detrimental by increasing the blood glucose levels [26].

A lot of *in vitro* tests have been utilized in order to monitor the putative antioxidant properties of molecules originated from plants and in most of the cases it has been shown that they are potent antioxidants. Although, prior to the validation of these results *in vivo* in order to study their potential therapeutic effectiveness, it has to be taken into account that the plant antioxidants pass through several physiological processes, such as metabolism when administered in living organisms. Therefore, the findings of *in vitro* and *in vivo* antioxidant potential assessment studies are not always accompanied by a biological result. Nevertheless, the results of *in vitro* assays are usually unsuitably extrapolated to organisms without undertaking sufficient number of *in vivo* experiments [27].

It has to be stressed here that the new paradigm that is now formulated in the field of toxicology is referred to the so-called real life exposure scenario [28–31]. According to this, the effects of routinely encountered xenobiotics when animals are exposed long-term, even to doses well below NOAEL (no-adverse-effects-level), are harmful for blood and tissues since they induce to toxicity and oxidative stress [32,33]. However, it is interesting to examine the impact of OO, when it is administered in an acute-like mode on oxidative stress under a toxicological frame. Based on the above, the health benefits of OO have not been fully revealed. In fact, we are only just in the beginning of understanding the countless ways that OO may improve human health and way of life. It is worth noting that the literature lacks experiments investigating the effect of olive phenolic compounds on the function of specific organs (e.g., spleen, pancreas, brain or heart) [34]. Moreover, the molecular mechanisms for the reinforcement of blood and tissue antioxidant defense due to the consumption of diet rich in OO are still being studied. Thus, the main objective of this study was to evaluate the potential positive or negative role of the administration of OO with high polyphenolic content on blood and tissue redox status taking into account the great debate concerning the ambiguous impact of antioxidants on human health. Furthermore, we have hypothesized that the effect of OO will be tissue dependent, meaning that it may be harmful for some tissues and beneficial for some others. Finally, we intended to assess whether the observed effects of OO are attributed to one or more particular phenolic compounds, or to the synergic effect of the total phenolic fraction together with the rest constituents of OO.

## 2. Materials and methods

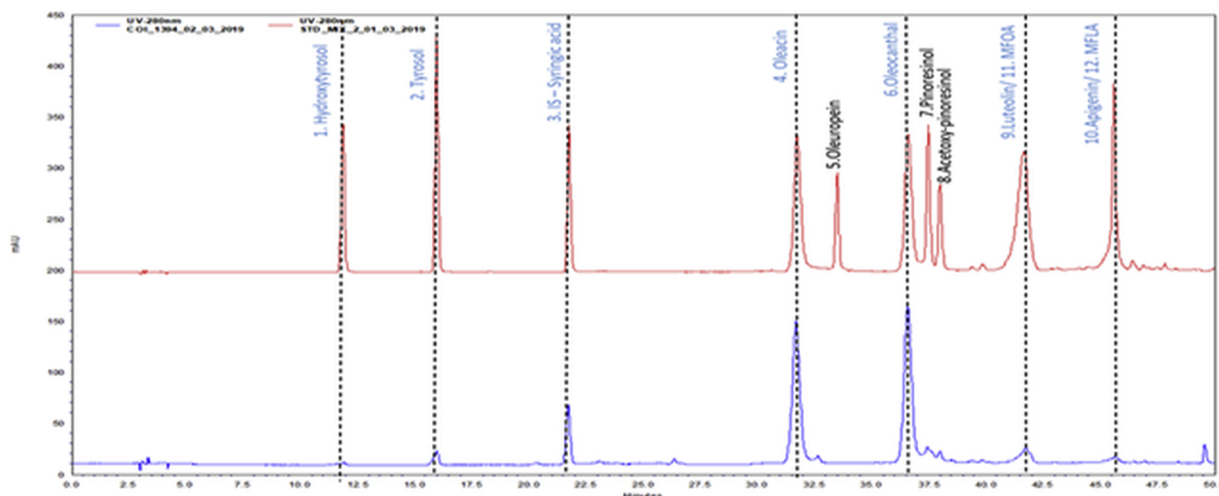
### 2.1. OO information

OO under study (OLE\_1394) has been produced in a two-phase OO mill. The drupes were collected during the harvesting period 2017–2018 from Arkadia region of Greece. The olive tree was of "Kalamon" variety and the cultivation practice was organic.

### 2.2. OO biophenols analysis

In a 10 ml test tube, 2.0 g of OO and 1 ml of the internal standard (IS) solution (syringic acid) were added and vortexed for 30 s. Then, 5 ml of the methanol/water (80:20, v/v) were added and vortexed for 1 min. The mixture was placed in an ultrasonic bath for 15 min at room temperature and then centrifuged for 25 min. An aliquot of the supernatant phase was taken, filtered and forwarded to HPLC-DAD analysis.

The HPLC-DAD method proposed from the International Olive Council (IOC) 2009 – COI / T.20 / Doc No 29 was performed [35]. Specifically, the separation was achieved on a reversed-phase Spherisorb Discovery HS C18 column (250 × 4.6 mm, 5  $\mu$ m; Supelco) using a mobile phase consisting of 0.2 % aqueous orthophosphoric acid (A) and methanol/acetonitrile (50:50 v/v) (B), at a flow rate of 1.0 ml/min and



**Fig. 1.** RP-HPLC-DAD chromatogram of the analyzed OLE\_1394 OO sample at 280 nm (blue line) and the chromatogram of the reference compounds solution at 280 nm (red line) (1: Hydroxytyrosol, 2: Tyrosol, 3: Syringic acid (I.S.), 4: Oleacein, 5: Oleuropein, 6: Oleocanthal, 7: Pinosesinol, 8: Acetoxypinosesinol, 9: Luteolin, 10: Apigenin, 11: MFOA, 12: MFLA).

ambient temperature. The applied gradient elution was as follows: 0 min, 96 % A and 4% B; 40 min, 50 % A and 50 % B; 45 min, 40 % A and 60 % B; 60 min, 0% A and 100 % B; 70 min, 0% A and 100 % B; 72 min, 96 % A and 4% B; 82 min, 96 % A and 4% B. The injection volume was set to 20  $\mu$ l. Chromatograms were monitored at 280 nm (Fig. 1).

### 2.2.1. Qualitative determination

For the qualitative determination of biophenols an analytical standard working solution consisted of the major biophenols, referred to in EFSA's health claim, was prepared and analyzed. Some of them were commercially available such as tyrosol, syringic acid, pinosresinol, hydroxytyrosol, luteolin, apigenin, oleacein, oleocanthal, and the others were isolated from OO in high-purity grade such as acetoxypinosresinol, Mono-Aldehydic Form of Oleuropein Aglycon (MFOA) and Mono-Aldehydic Form of Ligstroside Aglycon (MFLA). The qualitative determination of major biophenols was carried out by comparing the retention time and UV-spectra of the analytes detected to those of the corresponding reference compounds (Table 1, Supplementary Material SM\_Fig1).

### 2.2.2. Quantitative determination

For the quantitative determination of biophenols the method described in the COI / T.20 / Doc No 29 method, was followed. Specifically, biophenol content (lignans, flavonoids, phenolic acids,

**Table 1**

Reference compounds. The retention time (RT, min) and the wavelength (UV, nm) of maxima absorption of each analyte.

Standard Reference Compounds	Rt(min)	UV(nm)
1 Hydroxytyrosol	11.88	210-279
2 Tyrosol	15.96	220-275
3 Syringic acid (I.S.)	21.81	217-275
4 Oleacein	31.77	227-280
5 Oleuropein	33.52	233-280
6 Oleocanthal	36.52	226-276
7 Pinosesinol	37.51	228-279
8 Acetoxypinosesinol	38.03	229-279
9 Lyteolin	41.74	349
10 Apigenin	45.66	217-266-337
11 Monoaldehydic form of Oleuropein aglycon (MFOA)	41.72	230-280
12 Monoaldehydic form of ligstroside aglycon (MFOA)	45.66	227-280

secoiridoids, oxidative forms of oleuropein and ligstroside aglycones), expressed in mg Tyrosol/kg OO, was estimated by measuring the sum of the areas of the related chromatographic peaks and the relative response factor of external standard solutions of tyrosol and syringic acid (RRF syringic acid/ tyrosol) (Table 2).

Concentration levels of major biophenols have been also determined using regression analysis method. Specifically, standard calibration curves of Hydroxytyrosol (HT), Tyrosol (T), Oleacein (OLEA) and Oleocanthal (OLEO) were prepared and the analysis was performed at 280 nm. For the HT and T quantification, 9-points calibration curves were constructed (HT:  $y = 84028x + 39609$ ,  $R^2 = 0.9997$ ; T:  $y = 53933x - 11712$ ,  $R^2 = 0.9987$ ), while OLEO and OLEA were quantified according to their 8 & 10-points calibration curves respectively (OLEA:  $y = 32720x + 12723$ ,  $R^2 = 0.9997$  and OLEO:  $y = 18836x + 54185$ ,  $R^2 = 0.9982$ ). The results were expressed in mg analyte per kg of OO (Table 3). The corresponding calibration curves are given in the Supplementary Material (SM\_Fig2). HT and T reference standards were purchased from ExtraSynthase (Lyon Nord, France) while OLEO and OLEA were purchased from Pharmagnose SA (Oinofyta, Greece).

### 2.3. Animals

Eleven male Wistar rats (3 months old,  $318.5 \pm 18.4$  g) were housed in cages of 3 in the animal facility room with a 12 h light/dark cycle, controlled temperature ( $22 \pm 2$  C) and humidity (50 %). The experiment was performed in the animal facility of the laboratory of Clinical Pharmacology, School of Medicine, Aristotle University of Thessaloniki in accordance to the Helsinki Declaration and National standards (Permission code: EL-54-BIOexp-04). The experimental protocol was approved by the National Veterinary Administration authorities [License No.:438329(1842)]. All animals were treated in accordance with the guiding principles of the European Community Council Directive (89/609/EEC) for the care and use of laboratory animals.

**Table 2**

Quantitative determination of phenolic components.

Sample Code	RRF*	Determination of polyphenols (mg Tyr/Kg OO)
OLE_1394	4.88	580

RRF\*: Relative Response Factor for the expression of the result as tyrosol.

**Table 3**  
Quantitative determination of the major biophenols.

Sample Code	HT (mg/Kg)	T (mg/Kg)	OLEA (mg/Kg)	OLEO (mg/Kg)
OLE_1394	2.79	10.36	316	590

#### 2.4. Experimental design

The animals were randomly divided into 2 groups. The control group (n = 6), in which the rats were fed with standard commercial rat chow (containing corn, soybean meal, barley, bran, milk paste, molasses) purchased from Viozois (Thessaloniki, Greece) and the olive oil treated group (n = 5) including the rats that were fed with standard commercial rat chow plus the OO containing 900 mg biophenols/kg OO. The diet was administered through an appropriate gastrointestinal gavage (0.1 ml/rat/day corresponding to consumption of 20 g of OO/day, according to EFSA) for 14 consecutive days. The condition and health of the animals were observed daily and their body weight was measured at days 1, 7 and 14. Twenty four hours after the last administration the rats were anaesthetized with isoflurane (IsoFlo®, Abbot) and blood samples were drawn by cardiac puncture. Then, the stomach, the small and the large intestine, the liver, the pancreas, the spleen, the kidney, the lung, the heart, the quadriceps muscle and the brain were excised, snapped frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until further analysis.

#### 2.5. Blood and tissue preparation

Blood samples were centrifuged at 1370 g, 10 min,  $4^{\circ}\text{C}$ . The plasma was collected for the measurements of total antioxidant capacity TAC, thiobarbituric acid reactive substances TBARS and protein carbonyls CARB. At the erythrocyte pellet, distilled water 1:1 v/v was added. The samples were vortexed and centrifuged at  $4000 \times g$ , 15 min,  $4^{\circ}\text{C}$ . The supernatant, which is the red blood cell lysate (RBCL) was collected for the measurement of glutathione (GSH) and  $\text{H}_2\text{O}_2$  decomposition activity. Both plasma and RBCL were kept at  $-80^{\circ}\text{C}$ .

The results of the biomarkers measured in RBCL are expressed on the basis of mg of hemoglobin (Hb). Hemoglobin concentration was determined by the hemoglobin cyanide (HiCN) method using a commercial kit (Dutch Diagnostics, Zutphen, Holland). According to the manufacturer's instructions, 5  $\mu\text{l}$  of RBCL was added in 1 ml of working hemoglobin reagent (reagent R1). The reagent R1 (pH = 7.3) was consisted of potassium hexacyano ferrate (III) (0.607 mmol/l), potassium cyanide (0.767 mmol/l), potassium dihydrogen phosphate (1.030 mmol/l) and detergent 0.05 %. Then, the samples were vortexed and incubated in the dark for 10 min and the absorbance was measured at 540 nm. In each experiment, 1 ml of R1 was used as the blank. The final hemoglobin concentration was expressed as g/dl.

The tissue samples were homogenized with a homogenizer (T10 basic Ultra-Turrax, IKA T10 basic) in 0.01 mM PBS (138 mM NaCl, 2.7 mM KCl, 1 mM EDTA, pH = 7.4) containing a cocktail of protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany) after a brief sonication treatment (60 s, 70 % amplitude, 0.7 s cycle) on ice. Finally, the homogenate was centrifuged ( $10,000 \times g$ , 15 min,  $4^{\circ}\text{C}$ ), the supernatant was collected and stored at  $-80^{\circ}\text{C}$  in aliquots. The results of biomarkers in tissues are expressed on the basis of mg of total protein. Total protein was assayed using the Bradford reagent (Sigma-Aldrich, Munich, Germany).

#### 2.6. Spectrophotometric evaluation of blood and tissue redox status

Three biomarkers that reflect the antioxidant capacity, namely reduced glutathione (GSH), total antioxidant capacity (TAC) and  $\text{H}_2\text{O}_2$  decomposition rate were measured. Moreover, two biomarkers that are

representative of the oxidative damage of lipids and proteins, namely TBARS and CARB, respectively were also evaluated [36,37].

#### 2.7. Protocol for the determination of glutathione (GSH)

GSH was measured according to a slightly modified protocol of Reddy et al. [38], as described by Gerasopoulos et al. [39]. Specifically, the proteins of the RBCL or the tissue homogenate were precipitated with 5% trichloroacetic acid (TCA) in order to eliminate protein-linked -SH groups. Then, 20  $\mu\text{l}$  of the TCA treated RBCL or tissue samples was mixed with 660  $\mu\text{l}$  of sodium potassium phosphate buffer (67 mM, pH = 8) and 330  $\mu\text{l}$  of 5,5'-dithiobis-2 nitrobenzoate (DTNB, 1 mM) followed by incubation in the dark at room temperature (RT) for 15 min. The absorbance was monitored at 412 nm [40]. The calculation of GSH concentration were based on the molar extinction coefficient of DTNB ( $13.6 \text{ mM}^{-1} \text{ cm}^{-1}$ ).

#### 2.8. Protocol for the determination of total antioxidant capacity (TAC)

TAC determination was based on the method of Janaszewska and Bartosz [41] with slight modifications as previously described by Gerasopoulos [39]. Specifically, 20  $\mu\text{l}$  of plasma or tissue homogenate was added to 480  $\mu\text{l}$  of sodium potassium phosphate (10 mM, pH = 7.4) and 500  $\mu\text{l}$  of DPPH (2,2-diphenyl-1-picrylhydrazyl, 0.1 mM). The samples were incubated in the dark for 45 min at RT and then were centrifuged ( $20,000 \times g$ , 3 min,  $25^{\circ}\text{C}$ ). The absorbance was monitored at 520 nm. The results are displayed as mmol of DPPH reduced to 2,2-diphenyl-1-picrylhydrazine (DPPH:H) by plasma or tissue antioxidants.

#### 2.9. Protocol for the determination of catalase (CAT) and $\text{H}_2\text{O}_2$ decomposition rate

CAT and  $\text{H}_2\text{O}_2$  decomposition rate in RBCL and tissues, respectively were determined as previously described by Aebi [42]. According to the protocol, 4  $\mu\text{l}$  of erythrocyte lysate (diluted 1:10) or tissue homogenate was added to 2991  $\mu\text{l}$  of 67 mM sodium potassium phosphate (pH = 7.4) and the samples were incubated at  $37^{\circ}\text{C}$  for 10 min. Afterwards, 5  $\mu\text{l}$  of 30 %  $\text{H}_2\text{O}_2$  was added to the samples and the change in absorbance was immediately monitored at 240 nm for 130 s. The calculations were based on the molar extinction coefficient of  $\text{H}_2\text{O}_2$  ( $40 \text{ M}^{-1} \text{ cm}^{-1}$ ).

#### 2.10. Protocol for the determination of thiobarbituric acid reactive substances (TBARS)

For TBARS determination a modified assay of Keles et al. [43] was used according to [44]. In brief, 20  $\mu\text{l}$  of plasma or tissue homogenate was mixed with 500  $\mu\text{l}$  of Tris-HCl (200 mM, pH = 7.4) and 500  $\mu\text{l}$  of 35 % TCA and incubated for 10 min at RT. Afterwards, 1 ml of  $\text{Na}_2\text{SO}_4$  (2 M) and thiobarbituric acid (55 mM) solution was added and the samples were kept in the water bath at  $95^{\circ}\text{C}$  for 45 min. After the incubation, the samples were cooled on ice for 5 min and mixed with 1 ml of 70 % TCA. The samples were centrifuged ( $15,000 \times g$ , 3 min,  $25^{\circ}\text{C}$ ) and the absorbance of the supernatant was monitored at 530 nm. A blank, without the blood or tissue sample was also used. TBARS are expressed in terms of malondialdehyde (MDA) equivalents. The molar extinction coefficient of MDA is  $155 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ .

#### 2.11. Protocol for the determination of protein carbonyls

Protein carbonyls were measured according to Patsoukis et al. [45], as previously described [44]. Briefly, 50  $\mu\text{l}$  of 20 % TCA was mixed with 50  $\mu\text{l}$  of plasma or of tissue homogenate and this mixture was incubated in an ice bath for 15 min and centrifuged at  $15,000 \times g$  for 5 min at  $4^{\circ}\text{C}$ . The supernatant was discarded and 500  $\mu\text{l}$  of 10 mM DNPH (in 2.5 N HCl) for the sample, or 500  $\mu\text{l}$  of 2.5 N HCl for the blank, was

added to the pellet. The samples were incubated in the dark at room temperature for 1 h, with intermittent vortexing every 15 min, followed by centrifugation at  $15,000 \times g$  for 5 min at  $4^\circ\text{C}$ . The supernatant was discarded and 1 ml of 10 % TCA was added and the samples were vortexed and centrifuged at  $15,000 \times g$  for 5 min at  $4^\circ\text{C}$ . The supernatant was again discarded and 1 ml of ethanol-ethyl acetate (1:1 v/v) was added and the samples were vortexed and centrifuged at  $15,000 \times g$  for 5 min at  $4^\circ\text{C}$ . This step was repeated two more times. The supernatant was discarded and 1 ml of 5 M urea (pH = 2.3) was added and the samples were vortexed and incubated at  $37^\circ\text{C}$  for 15 min. Then they were centrifuged at  $15,000 \times g$  for 3 min at  $4^\circ\text{C}$  and the absorbance was monitored at 375 nm. The calculation of protein carbonyl concentration was based on the molar extinction coefficient of 2,4-dinitrophenylhydrazine ( $22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ ).

## 2.12. Evaluation of $\gamma$ -glutamylcysteine ligase, catalytic subunit (*gclc*), catalase (*cat*) and superoxide dismutase 1 (*sod1*) gene expression

The brain, spleen and pancreas were selected for further analysis regarding the expression levels of  $\gamma$ -glutamylcysteine ligase, catalase and superoxide dismutase genes using real-time PCR. The redox status of these tissues was significantly affected by the administered OO either beneficially or detrimentally. Thus, we assumed that this response could be attributed to altered gene expression levels of crucial antioxidant defense enzymes, such as the three tested herein. RNA was extracted from all tissues using an RNA isolation kit (PureLink™ RNA kit, Invitrogen, USA) according to the manufacturer's instructions. RNA was quantified and its purity was confirmed by measuring the  $\text{OD}_{260/280}$  with a value  $> 1.8$  indicating lack of protein contamination. Approximately, 10  $\mu\text{g}$  of the extracted RNA were treated with DNase (RQ1 RNase-Free DNase, 1U/ $\mu\text{l}$ , Promega, USA). DNA-free RNA was then reverse transcribed to obtain cDNA (Superscript II Reverse Transcriptase, Invitrogen, USA) using oligo (dT) 12–18 primers (Invitrogen, USA). Amplification of cDNAs for *cat*, *sod1* and *gclc* as well as the *actin* gene (housekeeping gene) was performed in 10  $\mu\text{l}$  reactions containing SYBR® Select Master Mix (2 $\times$ , applied biosystems, USA), 0.25  $\mu\text{M}$  of each primer, 50 nM ROX Low and 25 ng cDNA for the amplification of all tested genes. The utilized primers were based on the literature and are shown in Table 4 [46,47]. The thermocycling conditions used for the amplification of the aforementioned genes were the following: 3 min at  $95^\circ\text{C}$ , 45 cycles of 15 s at  $95^\circ\text{C}$ , 30 s at  $55^\circ\text{C}$ , followed by 30 s at  $72^\circ\text{C}$ . Finally, a melting curve was carried out from  $55^\circ\text{C}$  to  $95^\circ\text{C}$  to check the specificity of the products. All qPCR were performed on a  $\mu\text{x}3005 \text{ P}$  system (Stratagene, UK). Amplification efficiencies were  $> 89\%$  with  $r^2$  values  $> 0.987$  for all genes.

## 2.13. Evaluation of $\gamma$ -GCLC, CAT and SOD1 protein levels

The protein levels of  $\gamma$ -GCLC, CAT and SOD1 were measured in the same tissues (brain, spleen, pancreas) using western blot analysis. Tissue homogenate (as already described at blood and tissue preparation section) containing 30  $\mu\text{g}$  of protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at an

8% polyacrylamide gel. Proteins were then transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The membranes were blocked overnight with 5% non-fat milk in 13 mM Tris/150 mM NaCl, pH = 7.5, 0.2 % Tween-20. They were then probed with polyclonal goat anti-rat SOD1 1:600; Cat. no. sc-8637 or polyclonal rabbit anti-rat  $\gamma$ -GCLC 1:600; Cat. no. sc-28965; both from Santa Cruz Biotechnology Inc., Dallas, TX, USA or polyclonal goat anti-rat CAT 1:400; Cat. no. AF3398; from R&D Systems, Minneapolis, MN, USA primary antibodies for 1 h at RT. After 5-min washing steps the membranes were incubated with horseradish peroxidase-conjugated polyclonal goat anti-rabbit 1:5000; Cat. no. 31,462 or polyclonal donkey anti-goat 1:3000; Cat. no. PA1-28659; both from Thermo Scientific, Rockford, IL, USA secondary antibodies for 30 min at RT. All membranes were re-probed with polyclonal rabbit anti-human anti-mouse glyceraldehyde 3-phosphate dehydrogenase GAPDH; 1:1000; Cat. no. PA1-988; Thermo Scientific for normalization. The optical density of the protein bands monitored using Alpha View quantification software Alpha Innotech, San Leandro, CA, USA.

## 2.14. Statistical analysis

One-way ANOVA followed by Tukey's test was applied to compare the means between the two groups. The statistical significance level was set at  $p < 0.05$ . The results are expressed as mean  $\pm$  SEM (standard error of the mean) of three experiments ( $n = 6$ ). Statistical analyses were performed using the SPSS software (version 20.0; SPSS, Inc., Chicago, IL, USA).

## 3. Results

### 3.1. Redox biomarkers

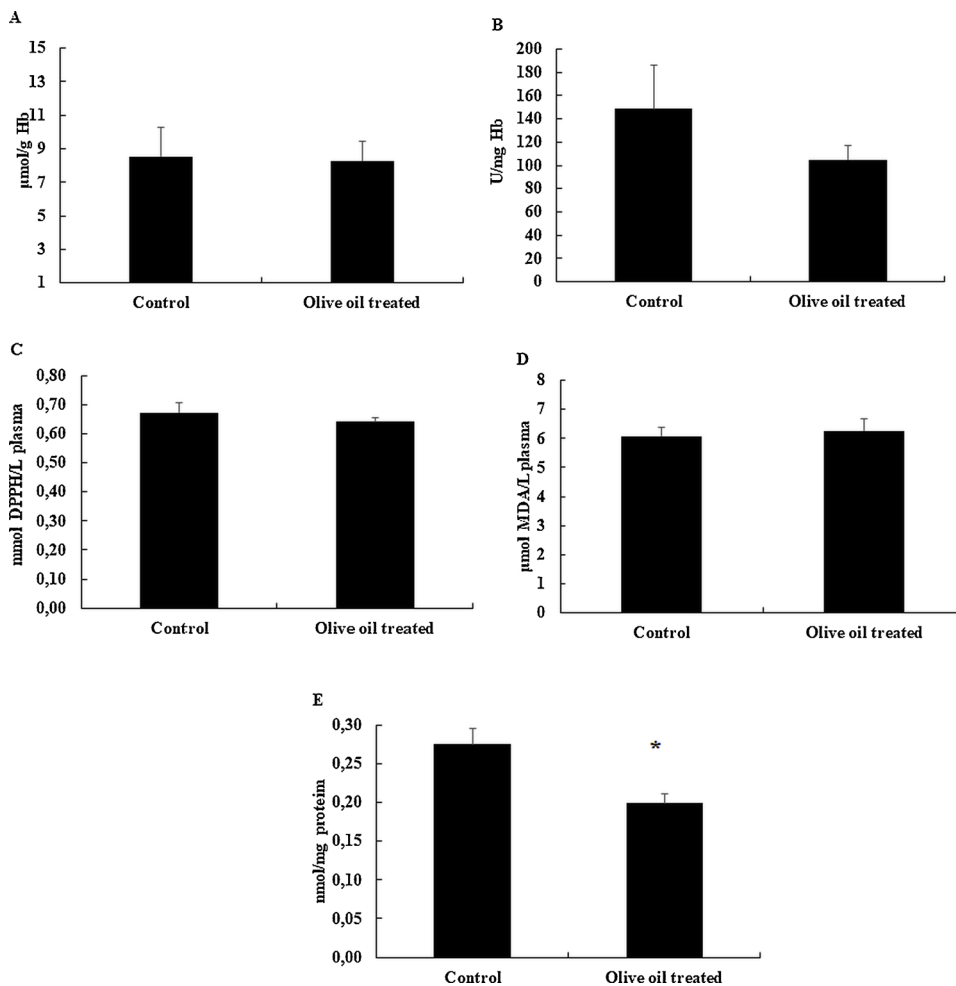
According to the results obtained, OO administration to rats induced contradictory effects regarding the tested redox biomarkers. Overall, the redox status of blood, brain, muscle and small intestine was affected beneficially, although, detrimental effects were observed in spleen, pancreas, heart and liver. Additionally, no distinct effects were observed in lung, colon and kidney, while, we do not report any effect of OO administration on stomach. Specifically, a reduction of protein oxidation levels was observed in the OO treated group compared with the control in blood (Fig. 2) and small intestine (Fig. 7), by 27.6 % and 57.7 %, respectively. Moreover, lipid peroxidation levels were decreased by 52.3 % in brain (Fig. 6) and GSH levels were increased by 41.1 % in muscle due to OO administration (Fig. 3).

Concerning the harmful effects of OO, spleen seems to be the most affected organ, since a reduction in GSH by 8% (Fig. 3),  $\text{H}_2\text{O}_2$  decomposition rate by 17.1 % (Fig. 4) and TAC by 10.4 % (Fig. 5) and an increase in protein oxidation levels by 67.4 % (Fig. 7) were found in the OO treated group compared with the control. Furthermore, pancreas lipid peroxidation (Fig. 6) and protein oxidation (Fig. 7) were enhanced after OO administration by 65.8 % and 8.0 %, respectively compared with the control. Meanwhile, liver GSH levels (Fig. 2) declined by 12.8 % and lipid peroxidation in heart was enhanced by 20.7 % (Fig. 6).

Moreover, our findings in lung, colon and kidney were not distinct. In more detail, GSH levels in lung (Fig. 3) were increased by 18.8 %. It seems that the increased GSH protected proteins from oxidation (Fig. 7), since protein carbonyl levels were declined by 39.3 %, however it failed to protect lung lipid oxidation (Fig. 6), because the TBARS levels were higher by 32.8 %. Finally, in colon and kidney, the  $\text{H}_2\text{O}_2$  decomposition rate (Fig. 4) was decreased by 22.3 % and 11.3 %, respectively and so was TAC (Fig. 5) by 23.8 % and 16.6 %, respectively. Interestingly, these results were accompanied by a decrease of lipid peroxidation levels (Fig. 6) (18.5 %) in colon and of protein oxidation (Fig. 7) levels (15.6 %) in kidney. Finally, the measured redox biomarkers in stomach were not affected.

**Table 4**  
The primer sequences used.

Gene	Gene ID	Primer (5'-3')
<i>cat</i>	24248	Forward: TTCTACACTGAAGATGGTAACTG
		Reverse: GAAAGTAACTGATGGAGAGAC
<i>sod1</i>	24786	Forward: AACCAAGTTGTTGGTTCAGGA
		Reverse: CTCTGAGAGTGAGATCACA
<i>gclc</i>	25283	Forward: CAGAGTATGGGAGTTACATGATTGAAG
		Reverse: TGTGTTGAACTCGGACATCGTT
<i>actin</i>	81822	Forward: AGCCATGTACGTAGCCATCC
		Reverse: TCGGAACCGCTCATTGCCG



**Fig. 2.** The effects of OO administration on redox biomarkers of blood. (A) GSH in RBCL, (B) CAT in RBCL. (C) TAC in plasma, (D) TBARS in plasma, (E) CARB in plasma. \*(p < 0.05): Statistically significant compared to the control group. GSH: reduced glutathione; TAC: total antioxidant capacity; TBARS: thiobarbituric reactive substances; CARB: protein carbonyls; RBCL: red blood cell lysate.

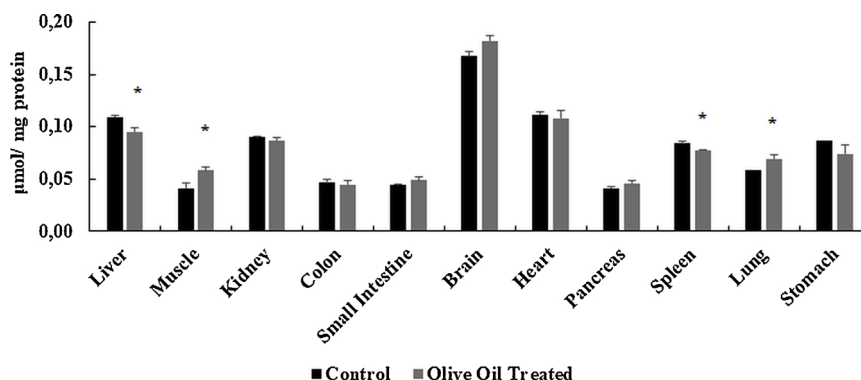
### 3.2. Gene and protein expression

According to our findings, the mRNA levels of  $\gamma$ -glutamylcysteine ligase were increased in brain and pancreas 1.32- and 1.4-fold, while they were decreased in spleen 0.69-fold. Superoxide dismutase levels were statistically significantly increased only in the brain (1.16-fold) and decreased in spleen (0.78-fold), while catalase levels were decreased both in spleen and pancreas 0.72- and 0.74- fold, respectively (Fig. 8) As for the respective protein expression,  $\gamma$ -glutamylcysteine ligase levels were increased in brain and pancreas by 31.10 % and

32.36 % compared with the control, respectively, while they were decreased in spleen by 66.43 %. Superoxide dismutase was increased in brain (30.79 %), but decreased in pancreas (59.53 %). Finally, catalase was decreased both in spleen and pancreas by 30 % (Fig. 9).

### 4. Discussion

We report herein that administration of an OO sample rich in biophenols in rats for two weeks in a dose that corresponds to the relevant EFSA health claim exerts controversial action on blood and tissue redox



**Fig. 3.** The effect of OO administration on GSH (reduced glutathione) levels of the rat tissues. \*: Statistically significant compared to the control group.

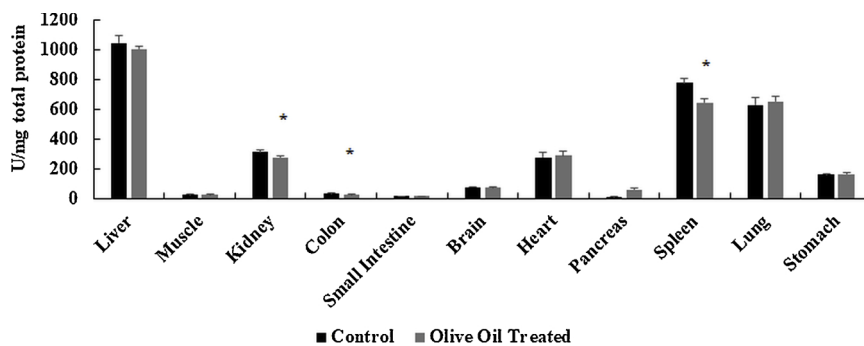


Fig. 4. The effect of OO administration on H<sub>2</sub>O<sub>2</sub> decomposition rate of the rat tissues. \*: Statistically significant compared to the control group.

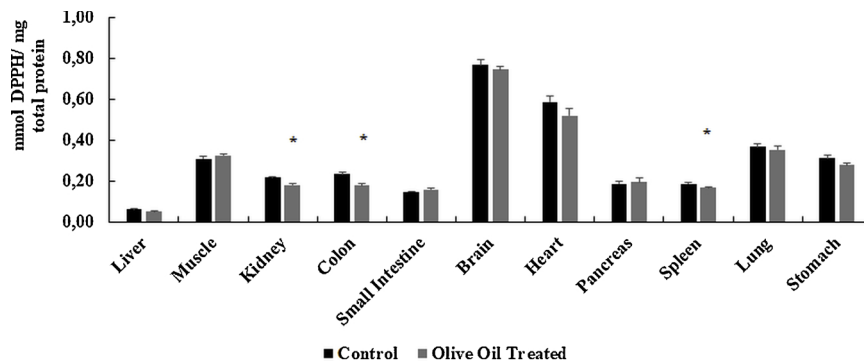


Fig. 5. The effect of OO administration on TAC (total antioxidant capacity) of the rat tissues. \*: Statistically significant compared to the control group.

status. Specifically, it was beneficial for blood, brain, muscle and small intestine, it induced oxidative stress in spleen, pancreas, liver and heart, whereas no distinct effects were observed in lung, colon and kidney. These results were reflected not only through the impairment of the levels of the antioxidant biomarkers, such as GSH and TAC but also through the increase in the concentrations of the biomarkers that indicate macromolecule oxidation, namely TBARS and CARB. Previous studies of our group have associated the biophenols contained in OO with potent *in vitro* antioxidant activity. The obtained OO polyphenol extracts were strong *in vitro* antioxidant agents and free radical scavengers, but also at highest concentrations, extracts causes the decrease of GSH, implying a prooxidant action [48–50]. However, experimental designs studying the effects of OO on redox status *in vivo* are either observational or lack in-depth analysis in order to provide an overall picture of the interaction between the biophenols content of OO and animal or human tissues. Thus, in the current study, the tested OO was selected following previous screening experiments of our research group [49]. Consequently, with the *in vivo* experiment described herein, we tried to shed light on whether *in vitro* observations actually

correspond to *in vivo* systems. This concept is of high importance since the promising beneficial effects of plant derived extracts *in vitro* often do not correlate to *in vivo* settings [51].

The first issue that must be clarified in order to assess a biological response after OO administration is the range of distribution and accumulation of OO biophenols in the body. According to Serra et al. 2012 [34], OO biophenols are absorbed, metabolized and distributed through the blood stream to all organs of the rat body, even across the blood–brain barrier. Sulphate conjugates of phenyl alcohols (mainly HT and T) were the main metabolites quantified in the plasma and tissues and free forms of some biophenols, such as oleuropein derivatives in the plasma and brain, luteolin in the kidney, testicle, brain and heart, or HT in the plasma, kidney and testicle were identified, proposing how OO exerts its biological response in specific organs [34]. In this end, numerous studies have demonstrated the beneficial health effects of OO biophenols [52]. Specifically, OO administration to rats offered protection against cadmium [53], TCDD [54], fluoxetine [55], 2,4 dichlorophenoxyacetic acid [56], aluminium and acrylamide [57] and paracetamol [58] induced toxicity, as assessed mostly in plasma and liver

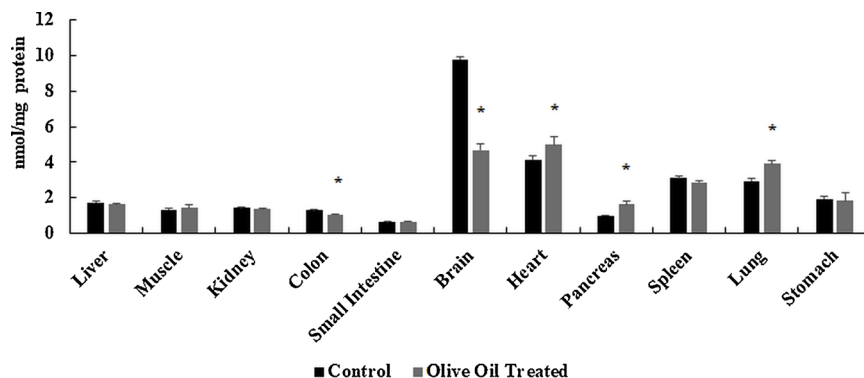


Fig. 6. The effect of OO administration on TBARS (thiobarbituric acid reactive substances) levels of the rat tissues. \*: Statistically significant compared to the control group.

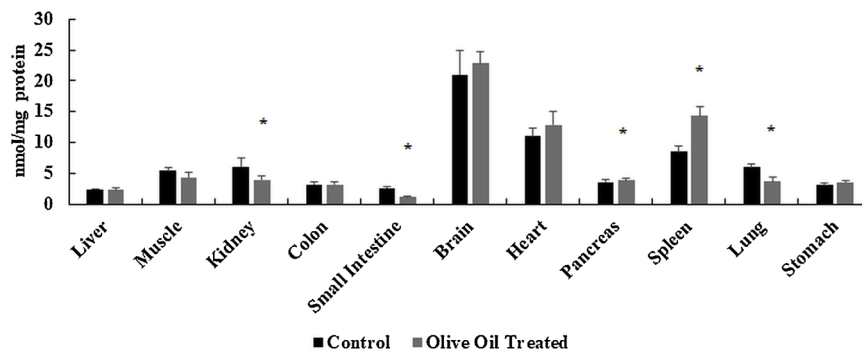


Fig. 7. The effect of OO administration on CARB (protein carbonyls) levels of the rat tissues. \*: Statistically significant compared to the control group.

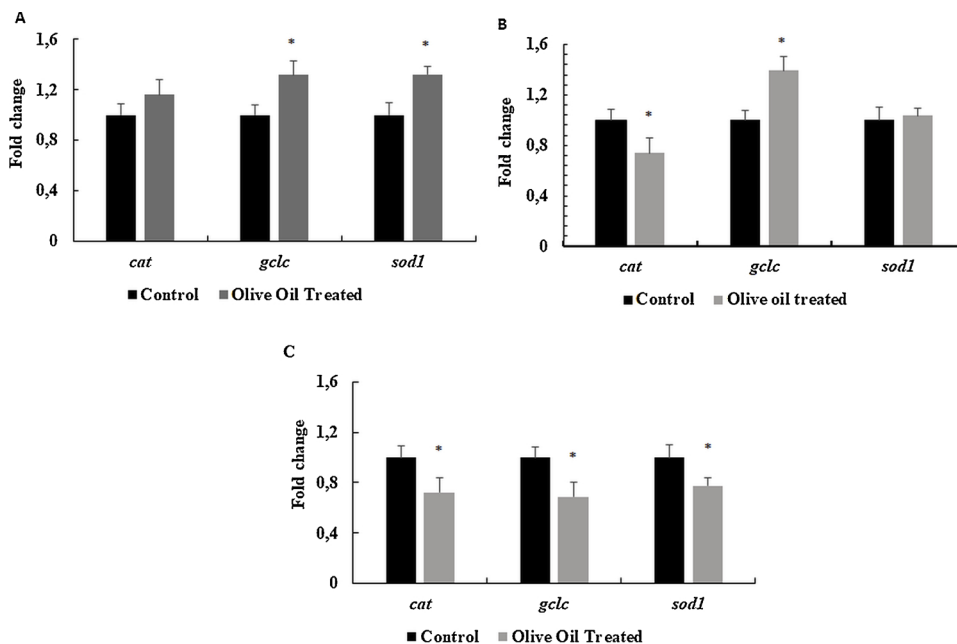


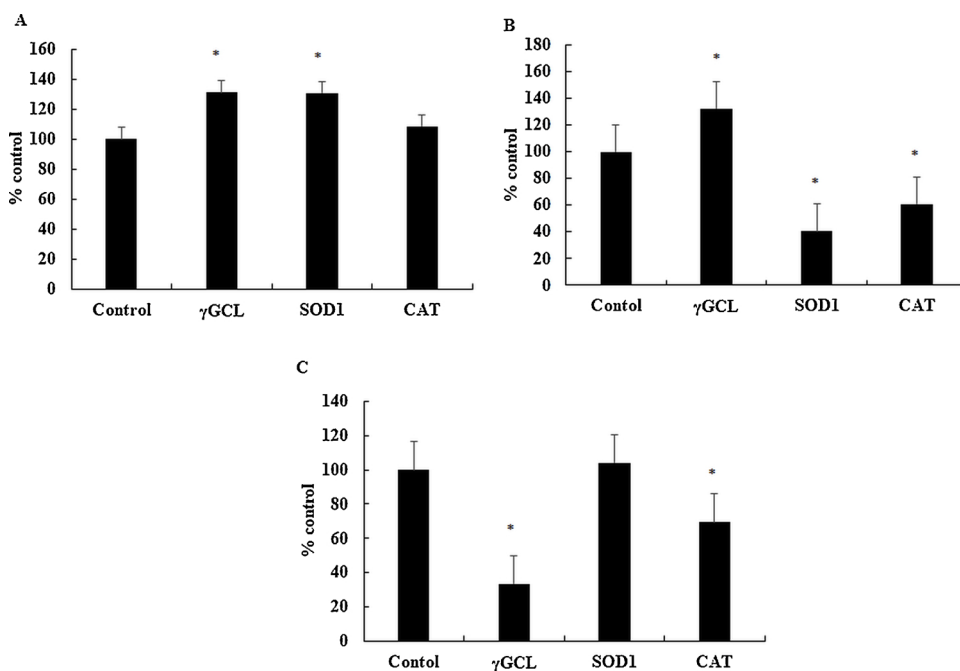
Fig. 8. The effects of olive oil administration on the expression levels of genes associated with the endogenous antioxidant defense mechanisms in selected rat tissues A) Brain, B) Pancreas, C) Spleen. \*: Statistically significant compared to the control group. The results are presented as mean fold change  $\pm$  SEM following normalization with the actin gene. gclc:  $\gamma$ -glutamylcysteine ligase, catalytic subunit; cat: catalase; sod1: superoxide dismutase.

redox (in terms of CAT, SOD, GPx, GR, NQO1, HO1, MDA and protein carbonyls,) and inflammatory (e.g TXB2 and LTB4) biomarkers. Also, EVOO administration to rats significantly decreased the levels of inflammation biomarkers with a concomitant increase of serum antioxidant capacity [59]. Moreover, human studies have indicated that sustained consumption of EVOO with high and moderate biophenolic content was more effective in protecting LDL from oxidation and in rising HDL cholesterol levels than other types of OO, proposing a dose-dependent protection mechanism against oxidative stress [60,61]. Additionally, OO protected DNA against oxidative damage and reduced cancer onset [62].

However, the current work is the first to investigate the alterations of redox status in the majority of tissues and not just blood or liver and also the endogenous redox state of rats without any administration of a toxic compound. Interestingly, our results for the first time point out a negative relation between OO biophenols and tissue redox status, especially spleen, pancreas, heart and liver, where a decrease at GSH and TAC levels was observed with a concomitant increase of lipid and protein oxidation levels. Faine et al. 2004 [63], showed that OO supplementation diminished catalase and GPx activities in myocardium. While, GPx activity has been inversely related to lipid peroxidation. Moreover, in another study [64], HT-sulfate was detected in spleen at low concentrations after the ingestion and remained there for a long time period, a fact that may account for the different antioxidant effectiveness of EVOO in spleen compared with other organs. Moreover, OO biophenols can exert direct antioxidant activity as well as modulate

detoxification enzymes [65]. Although, the molecular pathways/mechanisms for the reinforcement of blood and tissue antioxidant defense due to the consumption of diet rich in OO are still being under investigation. Even though, recent evidence has pointed out that the biophenolic components present in the OO are involved in the modulation of the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pathway. In turn, the activation of this pathway promotes the expression of antioxidant enzymes [5,49]. We have previously demonstrated that the polyphenolic compounds of the currently used OO increases the levels of both GSH and the mRNA and protein levels from the  $\gamma$ -GCL catalytic subunit ( $\gamma$ -GCLc) in endothelial, liver carcinoma, cervical cancer and myoblast cell lines, thus the altered enzyme expression levels may ascribed to the derepression of Nrf2 [49,50]. Although, the increased levels of  $\gamma$ GCLc observed herein in brain and pancreas that are not accompanied by the expression of other antioxidant enzymes. Also, elevated GSH levels can be attributed either to a higher biosynthetic rate (driven by  $\gamma$ -GCLc) or a higher recycling rate (driven by GPx and GR). According to our results,  $\gamma$ -GCLc levels were increased both in the mRNA and the protein level. Therefore, we have hypothesized that the current observations regarding GSH could be attributed to an enhanced biosynthetic rate. The differences of the availability, distribution, concentration and the time of persistence of the biophenols and their metabolites present in OO in different organs of the same animal may account for the differential antioxidant activity [64]. Indeed, studies of our laboratory have reported tissue-specific antioxidant effects of coffee biophenols on rats and OO mill wastewater in sheep, which





**Fig. 9.** The effects of olive oil administration on the protein levels of antioxidant enzymes in selected rat tissues A) Brain, B) Pancreas, C) Spleen. \*( $p < 0.05$ ): Statistically significant compared to the control group. The results are presented as a percent change of the levels of the respective protein in the control group  $\pm$  SEM following normalization with the GAPDH protein.  $\gamma$ -GCL:  $\gamma$ -glutamylcysteine ligase, catalytic subunit; CAT: catalase; SOD1: superoxide dismutase 1.

are not quite similar to those referred in this study [51,66] using the same experimental design. On the contrary, administration of polyphenol-rich grape pomace extract with strong antioxidant capacity in *in vitro* test in rats generally induced oxidative stress at rest and after exercise whereas exercise performance was not affected. These findings suggest that the polyphenol extract does not behave with the same way *in vitro* and *in vivo* [67]. Moreover, the route of administration (e.g., oral, intravenous, intraperitoneal) affects the rate and extent of absorption and, therefore, the anticipated biological action [68]. Thus, an important restriction for the miscarriage of antioxidants is the absence of standard route of administration, the non-optimal dosages and their bioavailability in targeted cell compartments, where ROS production is in excess [24].

Biophenols are mostly investigated for their antioxidant properties, but they may also act as prooxidants inducing oxidative stress. This is mainly observed when antioxidant molecules are administered individually and not as a part of the diet. The phenolic radical, which is created after neutralizing reactive species, may oxidize GSH generating a thiyl radical ( $RS\cdot$ ), which when reacts with GSH generates the disulfide radical ( $GSSG\cdot$ ). The latter reduces  $O_2$ , thus producing  $O_2^{\cdot-}$ . Moreover, the phenolic radical reduces ferric anion ( $Fe^{3+}$ ) to ferrous anion ( $Fe^{2+}$ ), which can produce the highly reactive  $OH\cdot$  by Fenton reaction [20]. Thus, “a protective effect of diet is not equivalent to a protective effect of antioxidants in diet” [19]. In 1995, Levander et al. [69], introduced a new term, the “dietary oxidative stress”. It signifies the disruption of cell redox homeostasis caused exclusively by diet via the excess uptake of oxidative load or the impaired availability of antioxidants. The use of the term “dietary oxidative stress” confirms the excessive role that has been given to nutrition from scientists, but ostensibly attends the norms of redox biology [14]. Likewise, the up regulation of antioxidant enzymes or the increased uptake of antioxidant compounds as dietary components or as nutritional supplements is not synonymous with an improved defense against the harmful effects of reactive species [14]. Apart from the term “dietary oxidative stress”, researchers have also introduced the term “antioxidative stress”, which occurs through the prooxidants effects from high levels of antioxidant compounds [70]. Reactive species production is not unavoidably an un-desirable condition, since its outcomes may be beneficial for many physiological processes in cells. On the contrary, there are potentially harmful effects of “antioxidative stress,” mostly in the

cases of overconsumption of antioxidants. Antioxidants can neutralize ROS and decrease oxidative stress; however, this is not always beneficial for disease onset or progression (e.g., cancer) or for delaying aging [70]. Recent studies suggest that antioxidant supplements do not offer sufficient protection against oxidative stress and damage or they do not increase lifespan. Moreover, the antioxidant therapy has no effect and can even promote mortality [71–75]. On the basis of the above, the obtained results from clinical trials, where the individuals received antioxidants failed to indicate beneficial outcomes. Schulz et al., found that nutrition antioxidants totally abrogate the outspread of lifespan by suspending an adaptive response to ROS named “mitohormesis” [76]. Under certain conditions, *in vitro* and *in vivo* trials showed that vitamin C and E, SOD, GSH and  $\beta$ -carotene caused “antioxidative stress” in addition to prooxidative stress [70].

Additionally, in the present study no distinct effects or no effects whatsoever were observed in lung, colon, kidney and stomach. It is worth to be mentioned that cell homeostatic machinery regulates the potential alterations of its antioxidant arsenal. Thus, administration of exogenous antioxidants possibly mitigates the synthetic rate or even the uptake of endogenous antioxidants, so that the total “cell antioxidant potential” remains unaltered, a phenomenon described by Cutler et al. 2003, as “the oxidative stress compensation model”. This model tries to explain why the antioxidants through diet fail to decrease oxidative stress levels and increase longevity [77]. It is important to highlight that cells are under strict homeostatic control by a complex antioxidant network (e.g., molecules, enzymes, recycling of endogenous antioxidants). Therefore, if an exogenous antioxidant affects the endogenous antioxidant mechanism, a compensatory change in the levels of other antioxidants may lead to unaltered overall antioxidant capacity [70].

According to the chemical composition of the administered OO sample, oleocanthal (OLEO) is present in high concentration equal to 590 mg/kg. OLEO, a fundamental constituent of OO has been associated with numerous activities that are beneficial for human health [78]. Indeed, it has been reported that OLEO inhibits cyclooxygenase-1 and -2 activities, thus exerting strong anti-inflammatory properties and, astonishingly, it is more potent than ibuprofen [79]. Furthermore, OLEO is also known for its antioxidant and antimicrobial action [78,80], whereas it has been demonstrated that it acts as an anticancer agent as well [81]. Nevertheless, this study sets an important issue

regarding the optimum OLEO concentration of OO. It is possible that the adverse effects of OO observed in specific tissues could be related to the concentration levels of OLEO and/or other contained polyphenols. This observation seems to come against the common notion that the higher the polyphenolic content of OO the most beneficial will be for human health. On this end, our results formulate a skepticism regarding the amounts of OO constituents that at the end of the day are not detrimental for blood and tissue redox profile.

It is worth mentioning here that the majority of the studies in the literature examine the protective effects of polyphenols against oxidative stress, however only few of them investigate their putative toxic action. From the available data it appears that acute oral administration of a grape seed proanthocyanidin or punicalagin extract to rats or mice did not induce toxicity (60). However, nephropathy was observed when high doses were added to their diet chronically. Linear extrapolation of these data indicates appreciable risk at normal dietary levels [82]. Even though OO is a safe product, meaning that practically no adverse effects are anticipated following its administration in *in vivo* settings, in the present study we report that OO consumption by rats caused oxidative stress in some tissues, meaning that these specific tissues did not exert useful adaptations after an antioxidant treatment. Although, as the real life scenario dictates, humans consume OO during their entire lives, which means that this is a chronic practice for them. On the basis of the relevant literature, we believe that after long term administration the OO will possibly counteract these detrimental effects and will reinforce the tissue defense system due to its high polyphenolic content. This scenario appears also in a study of our group where feed supplemented with polyphenolic additives from OO mill wastewater elicited positive effects on lamb muscle, heart and liver redox status after a long term exposure compared with the short term exposure [83]. It has also to be stressed that the opposite observation was mentioned in a recent article [32]. Interestingly, the chronic administration of a mixture of xenobiotics induced beneficial effects on antioxidant mechanism of rat tissues only after short term exposure, whereas long term exposure showed toxicity [32]. It appears, therefore, that the acute administration of a putatively beneficial compound (*i.e.*, OO) leads to the impairment of antioxidant defense, but chronic treatment may lead to beneficial outcome. Finally, it must be pointed out that exposure levels depend on the mode of presentation of the polyphenols [82]. The above mentioned studies clearly indicate that long term exposure to a dose of xenobiotics that is seemingly harmless induces toxicity and disrupts redox equilibrium in animals [32]. On the contrary, when the exposure is short term, the impact is beneficial since redox adaptations occur [32,33]. Under this frame, the negative results observed in the present study after OO administration in specific tissues are probably considered as adaptive responses. The antioxidant arsenal of these tissues is anticipated to be reinforced when OO, which is not a xenobiotic but an edible oil, is administered long term. Therefore, some tissues are benefited directly by the undisputed positive action of OO, whereas others need more time in order to surpass the first so-called shock of administration and to, finally, end with useful redox adaptations. Thus, it will be more realistic to evaluate the effects of the low-dose exposure, in order to clarify the regulatory background of different compounds, introducing new regulatory guidelines [84].

## 5. Conclusion

This is the first study to report seemingly adverse effects after administration of OO in specific organs, regarding their redox status, despite the numerous health benefits of OO biophenols. However, according to the real life exposure scenario that was analyzed above it is believed that the negative role of OO in the redox status of some tissues is actually an adaptive response that is anticipated to reinforce their redox profile after long term administration, as it has also been previously reported. An important innovation of the present study is the fact that the administered OO sample has a high polyphenolic content,

thus offering crucial insight in the composition-based debate over the biological actions of olive oil. The new idea of toxicology (*i.e.*, the long-term low-dose exposure regimen) is applied here. Therefore, it appears that the contribution of diet antioxidants to the protection against oxidative stress is a multi-factorial phenomenon and time of exposure is a decisive factor. Therefore, further studies are required in order to assess the chronic effects of OO administration, a situation that resembles to the real life exposure scenario.

## Data availability statement

All data, tables and figures in this manuscript used to support the finding of this study are original and are available upon request.

## CRediT authorship contribution statement

**Paraskevi Kouka:** Conceptualization, Methodology, Software, Formal analysis, Resources, Writing - original draft, Writing - review & editing, Visualization. **Fotios Tekos:** Formal analysis. **Zoi Papoutsaki:** Visualization. **Panagiotis Stathopoulos:** Formal analysis, Writing - original draft, Visualization. **Maria Halabalaki:** Conceptualization, Methodology, Resources, Writing - original draft. **Maria Tsantarliotou:** Conceptualization, Methodology, Resources, Writing - original draft. **Ioannis Zervos:** Formal analysis. **Charitini Nepka:** Software, Investigation. **Jyrki Liesivuori:** Resources. **Valerii N. Rakitskii:** Resources. **Aristidis Tsatsakis:** Resources, Data curation. **Aristidis S. Veskokakis:** Writing - original draft, Writing - review & editing. **Demetrios Kouretas:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - review & editing, Supervision, Project administration.

## Declaration of Competing Interest

The authors declare that they have no competing interests.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.toxrep.2020.02.007>.

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