Hydroxytyrosol Is the Major Anti-Inflammatory Compound in Aqueous Olive Extracts and Impairs Cytokine and Chemokine Production in Macrophages

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Key words
- polyphenols
- hydroxytyrosol
- olive
- chemokine
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- inflammation

Abstract
Substances in olive products contribute to improved health as suggested by epidemiological data. In this study we assessed the effects of hydroxytyrosol (HT) on inflammatory mediators, cytokines and chemokines, and identified anti-inflammatory constituents of aqueous olive extracts, i.e., olive vegetation water (OVW). Murine macrophages (RAW264.7 cells) were stimulated with lipopolysaccharide (LPS) in the absence or presence of substances; inflammatory mediators [nitric oxide (NO), prostaglandin E2 (PGE2), cytokines, interleukins, chemokines] were determined by the Griess reaction, ELISA, or multiplex ELISA (Luminex technology). Expression of inflammatory genes was determined by RT-PCR. Aqueous olive extracts were fractionated by preparative HPLC and the fractions investigated for their effects on NO and PGE2 production. Results were further analyzed by principal component analysis. HT inhibited production of NO and PGE2 with an IC50 of 11.4 and 19.5 µM, respectively, reflecting strong anti-inflammatory activity. HT and OVW diminished secretion of cytokines (IL-1α, IL-1β, IL-6, IL-12, TNF-α), and chemokines (CXCL10/IP-10, CCL2/MCP-1). HT and OVW concentration-dependently reduced the expression of genes of inducible nitric oxide synthase (iNOS), IL-1α, CXCL10/IP-10, MIP-1β, matrix metalloproteinase-9, and prostaglandin E2 synthase (PGES). The effects of HT were partly mediated via the NF-kB pathway, as shown by RT-PCR analysis. HT was identified as the main bioactive compound of OVW. The data provide a molecular basis for elucidating the effects of HT on inflammatory processes. The effects of HT on NO and chemokine production point to their impact on chronic inflammatory processes in endothelium or arthritis.

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

Introduction
Various epidemiological studies suggest that the Mediterranean diet is associated with an improved health status and in particular with a lower incidence of coronary heart diseases. Olive products are an integral part of this diet and might confer benefits to health due to their content in monounsaturated fatty acids and in phenolic compounds. These are predominantly contained in the aqueous phase of olives and exert diverse biological activities in endothelial and epithelial cells, platelets, neurons, cells of the immune system, and neoplastic cells (for reviews see, e.g., [1–4]). In contrast, dietary olive oil modulates the lipid membrane composition and the production of inflammatory mediators including prostaglandin E2 (PGE2) and nitric oxide (NO) or scavenger receptors in peritoneal macrophages [5–7]. The most abundant olive polyphenols are 2-hydroxytyrosol (HT), oleuropein, and tyrosol [8]. These substances are potent antioxidants and radical scavengers [9–11]. They reduce LDL oxidation [12, 13] and influence cellular events related to atheroma formation [14]. Recent studies revealed that aqueous olive substances reduced cytokine production in vivo and consequently prevented chronic inflammatory processes in arthritic diseases [15].HT is a product derived from oleuropein by progressive hydrolysis and undergoes metabolic transformation after absorption in the gastrointestinal tract [16–18]. In this study we analyzed the anti-inflammatory properties of HT and identified its pleiotropic effects on the expression and production of inflammatory mediators including...
eicosanoids, interleukins, cytokines, and chemokines. Furthermore, the compounds contained in freeze-dried olive vegetation water (OVW) were fractionated, and the anti-inflammatory activity of the fractions was determined. The data indicate that HT accounts for the biological activity of aqueous olive extracts.

Materials and Methods

Reagents and olive polyphenolics
HT (purity > 99%) was obtained from Cayman Chemicals or produced by DSM Nutritional Products Ltd. (DNP). Freeze-dried OVW was from CreAgri, Inc. or from DNP and prepared as described previously [15]. The composition of OVW is comparable but not identical to olive mill waste water [19]. A batch of OVW containing 2.5% HT (i.e., 42% of all phenolic compounds) was used in these experiments. Total phenols have been determined by a modified Folin-Ciocalteu method [International Standard ISO 14502-1, Reference number ISO 14502-1 :2005(E)].

DMEM, MEM nonessential amino acids (NEAA), and phosphate buffered saline (PBS) were from Invitrogen; fetal bovine serum (FBS); lipopolysaccharide (LPS, E. coli serotype 055:B5) and N(−)-nitro-L-arginine methyl ester (L-NAME, purity > 98%) were from Sigma. NS-398 (purity > 98%) was from Cayman Chemicals. Primers and probes for quantitative RT-PCR were designed using the Primer Express™ software (Version 1.0; Applied Biosystems) and were synthesized by Sigma.

Fractionation of OVW and analytical methods
OVW (100 mg) was extracted with methanol and methyl tert-butyl ether (MTBE)/methanol (9:1) to prepare a hydrophilic and a lipophilic extract, respectively. Extracts were separated on a 250 × 16 mm column at the following conditions: Sorbens, Select B 10 µm (Merck); eluent A: water/5 mM ammonium formate, 0.1% formic acid; eluent B: acetonitrile/methanol (1:1), 5 mM ammonium formate, 0.1% formic acid; flow rate: 15 mL/min; gradient: 20–50% eluent B for hydrophilic extract (retention time: 5–15 min of profiling run), 50–88% of eluent B for lipophilic extract (retention time: 16–30 min of profiling run). Detection was at 254 nm. Thirty-two fractions were obtained for each type of OVW extract. Fractions were collected in 96-well plates, lyophilized, and the respective material was dissolved in DMSO. Aliquots of the fractions were diluted in cell culture medium (final dilution: 200×) and used in cellular assays (see below).

In order to determine the HT contents in OVW and the different fractions by HPLC, HT standard solution (5–1000 µg/mL) was prepared in DMSO. For each standard and sample, 25 µL of the DMSO solution was diluted with 150 µL MeOH/water 1:1 (v/v), and 5 µL thereof was analyzed on a reversed-phase HPLC system (Agilent 1200 Series). Chromatography was performed on a Beta-Basic 150 × 3 mm, 3 µm column (Thermo Electron), using a binary flow gradient at 0.8 mL/min and 25°C: 0–1 min isocratic 5% B; 1–12.5 min linear gradient to 11.5% B; 12.5–13.5 min linear gradient to 20% B; 13.5–40.0 min linear gradient to 45% B; 40.0–46.0 min linear gradient to 100% B; 46.0–50.0 min isocratic 100% B; 50.0–51.0 min linear gradient to 5% B, 51.0–55.0 min isocratic 5% B. Solvent A was water containing 0.1% formic acid; solvent B was MeOH containing 0.1% formic acid. Detection was at 280 nm. HT was eluted at approximately 5.7 min.

Cell cultures
RAW264.7 cells were from ATCC and cultured in DMEM supplemented with 10% FBS, 50 units/mL penicillin, 50 µg/mL streptomycin, and 0.1 mM NEAA. Cells were seeded into 12-well or 96-well plates at 1 and 0.05 × 10⁶ cells per well, respectively, and used after 2 days of preculture. Cells were starved in DMEM containing 0.25% FBS 18 hours before the treatment and were then stimulated with LPS (1 µg/mL) for 4–24 hours in phenol red-free DMEM containing 0.25% FBS. Substances were usually tested in a concentration range from 50 to 0.2 µM or µg/mL in twofold dilution steps. All treatments of an experimental series were done in duplicate. Each experiment was repeated at least three times. Supernatants were harvested and tested immediately afterwards (i.e., for LDH) or stored at −80°C until use.

RNA preparation and quantitative RT-PCR
RNA was extracted from cells cultured in 12-well plates and reverse transcribed by procedures detailed previously [20]. For Taqman analysis, cDNA (corresponding to 30 to 50 ng of total RNA input) was amplified in a ABI PRISM® 7700 sequence detection system (Applied Biosystems), using the Taqman® universal PCR Master Mix (Applied Biosystems), 50 nM primers, and 100 nM probe (VIC-TAMRA labeled) for the 18S rRNA internal control, and 300 nM primers and 100 nM probe (FAM-TAMRA labeled) for the gene of interest. The final volume per PCR reaction was 50 µL. The cycle number at which the fluorescence exceeded the threshold of detection (Ct) for ribosomal RNA was subtracted from that of the target genes for each well (ΔCt). Messenger RNA levels were then indicated as 2−ΔΔCt where ΔΔCt returns to the ΔCt of unstimulated minus treated cells. Alternatively, the percentage change, relative to the stimulated cells, was defined as (2ΔΔCt ×100) where ΔΔCt equaled the ΔCt of treated cells minus ΔCt of the (stimulated + compound)-treated cells.

Multiparametric analysis of cytokines, chemokines, and interleukins
Multiparametric kits were obtained from BIO-RAD Laboratories and used in the LiquiChip Workstation IS 200 (Qiagen) according to the manufacturers’ instructions. We used the Bio-Plex Mouse Cytokine 23-Plex Panel (BIO-RAD): the data were acquired with the Lumexin IS 2.3 software and evaluated with the LiquiChip Analyser software provided by Qiagen.

Determination of nitric oxide and PGE2
Concentrations of nitrite which is generated from cell-released nitric oxide (NO) were determined by the Griess reaction [21]. PGE2 was determined by ELISA using the Prostaglandin E2 EIA kit purchased from Cayman Chemicals and used according to the manufacturer’s instructions. All measurements were done in duplicates and at various dilutions of the culture supernatants.

Statistical analysis
Gene expression data mining was performed with the Genedata Expressionist program (Version Pro 4.5.8). The relative standard deviation of expression signals was less than 6% for all genes measured. In principal component analysis (PCA), the default program settings were applied. For both t-test and ANOVA computation, the respective ratio of medians was applied. Statistical significance was derived by filtering according to p-value (p < 0.05), as well as – in the case of the t-test – also due to fold-change. Filtering cut-off values were selected, as specified per respective analysis.
Supporting information

Data regarding the influence of substances on cellular LDH contents, on concentration-dependent inhibition of nitric oxide and PGE2 in RAW264.7 cells, and on effects of substances at the level of protein secretion and gene expression are available as Supporting Information.

Results

Olive vegetation water (OVW) constituents were extracted with methanol and analyzed by HPLC. The polar compounds eluted as one major and several minor peaks (Fig. 1). HT formed the main peak and represented ~42% of the total phenol content (compound 1 in Fig. 1). The other peaks contained tyrosol and related phenols [22], whose identities were not determined except for tyrosol (compound 2 in Fig. 1). OVW extracts were fractioned as described in Materials and Methods. The peak corresponding to HT in the hydrophilic extract had a retention time of ~5.5 minutes.

Macrophages respond to inflammatory stimuli by producing inflammatory mediators including nitric oxide (NO) and PGE2 and are crucial to measure anti-inflammatory effects of compounds in a bioassay. Murine macrophage RAW264.7 cells were stimulated with lipopolysaccharide (LPS) that induced secretion of NO and PGE2; the inhibitory effect of HT and OVW was determined in this bioassay. None of the substances tested in these experiments significantly affected cell viability at any concentrations as determined by the LDH release assay (Table 1 in Supporting Information). L-NAME and NS-398 were used as pharmacological controls: they specifically inhibited NO and PGE2, respectively (Table 1). Furthermore, we determined the inhibitory activity of OVW. It reduced NO production more efficiently than that of PGE2 (IC50 of 85 mg/L and 176 mg/L, respectively). When the observed IC50 of OVW was converted into HT “equivalents” by taking into account the percentage of HT in total OVW, they were similar to those obtained for pure HT. This suggests that the HT component in OVW accounted for the anti-inflammatory properties of OVW.

Next, we analyzed the effect of olive phenolics on the secretion of chemokines and cytokines that are released by activated RAW264.7 cells. LPS induced the secretion of various proinflammatory cytokines and chemokines (CCL2/MCP-1, CCL4/MIP-1β, CCL5/RANTES, CXCL10/IP-10, TNF-α, IFN-γ, IL-1α, IL-1β, IL-6, IL-12p40, IL-12p70) (Table 2; Fig. 3 in Supporting Information). HT (at 25 µM) inhibited the secretion of CCL2/MCP-1, CXCL1/KC, TNF-α, IL-1α, IL-1β, IL-6, IL-12p40, IL-12p70 (Table 2; Fig. 3 in Supporting Information). HT (at 25 µM) inhibited the secretion of CCL2/MCP-1, CCL4/MIP-1β, CCL5/RANTES, and CCL11/eotaxin were not affected. We further compared the anti-inflammatory activity of HT (at 25 µM) with that of OVW (at 250 mg/L, which returns to ~40 µM of HT). Whereas CCL5/RANTES levels were slightly increased, all other chemokines and cytokines as well as GM-CSF were significantly reduced by HT and to a comparable extent by OVW (Fig. 2). Taken together, these data show that HT and OVW inhibited distinct types of cytokines and chemokines.

The MBTE-soluble material of OVW was separated into fractions (see Materials and Methods) and their effect on NO or PGE2 determined. Two distinct “hot spots” that inhibited NO production were identified (Fig. 3). Fractions 4 and 5 (position A) were found to contain HT (at 18 and 19 µM HT, respectively); i.e., at a

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**Table 1** IC50 values of HT, OVW, NS-398, and L-NAME for the inflammatory mediators nitric oxide (measured as nitrite in culture supernatants) and PGE2.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Units</th>
<th>Nitrite Mean ± SEM</th>
<th>PGE2 Mean ± SEM</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT µM</td>
<td>11.4 ± 1.9</td>
<td>5</td>
<td>19.5 ± 2.6</td>
<td>5</td>
</tr>
<tr>
<td>OVW Total extract mg/L</td>
<td>85.0 ± 12.0</td>
<td>5</td>
<td>175.8 ± 24.6</td>
<td>5</td>
</tr>
<tr>
<td>HT “equivalents” µM</td>
<td>13.8 ± 1.9</td>
<td>5</td>
<td>28.5 ± 4.0</td>
<td>5</td>
</tr>
<tr>
<td>NS-398 µM</td>
<td>&gt; 50</td>
<td>3</td>
<td>0.02 ± 0.00</td>
<td>3</td>
</tr>
<tr>
<td>L-NAME µM</td>
<td>141.6 ± 22.5</td>
<td>3</td>
<td>&gt; 200</td>
<td>3</td>
</tr>
</tbody>
</table>

* SEM, standard error of the mean; * N, number of experimental series
concentration which exceeds the IC₅₀ determined for pure HT (see Table 1); this is consistent with the observed complete inhibition of NO by these fractions. The nature of the compound at position B is unknown. Whereas the fractions 4 and 5 abrogated LPS-induced NO production, they did not significantly reduce the PGE₂ production. Instead, the inhibitory profile for PGE₂ was less pronounced and the evidence that the NF-κB pathway is influenced by HT.

### Table 2 Effect of HT on the secretion of proteins by macrophages.

<table>
<thead>
<tr>
<th>Category</th>
<th>Protein</th>
<th>Effect of LPS a  (n = 3)</th>
<th>% Inhibition by HT (25 µM) b (n = 3)</th>
<th>% Inhibition by OVW (250 µg/mL) c (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemokine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCL2/MCP-1</td>
<td>40 ± 23</td>
<td>48 ± 6 **</td>
<td>54 ± 20 **</td>
<td></td>
</tr>
<tr>
<td>CCL4/MIP-1β</td>
<td>23 ± 13</td>
<td>-5 ± 6</td>
<td>-3 ± 10</td>
<td></td>
</tr>
<tr>
<td>CCL5/RANTES</td>
<td>118 ± 88</td>
<td>-10 ± 3</td>
<td>9 ± 14</td>
<td></td>
</tr>
<tr>
<td>CCL11/Eotaxis</td>
<td>31 ± 2</td>
<td>3 ± 6</td>
<td>5 ± 11</td>
<td></td>
</tr>
<tr>
<td>CXCL1/KC</td>
<td>97 ± 1</td>
<td>27 ± 21 **</td>
<td>29 ± 21 *</td>
<td></td>
</tr>
<tr>
<td>Cytokine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>18 ± 15</td>
<td>22 ± 3</td>
<td>14 ± 5</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>4520 ± 916</td>
<td>25 ± 5 **</td>
<td>14 ± 7 **</td>
<td></td>
</tr>
<tr>
<td>IL-1α</td>
<td>2324 ± 440</td>
<td>61 ± 8 **</td>
<td>48 ± 4 **</td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>18 ± 24</td>
<td>31 ± 5 **</td>
<td>29 ± 6 **</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>226329 ± 34204</td>
<td>66 ± 8 **</td>
<td>72 ± 16 **</td>
<td></td>
</tr>
<tr>
<td>IL-12p40</td>
<td>11 ± 3</td>
<td>27 ± 6 **</td>
<td>32 ± 10 **</td>
<td></td>
</tr>
<tr>
<td>IL-12p70</td>
<td>267 ± 153</td>
<td>46 ± 8 *</td>
<td>38 ± 3</td>
<td></td>
</tr>
<tr>
<td>Cell differentiation factor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GM-CSF</td>
<td>6091 ± 739</td>
<td>38 ± 7 *</td>
<td>34 ± 11 *</td>
<td></td>
</tr>
</tbody>
</table>

* Numbers indicate the ratio of proteins secreted by LPS-stimulated cells versus unstimulated cells; % inhibition ± SEM; p < 0.1*, p < 0.01**, p < 0.001 ***

Fig. 2 HT and OVW modulate the production of secreted macrophage proteins. The values obtained from supernatants of LPS-stimulated cells are set at 100% (dotted line). Asterisks indicate significant differences (p < 0.05) compared to LPS-only stimulated cells.

Fig. 3 HT and/or OVW exerted any effects on inflammatory mediators by influencing their gene expression level. To this aim, mRNA levels in macrophages were determined by quantitative RT-PCR (Table 3). Previous experiments had revealed that LPS induced strong alterations in responsive genes between 1 to 8 hrs of macrophage stimulation, with significant responses for all genes found at 4 hours. All substances were tested at 10–50 µM of HT (or its “equivalent” concentration contained in OVW), i.e., at a range that encompassed the IC₅₀ values established for NO or PGE₂ production. The substances did not influence basal mRNA levels in unstimulated cells. LPS treatment significantly increased the expression of COX-2, iNOS, TNF-α, IL-1α, CCL4/MIP-1β, CCL5/RANTES, and CXCL10/IP-10 (Table 3). HT (at 25 µM) had distinct effects on the various groups of genes: expression of COX-2 was increased, whereas prostaglandin E synthase (PGES) mRNA was downregulated. Inducible nitric oxide synthase (iNOS) mRNA was reduced by HT. Similarly, proinflammatory IL-1α mRNA levels were diminished in HT-treated cells, whereas the anti-inflammatory IL-10 was increased. With regard to chemokines, HT downregulated CXCL10/IP-10 expression, but did not alter CCL5/RANTES and CCL4/MIP-1β. HT significantly diminished mRNA levels of matrix metalloproteinase (MMP)-9, which is mainly produced in macrophages, whereas the expression of CD14, a membrane receptor that binds LPS was slightly reduced in activated RAW264.7 cells. OVW (at 250 µg/mL) had virtually identical effects as HT (Table 2). Fig. 4 shows the comparison of the effects of HT and OVW on mRNA levels of inflammatory genes. For iNOS, COX-2, CCL5/RANTES, MMP-9, and 1-kBα, both substances had similar concentration-dependent effects on mRNA levels. Compared to OVW, HT more strongly modulated CXCL10/IP-10, CCL4/MIP-1β, and IL-1α. With regard to CCL5/RANTES and IL-1α, the effects observed at the gene expression level matched those observed at the protein level (Fig. 2).

From the analysis of expression levels of transcription factors (TF) of the NF-κB signalling pathway, we can deduce one possible mode of action of HT: LPS-stimulation of macrophages led to a marked increase of NF-κB1, NF-κB49, and 1-kBα (Table 3). Concomitant treatment of cells with HT had significant effects on two of these TF and also affected NF-κBp65 levels. This is strong evidence that the NF-κB pathway is influenced by HT.
Using principal component analysis, which was based on the expression analysis of 16 genes, we further compared the effect of HT with that of OVW. A good reproducibility was observed among biological samples, as well as a separation of samples according to substance (HT or OVW) and substance concentration. The effect of treatment concentration was mostly reflected by principal component 1, while samples according to substance treatment were distinguished mostly by principal component 2 (Fig. 5a). In agreement with the hypothesized biological effects, samples of higher treatment concentrations were found to be further distant to the control group (LPS). The effect of treatment concentration was larger than the effect of the substance. Principal component 1 was found to correlate significantly with the effective HT concentration ($R^2 = 0.9498$, Fig. 5b). Hence, the HT concentration was the main determinant that accounted for the observed differences in gene expression. In order to separate the effects of different treatment substances (HT and OVW) from those of treatment concentration, n-way ANOVA was applied (with $p < 0.001$). Collectively, this corroborated the observation made at the level of inflammatory mediators that OVW and HT had similar effects on the expression of inflammatory mediators in macrophages.

**Discussion**

We provide evidence for pleiotropic effects of HT and OVW, which contains HT in its natural matrix, on the inflammatory response induced by LPS in macrophages. While published data underscored the atheroprotective effect of HT [14, 23, 24], the impact of HT on inflammatory processes has been analyzed only in a few studies. Here, we have shown that HT or OVW exerted robust effects on several parameters of the in vitro inflammatory response in murine macrophages. Inflammatory processes are directly or indirectly modified by the compounds: both early events (i.e., induction of inflammatory genes) and late responses (e.g., production of PGE2, NO, or chemokines) were changed. Importantly, we show that both HT and OVW impaired expression levels of genes of the inflammatory cascade. In agreement with our findings, HT also impaired NO and PGE2 production in J774 macrophages although significant effects were detected only at relative high concentrations (> 100 µM) [25]. Presumably, RAW264.7 macrophages are more sensitive than J774 cells to the effects of phenolics. In good agreement with the data of the

![Fig. 3](image)

**Table 3** Effect of HT on gene expression in macrophages.

<table>
<thead>
<tr>
<th>Category</th>
<th>Gene</th>
<th>Effect of LPS$^a$</th>
<th>% Reduction of gene expression by HT (25 µM)$^b$</th>
<th>% Reduction of gene expression by OVW (250 mg/L)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostaglandin synthesis</td>
<td>COX-2</td>
<td>331 ± 23</td>
<td>13 ± 7</td>
<td>1 ± 20</td>
</tr>
<tr>
<td></td>
<td>PGE2</td>
<td>2.2 ± 0.1</td>
<td>13 ± 5*</td>
<td>16 ± 3*</td>
</tr>
<tr>
<td>Cytokine</td>
<td>TNF-α</td>
<td>37 ± 1</td>
<td>− 3 ± 3</td>
<td>− 5 ± 3</td>
</tr>
<tr>
<td>Interleukin</td>
<td>IL-1α</td>
<td>11 740 ± 20</td>
<td>68 ± 1***</td>
<td>68 ± 6***</td>
</tr>
<tr>
<td>Inflammatory mediator</td>
<td>iNOS</td>
<td>53 ± 2</td>
<td>32 ± 4***</td>
<td>56 ± 6***</td>
</tr>
<tr>
<td>CC chemokine</td>
<td>CCL4/MIP-1β</td>
<td>72 ± 1</td>
<td>24 ± 8***</td>
<td>21 ± 5***</td>
</tr>
<tr>
<td></td>
<td>CCL5/RANTES</td>
<td>353 ± 12</td>
<td>− 13 ± 20</td>
<td>30 ± 25*</td>
</tr>
<tr>
<td>CXC chemokine</td>
<td>CXCL10/IP-10</td>
<td>221 ± 5</td>
<td>49 ± 5***</td>
<td>20 ± 7***</td>
</tr>
<tr>
<td>Macrophage marker</td>
<td>CD14</td>
<td>2.2 ± 0.1</td>
<td>− 28 ± 6*</td>
<td>− 27 ± 15*</td>
</tr>
<tr>
<td>Matrix metalloproteinase</td>
<td>MMP-9</td>
<td>2.8 ± 0.3</td>
<td>45 ± 6**</td>
<td>35 ± 5**</td>
</tr>
<tr>
<td>Transcription factor</td>
<td>IκBα</td>
<td>4.9 ± 0.0</td>
<td>30 ± 2**</td>
<td>29 ± 2**</td>
</tr>
<tr>
<td></td>
<td>NF-κB1</td>
<td>8.6 ± 0.2</td>
<td>29 ± 0*</td>
<td>31 ± 3**</td>
</tr>
<tr>
<td></td>
<td>NF-κB49</td>
<td>5.0 ± 0.3</td>
<td>24 ± 9**</td>
<td>28 ± 10**</td>
</tr>
<tr>
<td></td>
<td>NF-κBp65</td>
<td>1.8 ± 0.1</td>
<td>0 ± 1</td>
<td>10 ± 6*</td>
</tr>
</tbody>
</table>

$^a$ Numbers indicate the ratio of gene expression levels in LPS-stimulated cells versus unstimulated cells (± SEM); N = 3; $^b$ p < 0.1*, p < 0.01**, p < 0.001*** (compared to gene expression in LPS-stimulated cells)
current study, HT was shown to inhibit proinflammatory cytokines and iNOS in human monocytic cells in an NF-κB-dependent way [26]. Conversely, oleuropein, which is another aqueous olive phenolic substance, increased NO production (at > 10 µM) in mouse peritoneal macrophages [27]. Plausibly, the structural differences confer distinct biological properties to HT and oleuropein.

In this study, we have identified one “hot-spot” in OVW by bioassay guided fractionation, and HT was found to be eluted at that position. Moreover, bioefficacy of the two treatments (HT and OVW) correlated well with the effective HT concentration. Taken together, anti-inflammatory properties of OVW are attributed to its intrinsic content of HT. Other olive phenolics like tyrosol, oleuropein, or verbascoside might be contained in OVW in minor quantities. Yet, they have only a marginal effect in inflammatory processes, since their IC50 values were significantly higher than that observed for HT (our unpublished results).

In macrophages, LPS also activates cytokines/interleukins and chemokines. HT and OVW significantly reduced the expression of CXCL10/IP-10 but did not affect CCL5/RANTES or even augmented CCL4/MIP-1β protein levels. We hypothesize therefore that those olive phenolics might impact pathophysiological conditions like allergic reactions, where CXCL10/IP-10 controls migration of activated T1H cells. The distinct effect of HT and OVW on CXCL10/IP-10 and CCL5/RANTES is presumably mediated by the regulatory elements of the two genes [28, 29]. Remarkably, CCL2/MCP-1 and CXCL10/IP-10, two chemokines involved in monocyte recruitment to nascent atheroma [30] are drastically suppressed by HT. The substance also reduced MMP-9 expression (Table 1), which in turn is related to thrombus stability [31].

We infer that the attenuating effect of HT on these key elements in atheroma formation contributes to the atheroprotective properties of olive compounds [4, 14, 24].

HT most likely exerts its biological activity via signaling pathways, and in particular the NF-κB pathway and the associated transcription factors (TF). As shown in Table 3, HT significantly altered the expression of NF-κBp65 and I-κBα TF genes. This observation is consistent with the findings reported by Maiuri et al., who showed that the activation of NF-κB but also STAT-1α and interferon regulatory factor-1 (IRF-1) was blocked by HT [25]. These transcription factors are central regulators of the expression of inflammatory genes including those analyzed in this study. HT and OVW most likely act at the transcriptional level; yet, in the case of TNF-α or COX-2, they did not modulate gene expression but impaired the secretion of the protein or related metabolite (TNF-α or PGE2; see Fig. 3S in Supporting Information). This strongly suggests regulation at the posttranscriptional level.

This and other in vitro studies show that relatively high concentrations of olive phenolics are required to show effects on cellular metabolism. The relevance of these results needs to be compared with plasma levels of olive compounds that are achievable by food consumption. A dietary intake of 40 mL of olive oil results in a peak concentration of ~ 17 µM of HT in plasma 1 hour postprandial [32]. This value is well within the IC50 of HT for inhibiting the NO and PGE2 production observed in vitro in this study; at these concentrations, gene expression is also significantly modulated. Therefore, HT is supposed to exert similar effects in vivo at least transiently. Exposure to these or lower levels of HT by regular dietary intake might repeatedly modulate physiological pa-
rameters that provide a systemic health benefit by olive phenolics. In addition, dietary phenols have been shown to influence other physiological parameters like the production of F2-isoprostanes, endothelial function, or inhibition of platelet aggregation [12, 33]. Collectively, this study provides new insights into the molecular and biochemical mode of action that HT and its natural polyphenols. In addition, dietary phenols have been shown to influence other physiological parameters like the production of F2-isoprostanes, endothelial function, or inhibition of platelet aggregation [12, 33]. Collectively, this study provides new insights into the molecular and biochemical mode of action that HT and its natural “counterpart”, OWV, exert in inflammatory processes.

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Conflict of Interest

The authors are employees of DSM Nutritional Products and have no conflict of interest.

References