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Unravelling the capacity of hydroxytyrosol and its lipophenolic derivates to modulate the H_2O_2-induced isoprostanoid profile of THP-1 monocytes by UHPLC-QqQ-MS/MS lipidomic workflow

Carolina Alemán-Jiménez [a,1], Raúl Domínguez-Perles [b,*,1], Federico Fanti [b,c], Juana I. Gallego-Gómez [a], Agustín Simonelli-Muñoz [d], Espérance Moine [e], Thierry Durand [e], Céline Crauste [e], Ángel Gil-Izquierdo [b,*], Sonia Medina [b]

[a] Departamento de Enfermería, Universidad Católica de Murcia, UCAM, 30107 Murcia, Spain
[b] Department of Food Science and Technology, Research Group on Quality, Safety and Bioactivity of Plant Foods, CEBAS (CSIC), Campus Espinardo, 30100 Murcia, Spain
[c] University of Teramo, Faculty of Bioscience and Technology for Food, Agriculture and Environment, 64100 TE, Italy
[d] Departamento de Enfermería, Fisioterapia y Medicina, Universidad de Almería, Spain
[e] Institut des Biomolécules Max Mousseron (IBMM), UMR 5247, CNRS, University of Montpellier, ENSCM, Faculty of Pharmacy, Montpellier, France

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ABSTRACT

Presently, the attention given to natural substances to counteract damage produced by oxidative stress (OS) has risen sharply. In this scenario, hydroxytyrosol (HT) derivatives, formed as a result of HT conjugation with fatty acids (FAs) (lipophenols), have been recently described in foodstuffs such as extra virgin olive oil, as being powerful bioactive compounds with a higher activity than the unesterified phenolic compound. The present work describes the capacity of HT lipophenols to act on the course of OS and secondary inflammatory processes, based on their capacity to modulate the isoprostanoid profile induced by H_2O_2 in THP-1 monocytic cells. A UHPLC-QqQ-ESI-MS/MS-based lipidomics workflow was applied over a range of 37 human oxylipins. The main outcomes retrieved suggest both HT and HT-lipophenols as regulators of the cellular redox balance, acting as pro-oxidants *in vitro*, which is highly dependent on the experimental conditions. Our outcomes suggest the anti-inflammatory potential of both HT and HT-lipophenols, where the type of the FAs on the HT core appears to be critical for defining the bioactivity of lipophenols, highlighting that a lipidomic approach, with the simultaneous analysis of multiple oxylipins, is critical for the understanding of the bioactivity of lipophenols on isoprostanoid generation and hence, on pathophysiological processes.

1. Introduction

Lipids are involved in numerous biological processes and are key elements for the proper development of essential cell functions. Perturbations in lipid homeostasis, along with micro-environmental conditions that favor oxidative stress (OS), are closely associated with an array of pathophysiological conditions, namely obesity, diabetes, cancer, neurodegenerative disorders, and autoimmune diseases [1–3]. As for lipid oxidation, a clear-cut distinction may be made between enzymatic and non-enzymatic mechanisms, with both oxidative pathways related to the human pathophysiological conditions mentioned previously, for which dietary habits and patterns of nutrition are key aspects

Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; BHA, butylated hydroxyanisole; COX, cyclooxygenase; DMSO, dimethyl sulfoxide; ECACC, European Collection of Cell Culture; ESI, electrospray ionization; EVOO, extra virgin olive oil; FAs, fatty acids; FBS, fetal bovine serum; HT, hydroxytyrosol; IL-12, interleukine-12; LA, linoleic acid; LC-MS, liquid chromatography-mass spectrometry; LOX, lipoxygenase; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; OA, oleci acid; OS, oxidative stress; PMS, phenazine methosulfate; PLA2, phospholipase A2; PG, prostaglandin; PUFAs, polyunsaturated fatty acids; ROS, reactive oxygen species; SPE, solid-phase extraction; THP-1, human monocytic cell line; UHPLC, ultra-high pressure liquid chromatography; XTT, sodium 30-[1-(phenylaminocarbony)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate.

* Corresponding authors.
E-mail addresses: rdperles@cebas.csic.es (R. Domínguez-Perles), angelgil@cebas.csic.es (Á. Gil-Izquierdo).

[1] These two authors contributed equally to the present work.

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of special interest because of their capacity to modulate cell metabolism [4]. The non-enzymatic oxidation of lipids can be produced by reactions triggered by a high concentration of reactive oxygen species (ROS), such as singlet O_2, hypochlorous acid (HOCl), and ozone (O_3) or by inorganic free radical species derived from nitric oxide (NO), superoxide ion (O_2^-), and hydrogen peroxide (H_2O_2) [5]. On the other hand, enzymatic lipid oxidation reactions are catalyzed by peroxidases, which are a group of proteins capable of oxidizing lipids by using H_2O_2 as a source of oxidizing equivalents. This group of enzymes includes the so-called cyclooxygenase (COX), also known as prostaglandin H synthase, which is mainly involved in the oxidation of "free" polyunsaturated fatty acids (PUFA), particularly arachidonic acid (AA), giving rise to prostaglandins and other lipid mediators [6].

Oxidative environments can trigger a complex cascade of molecular reactions within cells to protect critical biomolecules from oxidative modifications. Diverse studies on these mechanisms suggest that phenolic antioxidants present in plant-based foods, and especially in extra virgin olive oil (EVOO), such as hydroxytyrosol (HT), possess specific biological capacities that may contribute to protecting the structure of the lipids from oxidative damage [7–10], thus helping to preserve their functions and ensure normal cell metabolism.

Within the so-called bioactive compounds present in plant-based foods, lipophenols (or phenolipids) have been described in the last decade, which are (poly)phenolic compounds esterified with fatty acids (FAs). Several advantages have been attributed to these molecules relative to the native molecule (unesterified phenolics or FAs) regarding their antioxidant, anti-carbonyl stress potency, radical scavenging, and anti-inflammatory activities [11–14], which have attracted considerable attention in the fields of food science, nutrition, and health [12,15]. Particularly related to the antioxidant activity of lipophenols, to date, a parabolic behavior ("cut-off" effect) has been demonstrated resorting to *in vitro* models. This behavior is characterized by an initial enhancement of antioxidant efficiency, in parallel to the increase in the alkyl chain length, until a critical point is reached when the molecule experiences a slight drop in its biological power [16]. Nevertheless, the scientific literature about the improved antioxidant capacity conferred to (poly) phenols as a result of conjugation with PUFAs is still very scarce. The limited information available on this issue indicates that two quercetin lipophenols (esterified with α-linolenic acid (ALA) and linoleic acid (LA)), display a potent antioxidant activity, being even capable of reducing A2E-induced cell death more efficiently than unbound quercetin [11,12] and a more recent article described that quercetin esterified with FAs showed much-improved lipophilicity, higher cell membrane affinity, and enhanced cellular antioxidant activity than the parent quercetin [17]. Moreover, both the effect of the fatty acid chain length and the degree of unsaturation in the lipid part, on the modulation of the generation of oxidized lipid mediators by lipidomics workflows, remain poorly explored.

Recently, lipophenols of HT, esterified with ALA, LA, and oleic acid (OA) (HT-ALA, HT-LA, and HT-OA, respectively) have been identified in an array of foods and foodstuffs such as EVOO, refined olive oil, flaxseed oil, grapeseed oil, and margarine, in a wide range of concentrations [15,18]. These lipophenols have also been suggested as powerful bioactive compounds with higher activity than the native molecule (HT), presumably due to the combination of the bioactivities of both phenolic compounds and FAs, and the modification of their lipophilicity [15]. However, their actual capacity to modulate molecular changes in pro-oxidative and inflammatory environments in cells and tissues, under oxidant conditions, according to their ability to modulate the quantitative isoprostanoid profile representative of oxidative and inflammatory damage, has not been addressed. Thereby, in this scenario, the present study elucidates the capacity of HT lipophenols to modulate the oxidative response triggered by H_2O_2 in the THP-1 human monocytic cell line, in comparison with unesterified HT *in vitro,* by the application of a lipidomic approach for the high-throughput identification of down- and up-regulated oxidized lipids.

2. Experimental section

2.1. Chemicals and materials

The authentic standards of high purity HT-lipophenols (HT-ALA, HT-LA, and HT-OA) were synthesized and fully characterized (using NMR analysis) by Durand's team at the Institut des Biomolecules Max Mousseron (IBMM) (Montpellier, France), according to previously published procedures [15]. All lipophenols were dissolved in dimethyl sulfoxide (DMSO) from Sigma Aldrich (St. Louis, MO, USA). Hydroxytyrosol with a purity of 99.6% was provided by Seprox BIOTECH S.L. (Murcia, Spain). Acetone, butylated hydroxyanisole (BHA), β-glucuronidase, type H2 from *Helix pomatia*, and BIS-TRIS (Bis-(2-hydroxyethyl)-amino-tris (hydroxymethyl)-methane) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and all LC-MS grade solvents, such as H_2O, methanol, and acetonitrile were from J.T. Baker (Phillipsburg, NJ, USA). Hydrochloric acid was purchased from Panreac (Castellar del Vallés, Barcelona, Spain), and the Strata X-AW, 100 mg/3 mL solid-phase extraction (SPE) cartridges from Phenomenex (Torrance, CA, USA). A total of 37 oxylipins (21 prostaglandins (PGs), 15 isoprostanes (IsoPs), and one thromboxane (TX)) generated from different polyunsaturated FAs (arachidonic acid (AA), dihomo-gamma-linolenic acid (DGLA), and eicosapentaenoic acid (EPA), respectively) in the frame of diverse enzymatic and non-enzymatic synthesis pathways, were assessed in the current study (Table 1). Some of them were purchased from Cayman Chemicals (Ann Arbor, Michigan, USA), including 15-F_{2t}-IsoP-d_4 (8-*iso*-$PGF_{2\alpha}$-d_4 as internal standard (IS)) and others (2,3-dinor-15-F_{2t}-IsoP, 2,3-dinor-15-*epi*-15-F_{2t}-IsoP, 5-F_{2t}-IsoP, 5-*epi*-5-F_{2t}-IsoP, 15-*epi*-15-D_{2t}-IsoP, 8-F_{3t}-IsoP, and 8-*epi*-8-F_{3t}-IsoP) were synthesized by Durand's team at the Institut des Biomolecules Max Mousseron (IBMM) (Montpellier, France). The THP-1 cell line and the RPMI-1640 were purchased from the European Collection of Cell Culture (ECACC, Public Health England, Porton Down, Salisbury, UK). L-glutamine, sodium 30-[1-(phenylaminocarbony)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT), and phenazine methosulfate (PMS) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell culture and esterified and unesterified hydroxytyrosol treatment

The monocytic human (THP-1) cell line (ECACC® General Cell Collection-88081201) was purchased from the European Collection of Cell Culture (ECACC, Public Health England, Porton Down, Salisbury, UK). The THP-1 cells were grown according to the previously described methodology [18,19]. The passage number of the cells used in this study was between 17 and 20. Cells were treated with 20 μM of individual HT lipophenols (HT-ALA, HT-LA, and HT-OA) and 20 μM of the unesterified molecule (HT) in triplicate (n = 3) for 24-h according to the cytotoxicity results of HT lipophenols described by Medina et al. on THP-1 cells [18]. Afterward, the cells were exposed to a pro-oxidative stimulus (50 μM H_2O_2) and maintained under these conditions at 37 °C and 5% CO_2, for another 24-h. Due to the physicochemical stability of compounds is associated with their biological properties, culture media spiked with unesterified and esterified HT was assessed on the quantitative profile of the target analytes by mimicking the experimental conditions, thus ensuring the stability of compounds and discarding auto-oxidation processes of FAs (Fig. S1). The concentration of HT-ALA, HT-LA, and HT-OA assessed on the capacity to modulate the level of isoprostanoids, was selected based on previous studies, which detected HT lipophenols in EVOO [15,18]. This concentration allowed for setting their capacity to prevent oxidative and inflammatory damages in THP-1 cells, under oxidative conditions. In this regard, although the concentration tested could exceed the theoretical level achieved in target cells (which has not been previously described), especially because of the expected breakdown of dietary lipophenols during gastrointestinal digestion, the experimental design established in the present work was utilized to understand the biological potential of the HT lipophenols in comparison

Table 1
Oxylipins assessed in THP-1 cells pre-exposed to 20 μM of the unesterified hydroxytyrosol (HT) and molecules of HT esterified with fatty acids (α-linolenic acid (ALA), linoleic acid (LA), and oleic acid (OA)) for 24 h followed by 24 h co-exposure with 50 μM of oxidizing agent (H_2O_2).

Prostanoids generated from AA				
Pathway I	Pathway D	Pathway E	Pathway F	Thromboxane
6-keto-$PGF_{1\alpha}$	PGD_2 (*)	PGE_2 (*)	$PGF_{2\alpha}$ (*)	11-dehydro-TXB_2
2,3-dinor-6-keto-$PGF_{1\alpha}$	PGDM (*)	20-OH-PGE_2	20-OH-$PGF_{2\alpha}$ (*)	
	Tetranor-PGDM	Tetranor-PGEM	19(R)-OH-$PGF_{2\alpha}$	
	Tetranor-PGDM lactone	Tetranor-PGAM (*)	Tetranor-PGFM	
	11-β-$PGF_{2\alpha}$ (*)		15-keto-$PGF_{2\alpha}$ (*)	
	2,3-dinor-11β-$PGF_{2\alpha}$ (*)			
	Tetranor-PGJM			
PGs generated from DGLA		PGs generated from EPA		
PGE_1		17-*trans*-$PGF_{3\alpha}$		
$PGF_{1\alpha}$ (*)				
Isoprostanes				
IsoPs generated from AA	IsoPs generated from DGLA	IsoPs generated from EPA		
15-F_{2t}-IsoP	15-E_{1t}-IsoP	8-*epi*-8-F_{3t}-IsoP		
15-keto-15-F_{2t}-IsoP (*)	15-F_{1t}-IsoP	8-F_{3t}-IsoP		
15-*epi*-15-F_{2t}-IsoP				
ent-15-*epi*-15-F_{2t}-IsoP				
2,3-dinor-15-F_{2t}-IsoP				
9-*epi*-15-F_{2t}-IsoP				
2,3-dinor-15-*epi*-15-F_{2t}-IsoP				
5-F_{2t}-IsoP				
5-*epi*-5-F_{2t}-IsoP (*)				
15-keto-15-E_{2t}-IsoP				
15-*epi*-15-D_{2t}-IsoP(*)				

AA: arachidonic acid; DGLA: dihomo- γ-linolenic acid; EPA: eicosapentaenoic acid; IsoPs: isoprostanes; PGs: prostaglandins; TX: Thromboxane. (*) These oxylipins were quantified in the current assay.

with the native unesterified molecule to prevent changes in the quantitative profile of isoprostanoids after exposure to an oxidative environment.

2.3. Extraction of isoprostanoids and analysis by UHPLC-ESI-QqQ-MS/MS

The quantitative isoprostanoid profile was determined in THP-1 cells as the sum of the intracellular concentration (compounds taken by the cells) and the concentration of the compounds secreted into the growth medium to obtain comprehensive information about the metabolism of oxylipins after exposure to 50 μM H_2O_2 with HT and HT-lipophenols treatments in contrast to untreated cells. The cells and supernatants were collected after a 24-h exposure to H_2O_2 and processed according to the procedure described by Campillo et al. [19]. Briefly, cells were lysed using a specific lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, containing 0.005% BHA). MeOH/HCl 200 mM (0.5 mL) was added to the cell lysate and growth medium, and the extracts obtained were centrifuged at 1000×*g* for 5 min, at 4 °C. The supernatants were collected and stored at −80 °C until oxylipin extraction and analysis.

For the extraction of oxylipins, both the pre-processed growth medium and cell lysate extracts were first enzymatically hydrolyzed (β-glucuronidase Type HP-2 from *Helix pomatia*) to remove glucuronide and sulfate conjugates, according to the method described by Medina et al. [20]. The hydrolyzed extracts were cleaned up by SPE using Strata X-AW cartridges (100 mg/3 mL), and the eluents were analyzed by UHPLC-ESI-QqQ-MS/MS, following the previously published methodology from our research team [20,21]. The 15-F_{2t}- IsoP-d_4 was used as an internal standard due to its similar fragmentation pattern relative to the target oxylipins considered in the present work. Data acquisition and processing were performed using the MassHunter software version B.08.00 (Agilent Technologies, Walbronn, Germany). The concentration of the isoprostanoids was calculated according to standard curves that were freshly prepared each day of analysis. Additionally, the limit of quantification (LOQ), calculated as a signal/noise ratio of 10, was set at 8 pM, in agreement with the LOQ previously reported by several authors for quantifying lipid peroxidation in cellular systems [19,22].

2.4. Statistical analyses

All treatments and extractions were performed in triplicate (n = 3), and the data were expressed as the mean ± SD. Statistical tests were performed at a 5% significance level using the SPSS 27.0 software package (LEAD Technologies, Inc., Chicago, USA). Data were subjected to a one-way analysis of variance (ANOVA), and the fulfillment of the one-way ANOVA requirements, especially the normal distribution of the residuals and the homogeneity of variance, were tested with the Kolmogorov–Smirnov and Levene's tests, respectively. When statistical differences were identified, the variables were compared using the Tukey's HSD post-hoc test. Significant differences among means were considered at $p < 0.05$.

3. Results and discussion

As referred to before, this study aimed to evaluate unesterified HT and esterified with ALA, LA, and OA, as bioactive compounds present in olive oil (EVOO and virgin olive oil (VOO)) on their ability to modulate the isoprostanoids response triggered by the exposure of THP-1 cells to oxidative environments (H_2O_2). The THP-1 is a human leukemia monocytic cell line, characterized by a strong correspondence with the monocytic fraction of peripheral blood mononuclear cells. Although THP-1 cells may not express matching features relative to primary monocytes and their regulatory mechanisms, they are characterized by physiological properties characteristic of primary monocytes, *in vivo* [23]. Because of this, the THP-1 cell line has been used as a model to study the molecular pathways involved in the oxidative and inflammatory responses, as well as the capacity of food compounds to modulate such pathways and the molecular mechanisms responsible for, which are strongly assoicated with the course of an array of pathophysiological situations [19,24].

For this purpose, the physicochemical stability of lipophenols is imperative for research their biological properties, the outcomes from the assays performed in this matter proved the stability of esterified molecules at *in vitro* cell growth conditions (Fig. S1). At this juncture, the present work contributes to elucidate how specific lipophenols can modulate, *in vitro*, the OS and inflammation-dependent isoprostanoid profile induced by the exposure to oxidizing agents. The effect of oxidizing H_2O_2 on the generation of OS and pro-/anti-inflammatory markers (IsoPs and PGs, respectively) [25,26] has already been described in THP-1 cells, following a similar experimental design [19]. The most relevant strength of the current assay is represented by the

lipidomic workflow applied, which allows shedding light on the bioactivity of lipophenols *in vitro* through a range of OS and inflammation-related isoprostanoids. This approach allows overcoming the constraints associated with the monitoring of single IsoP or PG that provides incomplete information on the pathophysiological model, as several isoprostanoid markers are rapidly metabolized and their rates of metabolism may vary significantly [27].

3.1. Modulation of the quantitative isoprostane profile of THP-1 monocytic cells by unbound and esterified hydroxytyrosol under oxidative conditions

The capacity of HT and its lipophenolic derivatives to modulate OS was monitored by assessing 15 IsoPs derived from AA, DGLA, and EPA (Table 1), although only three IsoPs from AA (15-keto-15-F_{2t}-IsoP, 5-*epi*-5-F_{2t}-IsoP, and 15-*epi*-15-D_{2t}-IsoP) were found at levels higher than the limit of quantification (LOQ) of the method utilized in the current study (Fig. 1). All the oxylipins were expressed as the sum of the intra- and extra-cellular concentrations recorded, as both are secreted by cells and provide a complete picture of the IsoPs profile and metabolism, under particular conditions. As expected, the exposure of THP-1 cells to 50 μM H_2O_2 caused an increase in the IsoP level in comparison with basal conditions (untreated cells) (Fig. 1), although this trend was not statistically significant for 5-*epi*-5-F_{2t}-IsoP or 15-*epi*-15-D_{2t}-IsoP. This lack of significance regarding the increase of specific OS markers under oxidative growing conditions for most of them may occur because of the concentration of H_2O_2 used in the current assay to trigger OS in THP-1 cells (50 μM), as concentrations of the agent used to generate oxidative damage in cellular models are in the high micromolar range (10–1000 μM) [28]. The nominal concentrations of H_2O_2 used to condition the growth medium, as well as its cytotoxicity strongly, depends on diverse factors such as incubation time or the cell concentrations of different supplements featured by the radical scavenging capacity of the culture medium [29]. However, concentrations above 100 μM are disproportionally high concerning the physiological oxygen concentration (low micromolar) and may affect the viability of THP-1 cells. The concentration of 50 μM was selected based on previous reports which described no statistical differences between the 50 and 100 μM treatments used to induce OS in cells with H_2O_2, whilst 200 μM of the oxidizing agent significantly reduced the viability of cells [30]. Moreover, to the present date, it has been reported that both IsoPs and PGs can be identified and quantified in the supernatant of human THP-1 macrophages, where they are highly produced after a 12 h oxLDL treatment (50 μg/mL) as compared to native LDL [24].

Regarding the 15-keto-15-F_{2t}-IsoP, a metabolite of 15-F_{2t}-IsoP (8-*iso*-$PGF_{2\alpha}$), it was not detected in the untreated controls, whilst after the exposure to 50 μM H_2O_2, it was found at a concentration of 0.097 ng/mL. When monitoring the preventive effect of HT and its lipophenolic derivatives present in VOO and EVOO, it was observed that none of them (HT-ALA, HT-LA, and HT-OA) were competent enough to smooth the

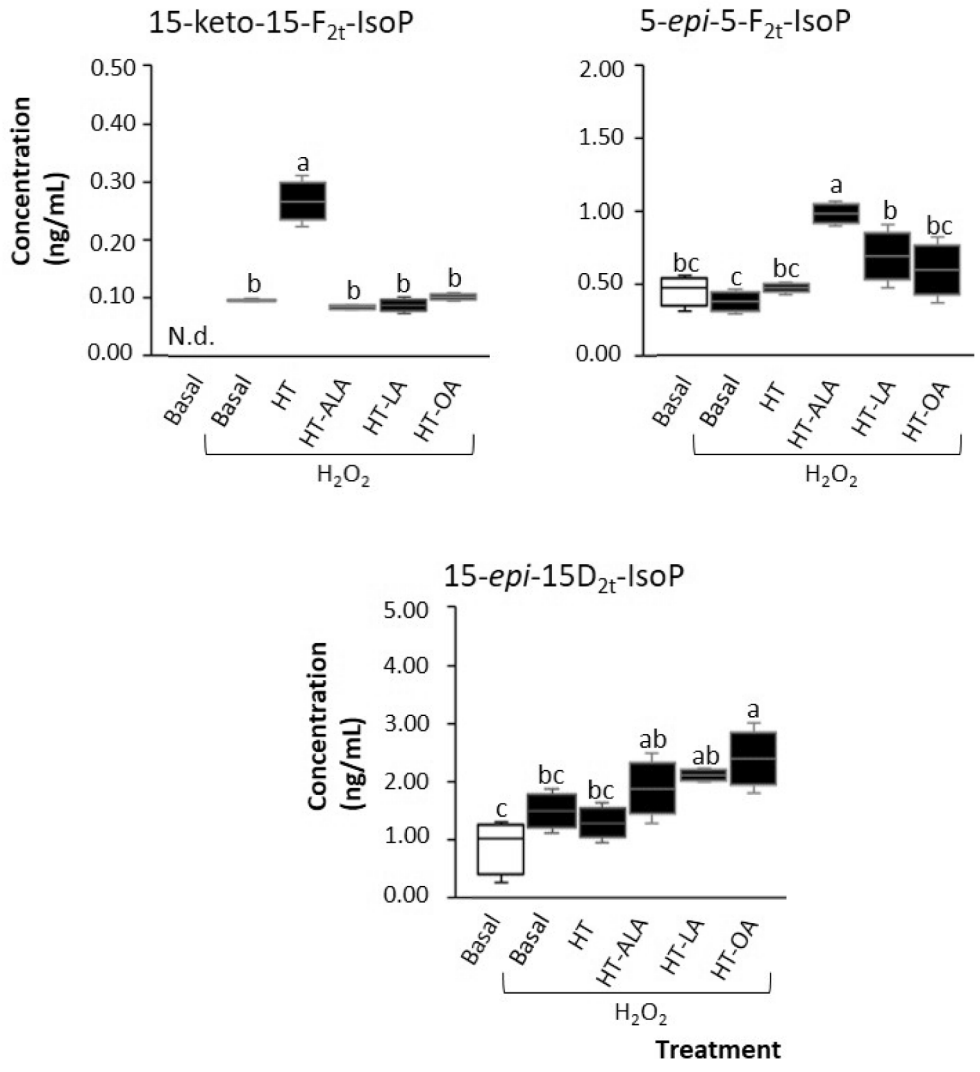

Fig. 1. Modulatory effect of the hydroxytyrosol (HT) and lipophenols of hydroxytyrosol (HT-ALA, HT-LA, and HT-OA) treatments on the quantitative isoprostane profile of H_2O_2-stimulated THP-1 monocytic cells. Isoprostanes were determined in THP-1 monocytic cells (cell lysate and growth media) pre-exposed to 20 μM of unesterified HT and lipophenolic derivatives of HT (α-linolenic acid (ALA), linoleic acid (LA), and oleic acid (OA)) and 50 μM H_2O_2. Values are shown as mean ± SD (n = 3). Different capital letters within each box and whisker plots indicate significantly different treatments at $p < 0.05$, according to a one-way analysis of variance (ANOVA) and Tukey's multiple range test. N.d.: not detected.

effect of the oxidative stimulus. However, the treatment with unbound HT caused a significant increase in the concentration of this OS marker up to 0.267 ng/mL (Fig. 1), thus evidencing a pro-oxidant effect at the concentration tested. Concerning 5-*epi*-5-F_{2t}-IsoP, the different concentrations induced by the array of treatments applied were not statistically significant relative to that recorded for basal and H_2O_2-treated cells, with HT-ALA being the only compound which induced a significant increase ($p < 0.05$) of 5-*epi*-5-F_{2t}-IsoP, up to the average concentration of 0.979 ng/mL (2.2-fold higher than controls).

To date, the major differences described in humans between the 5- and the 15-series of F_2-IsoPs are referred to as their concentration. Thus, the level of 5-F_{2t}-IsoP has been reported to reach concentrations up to 4 times higher than 15-F_{2t}-IsoP [31]. According to this previous information, the 5-series was found at a concentration 3.6 times higher than the 15-series within the F_2-IsoPs. This finding may provide valuable information about the pathophysiological meaning of OS and its relationship with an array of diseases. In this sense, to date, research on the further differences between these two series of IsoPs noted that 15-F_{2t}-IsoP is a vasoconstrictor and may be involved in the pathogenesis of coronary disorder, whilst 5-F_{2t}-IsoP is not able to develop vasomotor effects and as such, was not likely to be involved in the pathogenesis of vascular diseases or inflammatory processes, which account for the high involvement of vascular events [31]. This is very valuable information due to the close association between olive oil consumption and low mortality within the frame of cardiovascular disease [32].

Regarding the 15-series of D2-IsoPs, 15-*epi*-15-D_{2t}-IsoP displayed concentrations ranging from 0.904 ng/mL in untreated cells, to 2.405 ng/mL in cells exposed to HT-OA. Therefore, the outcomes in the present work suggest a possible pro-oxidant effect of HT-OA as compared with unesterified HT and control samples, tentatively due to a significant rise of the 15-*epi*-15-D_{2t}-IsoP concentration (Fig. 1).

Thus, according to the sum of the individual IsoPs, it is stressed that the level of total IsoPs in THP-1 basal cells (1.353 ng/mL) significantly increased ($p < 0.05$) in both H_2O_2-stimulated and HT-treated cells by 1.5-fold, on average. Also, the treatment with HT-esterified with FAs resulted in a significant rise in the IsoP concentration in comparison with basal level, to reach concentration values of 2.956, 2.892, and 3.098 ng/mL in cells pre-treated with HT-ALA, HT-LA, and HT-OA, respectively ($p < 0.05$, data not shown). These results would indicate a pro-oxidant effect that contrasts with the recognized antioxidant activity of unbound HT. Nevertheless, it may not be forgotten that under certain conditions, HT (as well as other (poly)phenols and their derivatives) may behave as 'pro-oxidant' agents, in a highly dependent fashion on the experimental conditions. This controversial situation, the so-called '(poly)phenols oxidative paradox' indicates that the same molecule may offer protection against highly reactive free radicals or act as a potentially toxic (oxidizer) compound [33]. In this regard, and concerning *in vitro* models, depending on the concentration, HT can generate H_2O_2 as a result of its auto-oxidation by O_2, thus influencing its biological activities [34,35]. Similarly, high HT doses (10–200 μM), in *in vitro* models have been shown to increase ROS generation within tumor cells, leading to their apoptosis [36,37]. On the other hand, research studies which resorted to preclinical models have provided evidence suggesting that high HT doses (300 mg/kg/d) could also induce a systemic pro-oxidant effect when it is supplemented during exercise training [38]. Also, a very recent study by Romana-Souza et al. described that the short-term treatment of neonatal murine dermal fibroblast (72-h) with olive oil extracts contributes to increasing the production of ROS-induced oxidative damage in lipids (as evidenced by the increase in the 15-F_{2t}-IsoP level secondary to the increase in NF-kB and COX-2 expression), which positively correlated with the content of OA [39]. These findings, although more research is necessary, could contribute to elucidate the mechanism of action of bioactive compounds, such as HT and its lipophenols, and their dual biological activity.

3.2. Modulation of the quantitative prostaglandin profile of THP-1 monocytic cells by unbound and esterified hydroxytyrosol under oxidative conditions

Previously, OS had been demonstrated as a powerful activator of molecular pathways responsible for the up-regulated expression of OS-related genes, as well as as a modulator of the production of PGs through the Mitogen-Activated Protein Kinases (MAPKs) pathway [40]. According to this background, the current study aimed at elucidating the capacity of HT and HT lipophenols (HT-ALA, HT-LA, and HT-OA) to modulate the quantitative prostanoid profile of THP-1 monocytic cells exposed to oxidative (50 μM H_2O_2) growing conditions. With this objective in mind, aside from monitoring changes in the IsoP profile (OS markers) referred to in the previous section, we assessed THP-1 lysates and growth media on the concentration of 21 PGs synthesized from diverse FAs through specific synthetic pathways (Table 1), thus providing a complete picture of the PG response.

3.2.1. Modulatory capacity of the prostaglandin D-pathway of hydroxytyrosol and its lipophenolic derivatives

Regarding the PGs synthesized within the frame of the D-pathway from AA, four compounds were identified and quantified in THP-1 cells (PGD_2, PGDM, 11-β-$PGF_{2\alpha}$, and 2,3-dinor-11-β-$PGF_{2\alpha}$), out of the seven monitored (Table 1 and Fig. 2). The exposure to 50 μM H_2O_2, as an oxidizing agent, gave rise to an increase that was only statistically significant ($p < 0.05$) for PGDM and 2,3-dinor-11-β-$PGF_{2\alpha}$ (up to 0.020 and 0.081 ng/mL, respectively). Also, when assessing the effect of HT and HT-lipophenols on the modulation of the PGD_2 concentration induced by H_2O_2, it was observed that all the compounds (esterified and unesterified) significantly enhanced the level of this PG, providing values ranging from 1.213 to 1.880 ng/mL. As for PGDM (a metabolite of PGD_2), its concentration increased to a higher extent in THP-1 samples treated with HT lipophenols in comparison with cells exposed only to the H_2O_2 stimulus and untreated control samples (not detected). Hence, HT-LA induced the highest concentration of PGDM (0.036 ng/mL), although the broad dispersion of the results did not allow identifying statistically significant differences with the increase induced by HT-ALA (Fig. 2). This finding agrees with a previous study, where PGDM was detected in macrophages after oxLDL stimulation, while PGDM was almost absent in control samples [24].

The primary metabolite of PGD_2, 11β-$PGF_{2\alpha}$, showed a rising trend that was represented by a non-significant higher concentration in THP-1 cells exposed to pro-oxidant growing conditions (3.679 ng/mL, 1.4-fold higher than untreated control cells). Similarly, THP-1 cells treated with 20 μM HT and HT-lipophenols (HT-ALA, HT-LA, HT-OA) significantly enhanced the concentration of 11β-$PGF_{2\alpha}$ (6.185, 7.827, 7.019, and 7.025 ng/mL, respectively).

Finally, in THP-1 cells, the presence of a metabolite of 11β-$PGF_{2\alpha}$, the so-called 2,3-dinor-11-β-$PGF_{2\alpha}$, was observed. Again, the concentration of this PG climbed up to 0.081 ng/mL under H_2O_2-induced oxidant conditions, whereas this metabolite was not detected in untreated control cells. Concerning the impact of pre-treating THP-1 cells with HT and its lipophenolic derivatives, only HT-ALA induced a significant increase, thus giving rise to the final concentration of 0.356 ng/mL (Fig. 2).

PGD_2 is the main PG produced by activated mast cells, and its activity is triggered by resorting to two main receptors (DP1 and DP2 (also called CRTH2)). By interacting with these receptors PGD_2 activates multiple signalling pathways that would depend on the activation of a downstream signalling cascade, as a result of the up- or down-regulation of secondary messengers, such as cAMP and Ca^{2+}. Thus, this PG seems to be responsible for putative biological functions, including anti-tumorigenic activity [41,42], vasodilation or antithrombotic properties [43], and both pro- and anti-inflammatory capacities [44]. In this scenario, the present work provides evidence on the capacity of HT lipophenols to boost the PGs D-pathway more efficiently than unesterified HT, perhaps due to the up-regulation of COX-2, which can shed

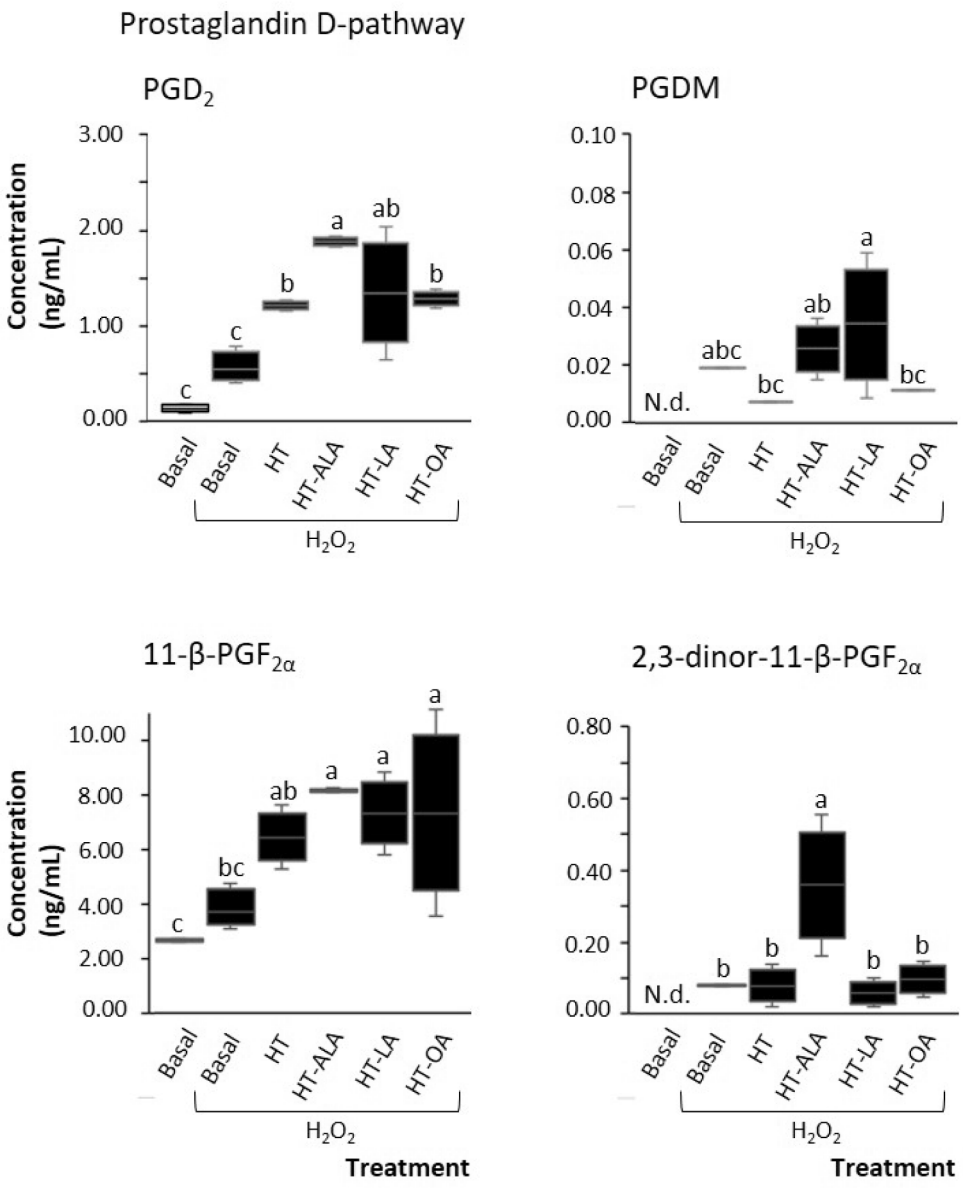

Fig. 2. Modulatory effect of the hydroxytyrosol (HT) and lipophenols of hydroxytyrosol (HT-ALA, HT-LA, and HT-OA) treatments on the prostaglandin D-pathway from arachidonic acid of H_2O_2-stimulated THP-1 monocytic cells. Prostaglandins were determined in THP-1 monocytic cells (cell lysate and growth media) pre-exposed to 20 μM of unesterified HT and lipophenolic derivatives of HT (α-linolenic acid (ALA), linoleic acid (LA), and oleic acid (OA)) and 50 μM H_2O_2. Values are shown as mean ± SD (n = 3). Different capital letters within each box and whisker plots indicate significantly different treatments at $p < 0.05$, according to one-way analysis of variance (ANOVA) and Tukey's multiple range test. N.d.: not detected.

some light on the diversity of biological activities that are specifically attributable to HT esterified with different FAs. Also, based on our outcomes referred to before, the type of FAs and the degree of unsaturation of lipophenols may be a critical feature of this class of bioactive compound, closely linked to their bioactivity, which merits further investigation. This is of special relevance, given that HT-OA provided a similar modulatory capacity of the PGs D-pathways as HT, although it was different as compared to HT-ALA and HT-LA. Accordingly, there is a need for additional experimental inputs for a sound comparison of the biological activities of the HT-FAs. This would allow selecting those plant-based foods (mainly, oleaginous matrices) with the most appropriate quantitative profile of HT lipophenols, in agreement with the diverse pathophysiological conditions related to OS and inflammation.

3.2.2. Modulatory capacity of the prostaglandin E-pathway of hydroxytyrosol and its lipophenolic derivatives

As mentioned above, ROS have been demonstrated to initiate a variety of molecular mechanisms in *in vitro* and *in vivo* eukaryotic cell models. Among them, ROS modulates the production of PGE_2 through the MAPKs pathway [40]. Moreover, PGE_2 is one of the most widely investigated PGs, which has been promoted as a biomarker of inflammation, informing about the state of a disease or therapeutic effectiveness. However, due to its rapid metabolism, the direct measurement of PGE_2 in biological samples is difficult, and is sometimes analyzed through its metabolites. In this regard, the major metabolite of PGE_2 is tetranor-PGEM. This serves as an indirect marker of PGE_2 biosynthesis, but again, it is also chemically unstable. When PGE_2 and tetranor-PGEM are not found, tetranor-PGAM, a dehydration product of tetranor-PGEM, can be measured as a surrogate PG to set the level of tetranor-PGEM in biological samples [45]. Therefore, a lipidomics workflow with the simultaneous analysis of multiple oxylipins is critical for understanding the activity of compounds (*e.g.*, lipophenols) on the generation of isoprostanoid and hence, on the curse of diverse pathophysiological processes [46]. Concerning the PG E-pathway from AA, in the current study, four PGs (PGE_2, 20-OH-PGE_2, tetranor PGEM, and tetranor-PGAM) were analyzed, but only two of them (PGE_2 and tetranor PGAM) were

detected in THP-1 cells (Fig. 3). The exposure to 50 μM H_2O_2 increased the concentration of PGE_2 in THP-1 cells 2.2-fold as compared to control (untreated) cells, whereas for tetranor-PGAM, it was not detected in neither under basal cells nor oxidative conditions.

The analysis of the effect of HT-ALA, HT-LA, and HT-OA on the concentration of PGE-pathway-based PGs provided evidence of the rising concentrations of both PGE_2 and tetranor-PGAM relative to controls, reaching 0.638, 0.520, and 0.676 ng/mL as the average concentrations of PGE_2, respectively. However, when THP-1 cells were treated with unesterified HT, the concentration of PGE_2 (0.393 ng/mL) was significantly lower than the one induced by HT-ALA and HT-OA (Fig. 3). On the contrary, regarding tetranor-PGAM, unesterified HT provided a significant increase of this metabolite (up to 1.871 ng/mL) as compared with HT lipophenols (1.166, 0.554, and 0.666 ng/mL for HT-ALA, HT-LA, and HT-OA, respectively). Thus, a joint analysis of the results retrieved for PGE_2 and tetranor-PGAM suggested that the decreased concentration of PGE_2 could be due to its metabolization to tetranor-PGAM. In this scenario, a cautious examination of these findings should be made to avoid misinterpretation, as PGE_2 is responsible for various biological activities, in many cases opposed, such as pro-/anti-inflammatory or pro-/anti-thrombotic, depending on their interaction with different E-prostanoid (EP) receptors (EP1, EP2, EP3, and/or EP4) [47].

PGE_2 is responsible for inflammatory symptoms but has also been associated with suppression of pro-inflammatory cytokines production (*e.g.*, IL-6). Moreover, specifically in monocytes, PGE_2 can interact with EP4, contributing to modulate TNF-α production and thus, controlling the progress of the inflammatory response [48]. In this sense, HT-OA has been described as a molecule with anti-inflammatory properties, which are developed in a concentration-dependent manner by suppressing PGE_2 production within 24-h, by inhibiting the COX-2 enzyme, as well as the expression of inducible NO synthase and the synthesis of interleukin-1β [49]. At this point, in the human oxylipin analyses, two main issues should be considered; firstly prostanoids have a very similar chemical structure and so, cross-reactivity may occur, especially with $PGF_{1\alpha}$ and $PGF_{2\alpha}$ when analyzing PGE_2 [45], and secondly, the measurement of a unique PG, particularly referring to PGs with dual biological activity, is not appropriate for the understanding of the inflammatory response, as it is composed of a very complex signaling cascade, including both pro-inflammatory and anti-inflammatory mediators. Consequently, a lipidomic approach plays an essential role in determining the biochemical mechanisms of lipid-related disorder processes through the identification of changes in the concentration of cellular lipid metabolites and their trafficking.

3.2.3. *Modulatory capacity of prostaglandin F-pathway of hydroxytyrosol and its lipophenolic derivatives*

Three out of the four PGs produced in the PG F-pathway were detected in the present work ($PGF_{2\alpha}$, 15-keto-15-$PGF_{2\alpha}$, and 20-OH-$PGF_{2\alpha}$) (Fig. 4). Concerning $PGF_{2\alpha}$, it was either detected in THP-1 cells under control conditions or after exposure to 50 μM H_2O_2, but no statistical differences were found between them. As shown in Fig. 4, the cells treated with HT, HT-LA and HT-OA induced a decrease in $PGF_{2\alpha}$ until undetectable amounts, and only HT-ALA produced an increase in this PG up to concentrations that were not statistically different when compared with untreated controls and H_2O_2-treated cells. There, the type of FA may be critical for the bioactivity of lipophenols. Specifically, $PGF_{2\alpha}$ may be synthesized from PGH_2 via the aldo–keto reductase (AKR) 1C3 enzyme, and may also be synthesized from PGE_2 via the AKR1C1 and AKR1C2 enzymes [50]. Thereby, the synthesis of this PG involves several substrates and different enzymes. The capacity to modulate the concentration of $PGF_{2\alpha}$ is of special relevance, it is a prostanoid with several biological activities, it is involved in vasoconstriction, and it contributes with the creation of the inflammatory environment by influencing the synthesis and secretion of IL1β, IL6, IL8, and TNFα [51]. Accordingly, our outcomes concerning $PGF_{2\alpha}$ strongly suggested the anti-inflammatory potential of both HT and HT lipophenols (mainly HT-ALA and HT-OA) as previously stated by [52,53], which highlighted HT-OA as an effective anti-inflammatory agent. Also, two products from $PGF_{2\alpha}$ were also detected; 15-keto- $PGF_{2\alpha}$, the first metabolite of $PGF_{2\alpha}$ in the 15-hydroxyprostagladin dehydrogenase (PGDH) pathway quantified in all samples, noting that, the exposure to H_2O_2 caused an increased concentration, with up to 3.8-fold higher values than untreated THP-1 cells (0.967 *vs.* 0.251 ng/mL) (Fig. 4). For this $PGF_{2\alpha}$ metabolite, both HT and its lipophenolic derivatives lowered the amount produced in THP-1 cells relative to that achieved when exposing cells only to the oxidative environment, although in any case, the decrease observed was statistically significant. Similarly, as related to 20-OH-$PGF_{2\alpha}$, a product of $PGF_{2\alpha}$ synthesized by cytochrome P450-catalyzed ω-oxidation, it was not detected in neither untreated controls nor H_2O_2-treated cells. HT-ALA gave rise to a smaller increase of this metabolite than HT, HT-LA, and HT-OA. It should also be underlined that the molecules responsible for the decrease in $PGF_{2\alpha}$ (HT, HT-LA, and HT-OA), were the most efficient in increasing the concentration of

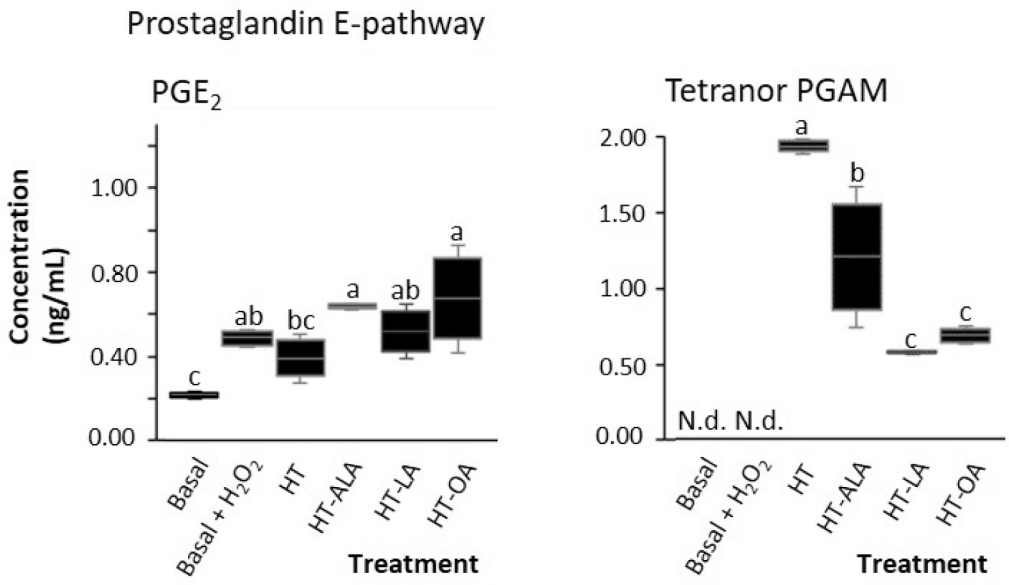

Fig. 3. Modulatory effect of hydroxytyrosol (HT) and HT lipophenols (HT-ALA, HT-LA, and HT-OA) treatments on the prostaglandin E-pathway from arachidonic acid of H_2O_2-stimulated THP-1 monocytic cells. Prostaglandins were determined in THP-1 monocytic cells (cell lysate and growth media) pre-exposed to 20 μM of unesterified HT and lipophenolic derivatives of HT (α-linolenic acid (ALA), linoleic acid (LA), and oleic acid (OA)) and 50 μM H_2O_2. Values are shown as mean ± SD (n = 3). Different capital letters within each box and whisker plots indicate significantly different treatments at $p < 0.05$, according to a one-way analysis of variance (ANOVA) and Tukey's multiple range test. N.d.: not detected.

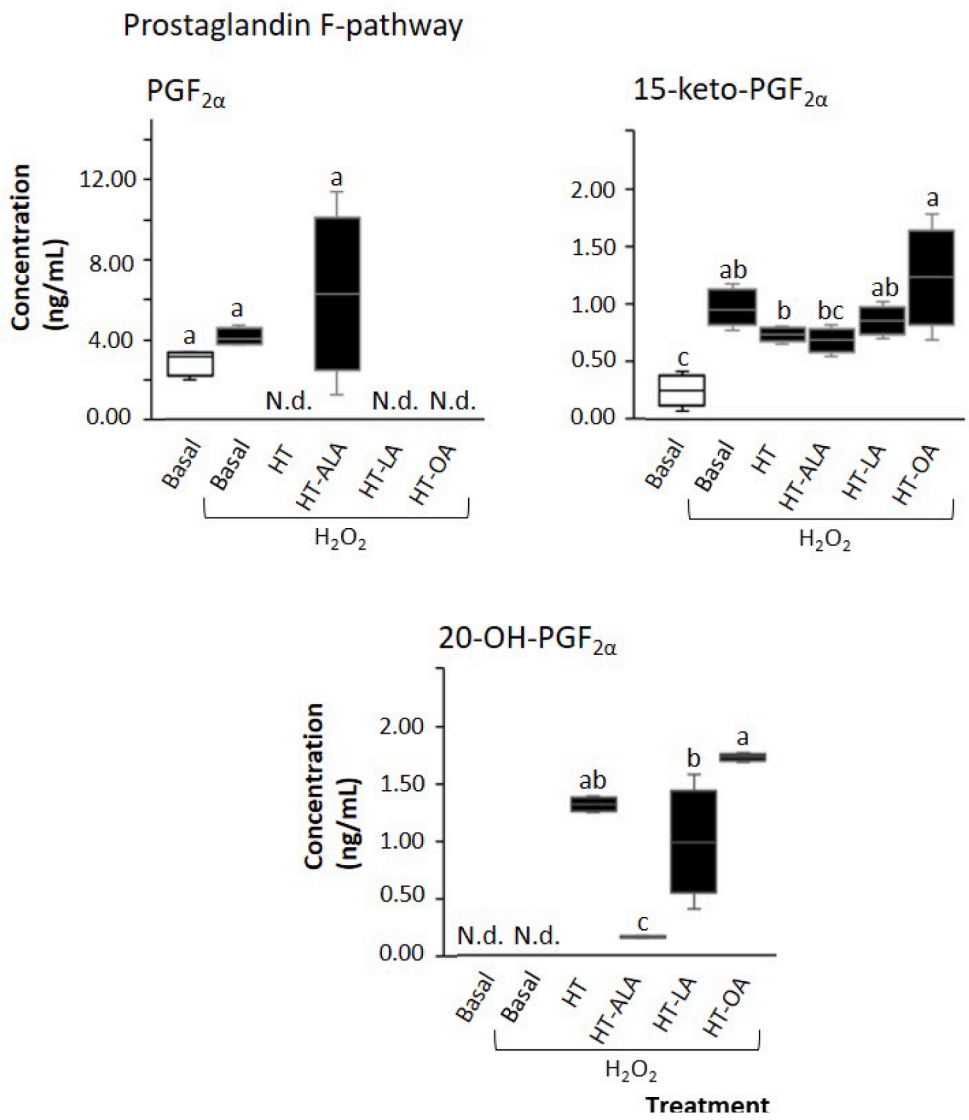

Fig. 4. Modulatory effect of hydroxytyrosol (HT) and HT lipophenols (HT-ALA, HT-LA, and HT-OA) treatments on the prostaglandin F-pathway from arachidonic acid of H_2O_2-stimulated THP-1 monocytic cells. Prostaglandins were determined in THP-1 monocytic cells (cell lysate and growth media) pre-exposed to 20 μM of unesterified HT and lipophenolic derivatives of HT (α-linolenic acid (ALA), linoleic acid (LA), and oleic acid (OA)) and 50 μM H_2O_2. Values are shown as mean ± SD (n = 3). Different capital letters within each box and whisker plots indicate significantly different treatments at $p < 0.05$, according to one-way analysis of variance (ANOVA) and Tukey's multiple range test. N.d.: not detected.

$PGF_{2\alpha}$ metabolites (15-keto- $PGF_{2\alpha}$ and 20-OH-$PGF_{2\alpha}$). Therefore, our outcomes reveal the importance of the concentration of the oxidizing agent, the degree of unsaturation of the FAs, and the esterification of the native molecules for the modulation action on the amount of PGs belonging to F-pathway.

3.2.4. Modulation of the level of prostaglandins from dihomo-gamma-linolenic acid by hydroxytyrosol and its lipophenolic derivatives

The C_{20} polyunsaturated FA, dihomo-gamma-linolenic acid (DGLA; 20:3 ω-6), is also a substrate for eicosanoid production and yields 1-series PGs (*e.g.*: PGD_1, PGE_1, and $PGF_{1\alpha}$), which are generally viewed as possessing anti-inflammatory and cytoprotective properties, and have been shown to modulate vascular reactivity [54]. However, DGLA may be indirectly converted to 2-series PGs through AA. As shown in Fig. 5, one of the two 1-series PGs from DGLA assessed in the current assay (PGE_1 and $PGF_{1\alpha}$) were quantified in all the samples ($PGF_{1\alpha}$). The treatment with the oxidizing agent caused an increase in the $PGF_{1\alpha}$ concentration, which was not statistically different from untreated samples (Fig. 5). For this $PGF_{1\alpha}$, only unesterified HT (1.012 ng/mL) exhibited the capacity to significantly attenuate the increase triggered by H_2O_2 (1.839 ng/mL), as compared with HT-OA (1.949 ng/mL). As mentioned above, it should be noticed that depending on the level of ROS, different redox-sensitive transcription factors may be activated and thus, cell-specific biological responses could be observed. In this context, both stimuli with H_2O_2 and HT-OA increased the levels of $PGF_{1\alpha}$, a PG with anti-inflammatory properties [54,55], a rise that may be due to a cellular defense mechanism. Nevertheless, to the present date, scarce information about the effects of the 1-series PGs on inflammation has been reported, and there is still an open discussion on their capacity to enhance the status of human health, in this case, regarding the inflammation processes.

4. Conclusions

Based on the main outcomes found for both HT and HT-FAs present in EVOO and VOO, they seem to actively participate in the redox balance of THP-1 cells, acting as pro-oxidants *in vitro*. This could be explained by the high concentration, cell type, and cell culture media, which are highly dependent on the experimental conditions. On the contrary, our outcomes strongly suggested the anti-inflammatory potential of both HT and HT-FAs. Although this activity, in the case of esterified molecules, appeared to depend on the type of FA, other factors such as the concentration of the oxidizing agent, could modulate the quantitative isoprostanoid profile. According to our results, unesterified and esterified HT molecules are defined by a different capacity to modulate the synthesis of isoprostanoids, depending on the pathway and FA involved.

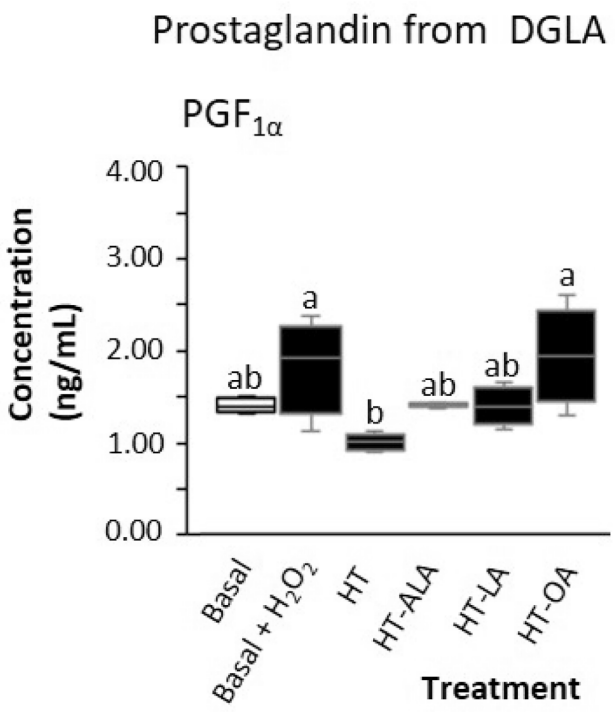

Fig. 5. Modulatory effect of hydroxytyrosol (HT) and HT lipophenols (HT-ALA, HT-LA, and HT-OA) treatments on the prostaglandin from dihomo-gamma-linolenic acid of H_2O_2-stimulated THP-1 monocytic cells. Prostaglandins were determined in THP-1 monocytic cells (cell lysate and growth media) pre-exposed to 20 μM of unesterified HT and lipophenolic derivatives of HT (α-linolenic acid (ALA), linoleic acid (LA), and oleic acid (OA)) and 50 μM H_2O_2. Values are shown as mean ± SD (n = 3). Different capital letters within each box and whisker plots indicate significantly different treatments at $p < 0.05$, according to one-way analysis of variance (ANOVA) and Tukey's multiple range test.

Accordingly, there is a need for additional experimental inputs for a sound comparison of the biological activities of esterified HT molecules, which could allow selecting those plant-based foods (mainly, oleaginous matrices, natural sources of HT) that have an adequate quantitative profile for HT-FAs, according to the diverse pathophysiological conditions associated with OS and inflammation. Also, further mechanistic studies are needed to shed light on the effect of gastrointestinal digestion to elucidate the bioaccessible and bioavailable fractions of HT-FAs, as well as the involvement of the diverse cytokines and chemokines as co-modulators of the inflammatory response that is composed of an elaborate cascade of both pro-inflammatory and anti-inflammatory mediators. Therefore, a lipidomics workflow with the simultaneous analysis of multiple oxylipins is of paramount importance for understanding the activity of lipophenols on isoprostanoid generation and hence, on pathophysiological processes.

CRediT authorship contribution statement

Carolina Alemán: Investigation, Formal analysis, Writing – original draft. **Raúl Domínguez-Perles:** Conceptualization, Investigation, Validation, Writing - review & editing. **Federico Fanti:** Formal analysis, Writing – original draft. **Juana I. Gallego-Gómez:** Investigation, Formal analysis, Resources. **Agustín Simonelli-Muñoz:** Investigation, Resources. **Espérance Moine:** Validation, Visualization. **Thierry Durand:** Writing - review & editing, Visualization, Supervision. **Céline Crauste:** Validation, Visualization. **Ángel Gil-Izquierdo:** Investigation, Writing - review & editing, Supervision, Funding acquisition. **Sonia Medina:** Conceptualization, Methodology, Investigation, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2021.106703.

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