Unravelling the capacity of hydroxytyrosol and its lipophenolic derivates to modulate the H2O2-induced isoprostanois profile of THP-1 monocytes by UHPLC-QqQ-MS/MS lipidomic workflow

Carolina Alemán-Jiménez, Raúl Domínguez-Perles, Federico Fanti, Juana Gallego-Gómez, Agustín Simonelli-Muñoz, Espérance Moine, Thierry Durand, Céline Crauste, Ángel Gil-Izquierdo, Sonia Medina

To cite this version:
Carolina Alemán-Jiménez, Raúl Domínguez-Perles, Federico Fanti, Juana Gallego-Gómez, Agustín Simonelli-Muñoz, et al.. Unravelling the capacity of hydroxytyrosol and its lipophenolic derivates to modulate the H2O2-induced isoprostanois profile of THP-1 monocytes by UHPLC-QqQ-MS/MS lipidomic workflow. Microchemical Journal, Elsevier, 2021, 170, pp.106703. 10.1016/j.microc.2021.106703. hal-03374014

HAL Id: hal-03374014
https://hal.archives-ouvertes.fr/hal-03374014
Submitted on 11 Oct 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Distributed under a Creative Commons Attribution 4.0 International License
Unravelling the capacity of hydroxytyrosol and its lipophenolic derivates to modulate the H$_2$O$_2$-induced isoprostanooid profile of THP-1 monocytes by UHPLC-QqQ-MS/MS lipidomic workflow

Carolina Alemán-Jiménez$^a,1$, Raúl Domínguez-Perles$^b,1$, Federico Fantib$^b,1$, Juana I. Gallego-Gómez$^a$, Agustín Simonelli-Muñoz$^d$, Espérance Moine$^e$, Thierry Durand$^e$, Céline Crauste$^1$, Ángel Gil-Izquierdo$^b,1$, 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

**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 isoprostanooid profile induced by H$_2$O$_2$ in THP-1 monocytes. 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 antioxidants *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 isoprostanooid generation and hence, on pathophysiological processes.

**Keywords:**
Hydroxytyrosol
Lipophenols
Isoprostanooids
Oxidative stress
Inflammation

**ARTICLE INFO**

**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-linoleic 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, oleic acid; OS, oxidative stress; PMS, phenazine methosulfate; PLA$_2$, phospholipase A2; PG, prostaglandin; PUFAs, polyunsaturated fatty acids; ROS, reactive oxygen species; SPE, solid-phase extraction; THP-1, human monocyte cell line; UHPLC, ultra-high pressure liquid chromatography; XTT, sodium 30-[1-(phenylaminocarbonyl)-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.

https://doi.org/10.1016/j.microc.2021.106703
Received 28 July 2021; Accepted 28 July 2021
Available online 6 August 2021

0026-265X/© 2021 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).
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$_2$O$_2$) [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$_2$O$_2$ 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" polysaturated 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 isoprostanoan 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$_2$O$_2$ 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.
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 (H₂O₂).

| Prostanoids generated from AA | Pathway I | Pathway D | Pathway E | Pathway F | Thromboxane |
|-----------------------------|-----------|-----------|-----------|-----------|-------------|
| 6-keto-PGF₁α                | PGE₂ (*)  | PGE₂ (*)  | PGE₂α (*) | 11-dehydro- | TXB₂        |
| 2,3-dinor-6-                 | PGD* (*)  | 20-OH-PGE₂| 20-OH-     | PGF₂α (*)  |             |
| keto-PGF₁α                  |           |           |           |           |             |
| Tetranor-PGD                | Tetranor-PGD| Tetranor-PGD| Tetranor-PGD| Tetranor-PGD|            |
| PGDM                        | PGEM      | PGF₂α (*) |           |           |             |
| Tetranor-PGD                |           |           |           |           |             |
| PGDM lactone                | PGAM (*)  |           |           |           |             |
| 11-β-PGF₂α (*)              |           |           | 15-keto-   |           |             |
| 2,3-dinor-11β-              |           |           |           |           |             |
| PGF₂α (*)                   |           |           |           |           |             |
| Tetranor-PGJ-cut             |           |           |           |           |             |
| PGs generated from DGLA     | PGE₁      |           |           |           |             |
| PGF₁α (*),                  |           |           |           |           |             |
| PGs generated from EPA      | 17-trans-PGF₂α|           |           |           |             |

Oxylipins after exposure to 50 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 (H₂O₂).

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 (H₂O₂).

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

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

The quantitative isoprostanooid 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 H₂O₂ with HT and HT-lipophenols treatments in contrast to untreated cells. The cells and supernatants were collected after 24 h exposure to H₂O₂ 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 Heliz 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-F₂α-isoP-d₄ 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 isoprostanooids 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 isoprostanooids response triggered by the exposure of THP-1 cells to oxidative environments (H₂O₂). 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 associated 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 isoprostanooid profile induced by the exposure to oxidizing agents. The effect of oxidizing H₂O₂ 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 isoprostanoic 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$_{2\alpha}$-IsoP, 5-epi-5-F$_{2\alpha}$-IsoP, and 15-epi-15-D$_{2\alpha}$-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$_2$O$_2$ 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$_{2\alpha}$-IsoP or 15-epi-15-D$_{2\alpha}$-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$_2$O$_2$ 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$_2$O$_2$ 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 disproportionately 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$_2$O$_2$, 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$_{2\alpha}$-IsoP, a metabolite of 15-F$_{2\alpha}$-IsoP (8-iso-PGF$_{2\alpha}$), it was not detected in the untreated controls, whilst after the exposure to 50 µM H$_2$O$_2$, 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 oxidative growing conditions for most of them may occur because of the concentration of H$_2$O$_2$ 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$_2$O$_2$ 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 disproportionately 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$_2$O$_2$, 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$_{2\alpha}$-IsoP, a metabolite of 15-F$_{2\alpha}$-IsoP (8-iso-PGF$_{2\alpha}$), it was not detected in the untreated controls, whilst after the exposure to 50 µM H$_2$O$_2$, 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$_2$O$_2$-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$_2$O$_2$. 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-F2t-IsoP, the different concentrations induced by the array of treatments applied were not statistically significant relative to that recorded for basal and H2O2-treated cells, with HT-ALA being the only compound which induced a significant increase (p < 0.05) of 5-epi-5-F2t-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 F2t-IsoPs are referred to as their concentration. Thus, the level of 5-F2t-IsoP has been reported to reach concentrations up to 4 times higher than 15-F2t-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 F2t-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 information about the pathophysiological meaning of OS and its relatives 

3.098 ng/mL in cells pre-treated with HT-ALA, HT-LA, and HT-OA, 1.5-fold, on average. Also, the treatment with HT-esterified with FAs (Fig. 2). This finding agrees with a previous study, where PGDM was statistically significant differences with the increase induced by HT-ALA 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 PGD2, 11β-PGF2α, 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-PGF2α (6.185, 7.827, 7.019, and 7.025 ng/mL, respectively).

Finally, in THP-1 cells, the presence of a metabolite of 11β-PGF2α, the so-called 2,3-dinor-11β-PGF2α, was observed. Again, the concentration of this PG climbed up to 0.081 ng/mL under H2O2-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).

PGD2 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 PGD2 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 Ca2+. 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

3.2. Modulation of the quantitative prostaglandin profile of THP-1 monocyctic 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 prostaglandin profile of THP-1 monocyctic cells exposed to oxidative (50 µM H2O2) 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 lyases 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 (PGD2, PGDM, 11-β-PGF2α, and 2,3-dinor-11-β-PGF2α), out of the seven monitored (Table 1 and Fig. 2). The exposure to 50 µM H2O2, 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-PGF2α (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 PGD2 concentration induced by H2O2, 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 PGD2), its concentration increased to a higher extent in THP-1 samples treated with HT lipophenols in comparison with cells exposed only to the H2O2 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].

Concerning the level of total IsoPs in THP-1 basal cells (1.353 ng/mL) significantly increased (p < 0.05) in both H2O2-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 controversy 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 H2O2 as a result of its auto-oxidation by O2, thus influencing its biological activities [34,35]. Similarly, high HT doses (10–200 µM), in vitro model systems 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/day) 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 fibroblasts (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-F2t-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.
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 tetrnanor-PGEM are not found, tetrnanor-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 iso prostanoid 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*, tetrnanor PGEM, and tetrnanor-PGAM) were analyzed, but only two of them (PGE*₂* and tetrnanor PGAM) were
detected in THP-1 cells (Fig. 3). The exposure to 50 µM H₂O₂ increased the concentration of PGE₂ 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 PGE₂ and tetranor-PGAM relative to controls, reaching 0.638, 0.520, and 0.676 ng/mL as the average concentrations of PGE₂, respectively. However, when THP-1 cells were treated with unesterified HT, the concentration of PGE₂ (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 PGE₂ and tetranor-PGAM suggested that the decreased concentration of PGE₂ 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 PGE₂, PGAM. In this scenario, a cautious examination of these findings should be made to avoid misinterpretation, as PGE₂ 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].

PGE₂ 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, PGE₂ 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 the expression of inducible NO synthase and the synthesis of intracellular NO (iNOS) in the 15-hydroxyprostaglandin dehydrogenase (PGDH) pathway quantified in all samples, noting that the exposure to H₂O₂ 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 PGF₂α 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-PGF₂α (a product of PGF₂α), a product of PGF₂α, it was not detected in untreated controls nor H₂O₂-treated cells. HT-OA were the most efficient in increasing the concentration of 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 (PGF₂α, 15-κeto-15-PGF₂α, and 20-OH-PGF₂α) (Fig. 4). Concerning PGF₂α, it was either detected in THP-1 cells under control conditions or after exposure to 50 µM H₂O₂, 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 PGF₂α until undetectable amounts, and only HT-OA produced an increase in this PG up to concentrations that were not statistically different when compared with untreated controls and H₂O₂-treated cells. There, the type of FA may be critical for the bioactivity of lipophenols. Specifically, PGF₂α may be synthesized from PGH₂ via the aldo-keto reductase (AKR) 1C3 enzyme, and may also be synthesized from PGE2 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 PGF₂α is of special relevance, as 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 PGF₂α 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 PGF₂α were also detected; 15-κeto- PGF₂α, the first metabolite of PGF₂α in the 15-hydroxyprostaglandin dehydrogenase (PGDH) pathway quantified in all samples, notting that the exposure to H₂O₂ 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 PGF₂α 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-PGF₂α, a product of PGF₂α, it was not detected in untreated controls nor H₂O₂-treated cells. HT-OA 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 PGF₂α (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 F-pathway from arachidonic acid of H₂O₂-stimulated THP-1 monocytes. Prostaglandins were determined in THP-1 monocytes (cell lystate 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 H₂O₂. 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.
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 $\omega$-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$_{2}$O$_{2}$ (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$_{2}$O$_{2}$ 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 H2O2-stimulated THP-1 monocytes. Prostaglandins were determined in THP-1 monocytes cells (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 H2O2. 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 modulators of the inflammatory response that is composed of an elaborate cascade of both pro-inflammatory and anti-inflammatory mediators. Furthermore, a lipidomics workflow with the simultaneous analysis of multiple oxylipins is of paramount importance for understanding the activity of lipophenols on isoprostanooid generation and hence, on pathophysiological processes.

CRediT authorship contribution statement

Carolina Alemán: Investigation, Formal analysis, Writing – original draft. Raúl Domínguez-Perlas: Conceptualization, Investigation, Validation, Writing - review & editing. Federico Fanti: Formal analysis, Writing – original draft. Juana I. Gallego-Gómez: Investigation, Formal analysis, Resources. Agustín Simonelli-Munoz: Investigation, Resources. Esperance Moine: Validation, Visualization. Thierry Durand: Writing - review & editing, Visualization, Supervision. Céline Crauste: Validation, Visualization. Angel 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.

Acknowledgements

This work was partially funded by the “Fundación Séneca de la Región de Murcia” Grupo de Excelencia 19900/GERM/15 and the Spanish project AGL2017-83386-R-AEI/FEDER, UE from the Spanish Ministry of Science, Innovation, and Universities. SM was supported by a Postdoctoral Contract (Saavedra-Fajardo 21078/SF/19) from the Agency for Science and Technology of the Region of Murcia – “Fundación Séneca” (Spain).

Appendix A. Supplementary data

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

References

[1] C. Hu, M. Wang, X. Han, Shotgun lipidomics in substantiating lipid peroxidation in redox biology: methods and applications, Redox Biol. 12 (2017) 946–955, https://doi.org/10.1016/j.redox.2017.04.030.
[2] X. Han, Lipid alterations in the earliest clinically recognizable stage of Alzheimers disease: implication of the role of lipids in the pathogenesis of Alzheimers disease, Curr. Alzheimer Res. 2 (2005) 65–77, https://doi.org/10.2174/156720505772786.
[3] S. Zalba, T.L.M. ten Hagen, Cell membrane modulation as adjuvant in cancer therapy, Cancer Treat. Rev. 52 (2017) 48–57, https://doi.org/10.1016/j.ctrv.2016.10.008.
[4] B. Sottero, G. Leonardiuzzi, G. Testa, G. Gargiulo, G. Poli, F. Biasi, Lipid oxidation derived aldehydes and oxysterols between health and disease, Eur. J. Lipid Sci. Technol. 121 (1) (2019) 1700047, https://doi.org/10.1002/ejst.2012.11010023110.ejst.201700047.
[5] W.L. Smith, R.C. Murphy, Oxidized lipids formed non-enzymatically by reactive oxygen species, J. Biol. Chem. 283 (23) (2008) 15513–15514, https://doi.org/10.1074/jbc.O800062200.
[6] Y.Y. Tyurina, C.M. St, A. Kisin, O. Vlasova, D.V. Krysko, A.A. Krysko, H.H. Kapralov, V.A. Dar, A.A. Tyurin, E.N. Amsnecato, S.B. Popova, P.S. Bolievich, J.A. Timashov, S.E. Kellum, P.K. Wenzel, J.S. Mallampalli, H. Greenberger, A.A. Bayar, V.E. Shvedova, Redox (phospho)lipidomics of signaling in inflammation and programmed cell death, J. Leukoc. Biol. 106 (2019) 57–81, https://doi.org/10.1002/jlb.2019.1001002.
[7] V.R. Gutierrez, R. De La Puerta, A. Catalá, The effect of tyrosol, hydroxytyrosol and oleuropein on the non-enzyme lipid peroxidation of rat liver microsomes, Mol. Cell. Biochem. 217 (2001) 35–41, https://doi.org/10.1023/A:1007219913090.
[8] M. Deiana, A. Incani, A. Rosa, G. Corona, A. Atzeri, D. Loru, M. Paola Melis, M. Asunta Dossi, Protective effect of hydroxytyrosol and its metabolite homovanillic alcohol H2O2 induced lipid peroxidation in renal tubular epithelial cells, Food Chem. Toxicol. 46 (2008) 2984–2990, https://doi.org/10.1016/j.fct.2008.05.037.
[9] Z. Zhang, J. Cao, L. Zhong, Hydroxytyrosol inhibits pro-inflammatory cytokines, iNOS, and COX-2 expression in human monocyte cells, Naunyn. Schmiedebergs. Arch. Pharmacol. 379 (6) (2009) 581–586, https://doi.org/10.1002/jn.200109.0399.
[10] Z. Peng, B. Zhang, J. Yao, D. Duan, J. Fang, Dual protection of hydroxytyrosol, an olive oil polyphenol, against oxidative damage in PC12 cells, Food Funct. 6 (6) (2015) 2091–2100, https://doi.org/10.1039/C5FO00097A.
[11] E. Moine, P. Barbet, L. Guillou, T. Durand, J. Vercauteren, C. Crauste, New lipophenol antioxidants reduce oxidative damage in retina pigment epithelial cells, Antioxidants 7 (2018) 197, https://doi.org/10.3390/antiox7120197.
[12] E. Moine, M. Boukhallal, D. Gia, N. Jacquemet, L. Guillou, T. Durand, J. Vercauteren, P. Barbet, C. Crauste, New lipophenol antioxidants prevent carbonyl and oxidative stresses involved in macular degeneration, Free Radic. Biol. Med. 162 (2021) 367–382, https://doi.org/10.1016/j.freeradbiomed.2020.10.316.
[13] Y. Zhong, F. Shahidi, Lipophilized Epigallocatechin Gallate (EGGC) derivatives as novel antioxidants, J. Agric. Food Chem. 59 (12) (2011) 6526–6533, https://doi.org/10.1021/jf2010505.
[14] Y. Zhong, Y.-S. Chiu, M.-H. Pan, F. Shahidi, Anti-inflammatory activity of lipophilic epigallocatechin gallate (EGGC) derivatives in LPS-stimulated murine macrophages, Food Chem. 134 (2) (2012) 742–748, https://doi.org/10.1016/j.foodchem.2012.02.172.
