Protective effect of hydroxytyrosol and its predominant plasmatic human metabolites against endothelial dysfunction in human aortic endothelial cells

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2 **metabolites against endothelial dysfunction in human aortic endothelial cells**

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43   **Abbreviations**

44   **ATCC:** American type culture collection

45   **CM:** complete medium

46   **DMEM:** Dulbecco's modified eagle's medium

47   **ESI:** electrospray ionization

48   **HAEC:** human aortic endothelial cells

49   **HBSS:** Hanks' balanced salt solution

50   **HT**: hydroxytyrosol

51   **HVAlc:** homovanillic alcohol

52   **HVac:** homovanillic acid

53   **ICAM-1:** intercellular cell adhesion molecule-1

54   **MCP-1:** monocyte chemotactic protein-1

55   **OO:** olive oil

56   **μSPE:** micro-elution solid-phase extraction

57   **SRM:** selected reaction monitoring

58   **sulfHT:** hydroxytyrosol sulfate

59   **sulfHVAlc:** homovanillic alcohol sulfate

60   **sulfHVac**: homovanillic acid sulfate

61   **TEER:** transepithelial electrical resistance

62   **TNF-α:** tumor necrosis factor-alpha

63   **VCAM-1:** vascular cell adhesion molecule-1

64   **VOO:** virgin olive oil

65

66   **Keywords**: atherosclerosis / Caco-2 / human aortic endothelial cells / hydroxytyrosol /

67   hydroxytyrosol metabolites

68

69   **Abstract**

70   **Scope:** Hydroxytyrosol (HT) is the major phenolic compound in virgin olive oil (VOO)

71   that may exert health benefits against atherosclerosis. The native form of HT is

72   undetectable in plasma due to an extensive first pass phase II metabolism. Therefore, it

73   is necessary to find strategies to obtain HT metabolites and to demonstrate their

74   protective role against the endothelial dysfunction.

75   **Methods and results:** Biosynthesis of the main plasmatic HT metabolites was

76   performed through Caco-2 cells. The bioactivity of HT and the mixture of metabolites

77   was tested at physiological concentrations (1, 2, 5 and 10 μM) in human aortic

78   endothelial cells (HAEC) co-incubated with TNF-α (10 ng/mL) for 18 and 24 h. After

79   the incubations, cells and media were analyzed to test possible deconjugation of

80   metabolites or conjugation of HT. Both HT and HT metabolites significantly reduced

81   the secretion of E-selectin, P-selectin, ICAM-1 and VCAM-1, but only HT metabolites

82   further reduced MCP-1 at 24 h. HT underwent a conjugation process after incubation

83   leading to its main metabolites in a dose-dependent manner.

84   **Conclusion:** Physiological HT metabolites, synthetized for the first time by using an

85   intestinal cell model, might be the responsible in part for the protection against

86   endothelial dysfunction.

87

1. Introduction

Virgin olive oil (VOO) a key component of the Mediterranean Diet (MD) has shown to

contribute to cardiovascular disease (CVD) prevention. Part of this effect has been

associated to VOO phenolic content [1,2]. Results from the PREDIMED study, the first

randomized clinical trial designed to assess the beneficial effects of the MD on the

primary prevention of CVD, reported that the MD supplemented with VOO has a dual

effect on the prevention of CVD, reducing classical cardiovascular risk factors and also

having an intense anti-inflammatory effect through the down-regulation of endothelial

dysfunction markers related to atherosclerosis, such as vascular cell adhesion molecule-

1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), E- and P-Selectin [3].

Hydroxytyrosol (HT) is the major phenolic compound in VOO in both free and

conjugated form, mainly as oleuropein aglycone structures commonly named

secoiridoids. HT has shown showing a wide range of biological functions, such as

antioxidant, anticancer, anti-inflammatory, and neuroprotective activities, as well as

having beneficial effects on the cardiovascular system [4,5]. The potential health

benefits of HT have stimulated intense research on the bioavailability and metabolism

of VOO phenolic compounds, a requisite to support their potential benefits for human

health. Previous studies have shown that VOO phenolic compounds undergo rapid

hydrolysis under gastric and intestinal alkaline conditions resulting in significant

increases in the amount of free HT entering the small intestine [6,7]. Thus, HT becomes

the major phenolic compound absorbed from the intestinal tract, which is further

subjected to an extensive first pass metabolism, both in the intestinal epithelium and the

liver, leading to the formation of phase II metabolites, mainly sulfate, methyl and

methyl-sulfate conjugates and glucuronides of HT [8–11].

Consequently, after the consumption of normal doses of VOO (less than 50 g / day) the

113   native form of HT is not detected in plasma and the potential health benefits of VOO

114   phenolic compounds could be either attributed to the biological activity of phase II

115   metabolites or to free HT generated at cellular level by enzymatic deconjugation. So, it

116   becomes essential to obtain the plasmatic HT metabolites, ~~and~~ test them in cell-based

117   experimental models at physiological concentrations and examine a possible

118   deconjugation process of these metabolites at the cellular level.

119   The physiological significance of most of the published articles on polyphenols

120   bioactivity has been questioned as they still describe the effects of molecules that are

121   only present in *planta* or in food. Nevertheless, detailed studies applying plasmatic and

122   microbial metabolites of phenolic compounds are starting to establish reasonable

123   mechanisms through which these physiological metabolites may exert beneficial effects

124   [12]. In the case of VOO phenolics, a previous study tested the *in vitro* radical

125   scavenging capacity of some of the HT metabolites observing that homovanillic alcohol

126   (HVAlc) and the 3-*O*-glucuronide conjugate appeared to be active in terms of

127   antioxidant activity [13]. However, further observations suggested that none of the

128   glucuronide conjugates of HT displayed antioxidant activity to protect LDL against

129   oxidation [14].

130   Regarding the antiatherogenic effects, only HT in its native form has been tested

131   on preclinical experimental models describing its protective effects against

132   atherosclerosis [15,16] and no data has been published in relation to its biological

133   metabolites. As reported by Valls *et al*., these metabolites could be involved in reducing

134   the endothelial dysfunction in humans [17]. However, the mechanisms have not been

135   demonstrated.

136   Endothelial dysfunction is involved in the early stages of atherosclerosis and is a

137   key process in the development of CVD [18]. Briefly, the endothelial dysfunction in the

138   early stages of atherosclerosis includes rolling (mediated by E- and P-selectin) and firm

139   adhesion (mediated by VCAM-1 and ICAM-1) of blood leukocytes to the activated

140   endothelial monolayer and directed migration (mediated by the monocyte chemotactic

141   protein-1; MCP-1) of the bound leukocytes into the intima [18,19]. So the endothelial

142   dysfunction biomarkers E-selectin, P-selectin, VCAM-1, ICAM-1 and MCP-1 were

143   selected in the present study as they have been considered as useful predictive

144   biomarkers of atherosclerosis [20]. The surface and soluble expression of these

145   molecules is greatly increased in atherosclerotic lesions as a result of stimulation by

146   pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α). This cytokine

147   was used in the present study as a stressor molecule for assessing the underlying

148   mechanisms as it induces the release and surface expression of cell adhesion molecules

149   [21,22].

150   So, the main aim of the present study was to optimize the use of Caco-2 cell line

151   as an alternative approach to obtain biological metabolites of HT previously detected in

152   human plasma, and to evaluate their *in vitro* endothelial protective effect compared to

153   HT alone at physiological concentrations [10].

154   **2. Material and methods**

155   *2.1. Solvents and reagents*

156   HT (99.51% of purity, synthetic) was provided by Seprox Biotech (Madrid, Spain).

157   Catechol, as the internal standard, (IS) was purchased from Sigma-Aldrich (St. Louis,

158   MO, USA). Hydroxytyrosol-3-*O*-sulfate was custom-synthesized by Toronto Research

159   Chemicals Inc. (Toronto, ON, Canada). Methanol (HPLC-grade), acetonitrile (HPLC-

160   grade), ethanol and dimethyl sulfoxide (DMSO) were purchased from Scharlab

161   (Barcelona, Spain) and orthophosphoric acid (85%) from Panreac (Barcelona, Spain).

162 Milli-Q water was obtained from a Milli-Q water purification system (Millipore Corp.,

163 Medford, MA, USA).

164 *2.2. Caco-2 cell culture treatments to obtain HT metabolites*

165 Caco-2 cell line was purchased from ATCC (American Type Culture Collection, US).

166 Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) high glucose

167 (HyClone, Thermo scientific, Logan, Utah) supplemented with 10% of fetal bovine

168 serum (Sigma-Aldrich, Madrid, Spain), 1% L-glutamine (HyClone, Thermo scientific,

169 Logan, Utah), 1% (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES)

170 (HyClone, Thermo scientific, Logan, Utah), penicillin and streptomycin (100mg/mL)

171 (Gibco, Spain). Cells were cultured at 37°C, in a 5% CO_2 humidified incubator. Media

172 culture was replaced every 2 days and phosphate buffered saline (PBS; HyClone,

173 Thermo Scientific, Logan, Utah) was used to wash the cells. In preliminary

174 experiments, the stability of HT was evaluated over time (6, 12, 24 and 48 h) with

175 DMEM or Hanks' Balanced Salt Solution (HBSS; HyClone, Thermo scientific, Logan,

176 Utah) media and finally, HBSS was chosen to perform all the experiments due to a

177 better stability of HT over the time in these media.

178   In order to select the optimal cell treatment to obtain HT metabolites, T75 flask

179 and transwell inserts (both from Corning Costar, Netherlands) were tested as two

180 different culture conditions (**Figure 1A**). On the one hand, Caco-2 cells were seeded in

181 T75 flasks at a density of 6.6 x 10^4 cells/mL and their HT conjugation rate was

182 evaluated at two differentiation states: three days after seeding (A treatment) or after 21

183 days of culture (B treatment). On the other hand, Caco-2 cells were seeded in the apical

184 side of transwell inserts at a density of 4.6 x 10^4 cells/mL and cultured for 21 days (C

185 treatment). D treatment consisted of 18 days of Caco-2 culture (4.6 x 10^4 cells/mL) in

186 the apical side of transwell inserts and, after 18 days, an additional co-culture of Caco-2

8

187 cells was seeded in the basolateral side (5×10^4 cells/ mL) for three more days, making

188 a total of 21 days. Monolayer formation in transwell inserts was evaluated by

189 transepithelial electrical resistance (TEER) measurement with a voltohmmeter

190 (Millipore Corp., MA) selecting cells with TEER > 250 Ω/ cm^2. For all treatments (A,

191 B, C and D), the initial concentration of HT in the culture medium was 100 μM (HT

192 stock solution 100 mM in methanol) and three exposure times were tested (6, 12 and 24

193 h). To preempt cytotoxicity, methanol concentrations never exceeded 0.1% (v/v) in

194 culture media. Finally, supernatants of T75 flasks (A and B treatments) and the sum of

195 the apical and basolateral supernatants from transwell inserts (C and D treatments) were

196 collected. Prior to chromatographic analysis (see section 2.4), HT and its metabolites

197 were extracted from the cell media using micro-elution solid-phase extraction (μSPE)

198 plates with OASIS HLB μElution Plates 30 μm (Waters Corp., Milford, MA, USA)

199 according to the previous study [23].

200 　　　　After the selection of 21-day cultured cells in T75 flasks with 24 h of HT

201 incubation (B treatment) as the optimal treatment (see section 3.2), different HT

202 concentrations (10, 15, 20, 25, 50 and 100 μM) in the culture medium were assayed in

203 order to test the effect of the HT concentration on the transformation yield of HT into its

204 metabolites.

205 *2.3. Purification of HT metabolites from Caco-2 culture medium*

206 Once the B treatment (21-day cultured cells in T75 flasks) incubated with 100 μM HT

207 for 24 h was selected as the optimal treatment for obtaining HT metabolites, the μSPE

208 plates were replaced by SPE cartridges with a higher sorbent size (OASIS HLB Elution

209 Plates 6 g, 35 cc, Waters, Milford, MA, USA) to obtain major amounts of HT

210 metabolites. The conditioning of the SPE cartridges was done by adding sequentially 35

211 mL of methanol and 40 mL of milli-Q water acidified at pH 2 with acetic acid (**Figure**

212   **1B**). Extractions were done by loading 50 mL of Caco-2 supernatant, which had been

213   previously mixed with 50 mL of phosphoric acid at 4%. The loaded cartridges were

214   washed with 30 mL of milli-Q water and 20 mL of methanol 5%. Finally, the retained

215   phenolic compounds were eluted using 60 mL of methanol (100%). This elution solvent

216   was evaporated to dryness under a nitrogen stream in an evaporating unit at 30 ºC

217   (PIERCE Model 18780, IL, USA). In order to obtain a more concentrated solution, after

218   evaporation, eluates from several cartridges were collected and consecutively

219   evaporated in the same tube. The residue was reconstituted with 1mL of methanol. The

220   extract was filtered through a 0.22 μm nylon syringe filter (Teknokroma, Barcelona,

221   Spain) and transferred to the autosampler vial before the quantification of HT

222   metabolites by liquid chromatography. This stock solution of HT metabolites was used

223   to study their effectivity on endothelial dysfunction model in HAEC.

224   *2.4. Chromatographic analysis of HT metabolites*

225   HT metabolites were analyzed by AcQuity UPLC^{TM} coupled to PDA detector and a

226   triple quadrupole detector (TQD)^{TM} mass spectrometer (Waters, Milford, MA, USA).

227   The analytical column was a High Strength Silica (HSS) T3 column (100 x 2.1 mm, 1.8

228   μm). During the analysis, the column was kept at 30 ºC and the flow rate was 0.3

229   mL/min using 0.2% acetic acid as solvent A and methanol as solvent B. The elution

230   started at 3% of eluent B and was linearly increased to 15% of eluent B in 6 min. Then,

231   it was linearly increased to 70 % in 8 min and further increased to 100 % in 3 min.

232   Then, it was returned to the initial conditions in 1 min, and the re-equilibration time was

233   2 min. The injection volume was 2.5 μL.

234   A triple quadrupole detector (TQD)^{TM} mass spectrometer was used as the

235   detector system. Ionization was done by electrospray (ESI) in the negative mode and the

236   data were collected in the selected reaction monitoring (SRM) mode. The ionization

237 source parameters were capillary voltage of 3 KV, source temperature of 150 ºC, and

238 desolvation temperature of 400 ºC with a flow rate of 800 L/h. Nitrogen (99% purity,

239 N2LCMS nitrogen generator, Claind, Como, Italy) and argon (≥99.99% purity,

240 Aphagaz, Madrid, Spain) were used as the cone and collision gases, respectively. Two

241 transitions were acquired for HT and the metabolites generated, one for quantification

242 and a second for confirmation purposes. The optimized SRM conditions are presented

243 in **Table S1** of **Supporting Information**. The software used was MassLynx 4.1. HT

244 and HT-3-*O*-sulphate were quantified with their own calibration curves, and the other

245 HT metabolites were tentatively quantified by using the calibration curve of HT. The

246 calibration curves were constructed by using cellular medium free of HT metabolites.

247 *2.5. HAEC cell culture for testing HT and HT metabolites activity*

248 HAEC (Cascade Biologics™, Portland, Oreg., USA) at the 5th passage were seeded on

249 Nunclon™ Δ surface 12-well plates at a density of approximately $44*10^3$ of viable

250 cells/mL (1 mL / well). For the first 24 h, cells were maintained in complete cell culture

251 medium (CM) composed of M-200 medium supplemented with 2% (v/v) low-serum

252 growth supplement, 10 mg/mL gentamicin, 0.25 mg/ml amphotericin B (purchased

253 from Gibco by Life Technologies, Madrid, Spain), 100 U/mL penicillin and 100 mg/mL

254 of streptomycin (from Biowest-Labclinics, Barcelona, Spain). The cells were grown to

255 confluence at 37°C in a humidified incubator (Heracell 150; Madrid, Spain) with an

256 atmosphere containing 5% CO_2. The medium was replenished every 2 days with fresh

257 CM. Viewed under an IMT2 microscope (Olympus, Barcelona, Spain), confluent

258 monolayers displayed a typical monolayer phenotype of quiescent endothelial cells after

259 5 days in culture.

260 Experiments of time and dose-response were conducted in order to establish the

261 final conditions of TNF-α as a stimulant. Stock solutions used for the experiments were:

262 5 mM of HT (commercial) dissolved in sterile H_2O and 10 mM of the purified solution

263 of HT metabolites dissolved in methanol (Panreac, Madrid, Spain). Appropriate

264 dilutions were prepared until the final concentrations to be tested in the culture media

265 (1, 2, 5 and 10 µM) were obtained. To preempt cytotoxicity, methanol concentrations

266 never exceeded 0.1% (v/v) in culture media. Then, HAEC were co-incubated with HT

267 or HT metabolites at 1, 2, 5 and 10 µM and TNF-α (10 ng/mL; Calbiochem, Darmstadt,

268 Germany), the respective vehicle control alone (sterile H_2O for HT or absolute methanol

269 for HT metabolites) or TNF-α alone (10 ng/mL) for 18 or 24h. Additional experiments

270 were performed to test HT at lower concentrations (<1 µM) equivalent to 9% of the

271 residual HT remaining in the HT metabolites mixture at the concentrations tested (0.09,

272 0.18, 0.45 and 0.9 µM).

273   After co-incubation, supernatants were collected and stored at –20°C for batched

274 measurements of the soluble forms of the selected cell adhesion molecules (VCAM-

275 1/CD106, E-selectin/CD62E, P-selectin/CD62P and ICAM-1/CD54) or chemokine

276 (MCP-1/CCL2) and for the activity of the lactate dehydrogenase (LDH) released. Cells

277 were lysed with NaOH 0.5M and stored at –80°C for measuring total cellular protein

278 amount in order to adjust cell adhesion molecules and chemokine measurements.

279   In order to study the possible deconjugation of HT metabolites or conjugation of

280 HT during the incubation with HEAC cells, the metabolite pattern in the cells and also

281 in the culture media at the end of the incubation (24 h) was performed according to the

282 same chromatographic method described in Section 2.4.

283 *2.6. Cytotoxicity assays*

284 The cytotoxicity effect of HT (0-200 µM) on Caco-2 cells was tested using the 3-(4,5-

285 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [24]. 200,000

286 Caco-2 cells/mL were seeded in 24-multiwell plates and incubated with DMEM. After

287 24 hours, DMEM media was replaced with the same media enriched with different

288 concentrations of the HT for 24 h at 37 °C and 5% CO_2. Cells were then washed twice

289 with PBS. DMEM enriched in 0.5 mg/mL of MTT (Sigma-Aldrich, Madrid, España),

290 was added to each well and incubated for 3 h. To determine the formazan production,

291 once supernatants discarded, cells were lysed using an extraction solution composed of

292 DMSO and ethanol at a concentration of 1:1. A colorimetric assay was performed at

293 570 nm optical density.

294   The cytotoxicity effect of HT or HT metabolites on HAEC was assessed with a

295 Cytotoxicity Detection Kit LDH (Roche Applied Science, Mannheim, Germany) as

296 previously described [25]. For these experiments, TNF-α treatment was considered the

297 maximum stress condition for the cells. The activity of the LDH released from the cells

298 was measured in cell-free supernatants collected at the end of the experiment. Results

299 are expressed as the mean optical density (OD: 492 nm) and standard deviation (SD;

300 error bars) of LDH produced by the cells under each treatment condition.

301 *2.7. Total cellular protein quantification*

302 In order to adjust the soluble cell adhesion molecules (E-selectin, P-selectin, VCAM-1

303 and ICAM-1) and chemokine (MCP-1) total cellular protein concentration from cell

304 lysates was determined using the Bradford protein assay from Bio-Rad with bovine

305 serum albumin (BSA). Standard concentrations of BSA were used as the calibration

306 standards across the protein assay. The absorbance was measured at 595nm. The results

307 were expressed in mg of total cellular protein per milliliter compared to the maximum

308 stimulation with TNF-α (10 ng/mL).

309 *2.8. Measurement of soluble cell adhesion molecules and chemokine protein secretion*

310 *by HAECs stimulated with TNF-α*

311   Luminex® Performance Assay was used to determine VCAM-1, E-selectin, ICAM-1

312   and P-selectin protein secretion and DuoSet® enzyme-linked immunosorbent assays

313   (ELISA) was used to detect MCP-1/CCL2 chemokine protein secretion. Both protein

314   detection kits were from R&D Systems (Vitro, Madrid, Spain) and the tests were

315   conducted according to manufacturer's protocol.

316   All experimental data were compared to the outcomes in the TNF-α-alone

317   incubation, since this achieved the maximal secretion of soluble cell adhesion molecules

318   or chemokine. A vehicle-alone control (sterile H_2O for HT or absolute methanol for HT

319   metabolites) was run in parallel and used as the control in the experiments. Results are

320   expressed as the percentage of soluble cellular adhesion molecules or chemokine

321   protein secretion adjusted by total cellular protein and standard error of the mean (SEM;

322   error bars) compared to the maximum stimulation with TNF-α (10 ng/mL).

323   *2.9. Statistical Analyses*

324   Unless otherwise stated, all experiments were performed at least twice and each

325   incubation condition was set up in duplicate. One-way analysis of variance (ANOVA)

326   followed by the Fisher's least significant difference (LSD) post-hoc test was used for

327   multiple comparisons. A value of $p < 0.05$ was considered statistically significant.

328   A requisite for the analytical quality of the model was the control of several aspects

329   involved in the cellular process and analytical performance of measurements. Thus, we

330   evaluated the precision of the model by calculating the SD, SEM and the coefficients of

331   variation (CV). All the results were analyzed with the Statistical Package for the Social

332   Sciences (SPSS) software (version 22.0).

333   **3. Results**

334   *3.1. Cytotoxicity*

335 Results from the Caco-2 MTT cytotoxicity assay showed that HT was not cytotoxic at

336 doses from 50 to 150μM (data not shown).

337 The cytotoxicity assay of the supernatants taken at the end of the study corresponding to

338 the co-incubation of HT or HT metabolites at different concentrations (1, 2, 5 and 10

339 μM) and TNF-α (10 ng/mL) for different exposure times (18 and 24 h), measured as the

340 activity of LDH release showed no difference compared to the TNF-α (10 ng/mL)

341 treatment alone, confirming that HT and HT metabolites are not cytotoxic at the

342 concentrations and times tested (**Figure S1** of **Supplementary Information**). Lower

343 concentrations of HT (<1μM) did not show any toxicity (Data not shown).

344 *3.2. Effect of Caco-2 cell pretreatment on HT metabolite production*

345 **Table 1** shows the metabolism yield after exposing HT (100 μM) to different Caco-2

346 cell pre-treatment models (A, B, C and D) at different exposure times (6, 12 and 24 h).

347 Comparing the T75 flask and transwell treatments (A and B *versus* C and D,

348 respectively), the results showed that HT was much less stable in transwell inserts and

349 also showed less metabolic activity compared to cells cultured in T75 flasks. Regarding

350 the different exposure times, the highest metabolic transformation was obtained after 24

351 h. Longer exposure times were tested, but more than 24 h did not increase the

352 metabolism yield and the total amount of metabolites obtained decreased with the time,

353 probably due to a degradation process.

354 Significant differences (*p* < 0.05) were observed on the biotransformation of HT

355 into its main metabolites between the two T75 flasks models (A and B) at all-time

356 points studied (6, 12 and 24 h), observing that Caco-2 cells under B treatment (21-days

357 cultured cells) had a significantly higher metabolic activity compared to A treatment (3-

358 days cultured cells). These results confirmed that differentiated Caco-2 cells can exert a

359 higher conjugation enzymatic activity (catechol-*O*-methyltransferase, UDP-

360   glucuronosyltransferase and sulfotransferase) compared to non-differentiated cells and

361   thus, they appeared to be a more appropriate approach to obtaining HT phase II

362   metabolites. In the case of the transwell models, no significant differences were

363   observed in the production of any HT metabolite when comparing C and D treatments.

364   This could be attributed to the low enzymatic activity of the non-differentiated co-

365   cultured cells over the 3 days used in D treatment.

366   Based on the biotransformation yield of HT into phase II metabolites, the

367   differentiated cells in T75 flasks (B treatment) after 24 h of HT exposure was selected

368   as the best approach to obtaining HT metabolites with the lowest concentration of

369   residual free HT in the culture medium. Apart from being more efficient in terms of

370   metabolic activity, T75 flasks are much cheaper, easier to handle and have a bigger

371   surface compared to transwell inserts, which allows higher amounts of metabolites to be

372   obtained.

373   Moreover, different HT concentrations in the culture medium were assayed in order to

374   test the effect of the initial HT concentration on its transformation yield into phase II

375   metabolites. Results showed that the metabolic transformation was similar in all

376   concentrations tested (10, 15, 20, 25, 50, 100 µM) (**Table S2** of **Supplementary**

377   **Information**). Based on these results and considering that after the incubation of 100

378   µM HT a major amount of HT metabolites was obtained, 100 µM HT was selected to

379   produce the stock solution of HT metabolites.

380   *3.3. Effect of HT and HT metabolites on E-selectin, P-selectin, VCAM-1, ICAM-1 and*

381   *MCP-1 protein secretion*

382   After 18 h of co-incubation, significant reductions were observed with HT at all tested

383   doses in E-selectin levels ($p<0.05$), with reductions ranging from 32.7 to 45.9%

384   compared to TNF-α alone. HT metabolites significantly reduced E-selectin protein

385 secretion by 36.6% ($p<0.05$) only at 10 μM (**Table S3** and **Figure S2** of

386 **Supplementary Information**).

387   After 24 h of co-incubation the reduction trend observed at 18 h was confirmed.

388 HT reduced E-selectin, P-selectin, VCAM-1 and ICAM-1 at all tested doses (1, 2, 5 and

389 10 μM) compared to TNF- α alone ($p<0.05$) but the reductions did not follow a dose-

390 dependent response (**Figure 2**). HT metabolites reduced E-selectin, P-selectin, VCAM-

391 1 and ICAM-1 at 2, 5 and 10 μM and additionally, they also reduced MCP-1

392 significantly at 5 and 10 μM compared to TNF-α alone. In this case, the molecules were

393 reduced in a dose-dependent manner ($p<0.05$; **Figure 2**).

394   It is noteworthy that at 10 μM, HT metabolites were more effective than HT at

395 reducing E-selectin, P-selectin, VCAM-1, ICAM-1 and MCP-1 with respective

396 reductions of 62.4, 61.9, 55.4, 57.0 and 39.0% compared to HT (53.6, 48.6, 48.7, 40.6

397 and 11.7 %, respectively (**Figure 2** and **Table S3** of **Supporting Information**).

398 However, these reductions in HT metabolites compared to HT were only significant for

399 the MCP-1 molecule.

400   To exclude the effects of the residual HT present in the HT metabolite mixture,

401 lower concentrations of HT (0.9, 0.45, 0.18 and 0.09 μM) equivalent to the 9% of the

402 tested doses of HT metabolites (10, 5, 2 and 1 μM, respectively) were tested to evaluate

403 the possible activity of the residual concentration of free HT (**Figure 3**). Results showed

404 that concentrations of 0.45 and 0.9 μM were effective in the reduction of VCAM-1,

405 ICAM-1, P-selectin and E-selectin indicating that the activity of the metabolite mixture

406 could be due in part to the residual HT in the case of the highest doses (5 and 10 μM).

407 However, when testing 0.18 μM HT any significant effect was observed in the

408 inhibition of the adhesion molecules, whereas the concentration of 2 μM of the HT

409 metabolites mixture exerted a significant effect in the reduction of all adhesion

410   molecules. The reduction percentages observed after testing 0.45 and 0.9 µM of HT

411   were 10% lower compared to their respective HT metabolites mixtures (10 and 5 µM).

412   These results indicate that HT metabolites are in part responsible for the attenuation of

413   the inflammation-induced expression of adhesion molecules. In the case of the

414   chemokine MCP-1, lower concentrations assays confirmed that HT has no effect on

415   reducing MCP-1 at any concentration.

416   Vehicle control condition for HT (sterile H_2O) or HT metabolites (absolute

417   methanol) are not shown due to their low values of E-selectin, P-selectin, VCAM-1,

418   ICAM-1 and MCP-1 protein secretion concentrations. They were not within the limits

419   of detection of the techniques used for determining these molecules in this study, so

420   they were assumed as practically zero.

421   *3.4. Analysis of HT and metabolites in cells and culture media after incubation*

422   The metabolite pattern in the cells and also in the medium at the end of the

423   incubation was analysed. Unexpectedly, results showed that after 24 h of incubation

424   with HT, the native compound underwent a conjugation process leading to its main

425   metabolites in a dose-dependent manner (**Figure 4**), revealing that HEAC cells have

426   conjugation enzymatic capabilities, mainly catechol-*O*-methyltransferase and

427   sulfotransferase activity (**Table S4 Supplementary Information**). On the other hand,

428   after incubating the HT metabolite mixture with HEAC cells, small amounts of free HT

429   were detected in cells and HT did not increase in parallel to the tested dose, indicating

430   that the metabolites were not hydrolysed in cells during the assay and probably HT was

431   degraded during incubation. Results from culture media presented a very similar profile

432   compared to cells (**Figure 4**).

433   **4.   Discussion**

434   The present study is among the first to obtain a mixture of physiological phase II

435   metabolites of HT and demonstrating that they are able to exert an effect in the

436   reduction of the endothelial dysfunction biomarkers in an *in vitro* endothelial cell model

437   at plasmatic concentrations achievable following ingestion of olive oil [10]. The

438   remaining amount of unaltered HT in urine is very low (<1%) [8,11] and undetectable

439   in plasma after the consumption of nutritional amounts of VOO phenols and, therefore,

440   the role of HT phase II metabolites in preventing atherosclerosis must be considered.

441   However, *in vitro* studies with endothelial cell models carried out to date used HT

442   [26,27] rather than the majority forms that appear in human plasma.

443   So, in order to perform mechanistic studies, firstly, it is necessary to obtain the

444   HT metabolites, which are not commercially available. For this proposal, in the present

445   study Caco-2 cells were incubated with HT in order to produce a mixture of HT

446   metabolites resembling the human plasma after the intake of virgin olive oil. Previous

447   studies have used different approaches to obtaining phase II metabolites of phenolic

448   compounds [28–31]. The chemical synthesis approach could be considered the cheapest,

449   but its main drawback is that most chemical procedures yield mixtures of α- and β–

450   anomers along with side-products [32]. Biocatalysis-assisted synthesis using tissue

451   homogenates or recombinant conjugating enzymes (glucuronosyltransferases and

452   sulfotransferases) is an alternative strategy for preparing phenolic conjugates [33].

453   However, enzymatic synthesis requires the use of either expensive enzymes, co-factors

454   (uridine 5'-diphospho-glucuronic acid and adenosine-5'-phosphosulfate) or liver

455   microsomes and the degree of conversion is also very low [28]. Another alternative

456   could be the use of biological samples to obtain phenolic phase II metabolites, which

457   have been reported previously for the isolation of resveratrol glucuronide conjugates

458   from human urine [30], thymol sulfate from rat urine [29] or catechin and quercetin

459 from rat plasma [31]. The main drawback of this approach is that it requires the

460 performance of an *in vivo* study and the amounts obtained are very small.

461   In relation to VOO phenolic metabolites, a previous study attempted to

462 synthetize HVAlc and the glucuronic forms of HT using porcine liver microsomes [34].

463 In another study the potential hepatic metabolism of VOO phenolic compounds by

464 HepG2 cell lines was tested, and the glucuronide, methylglucuronide, and methyl

465 conjugates of HT were the main metabolites produced [35]. In both studies no sulfate

466 conjugates were produced, which could be attributed to the low sulfotransferase activity

467 of HepG2 cell line and its very low/partial expression of cytochrome P-450 [36]. Our

468 previous studies in humans indicate that sulfation constitutes the major pathway for HT

469 metabolism in humans [9,10], and up to date, any effective and simple strategy to obtain

470 a pool of the real phase II circulating HT metabolites has been developed.

471   In the present study, we demonstrate that the use of Caco-2 cells as bioreactors

472 for the generation of phase II metabolites of HT is a very appropriate tool given their

473 availability, long life-span, stable phenotype, high metabolic efficiency, ease of

474 handling and the fact that they do not require the use of human volunteers or animals.

475 Caco-2 cells, which are usually used as a model for studying the intestinal transport and

476 metabolism of some dietary compounds [37], are obtained from human colon

477 adenocarcinoma and spontaneously differentiate in a confluence culture as intestinal

478 absorptive cells due to changes in their morphology [38]. Once they are differentiated,

479 Caco-2 cells exert phase II conjugate enzymes and cytochrome P-450 isoforms, and are

480 useful for studying their metabolic activity and characterizing human circulating

481 metabolites, as reported in previous studies in which the metabolic profile was

482 determined after a polyphenol exposure [7,37].

483   This approach also allowed to obtain a metabolic profile very similar to the

484   human plasma after the consumption of nutritional amounts of VOO phenols [10]. In

485   this previous study the main metabolites detected in plasma after a sustained intake of

486   25 mL of VOO with high phenolic content (500 mg total phenolic compounds/ kg oil)

487   were the sulfated forms of HT (79%), homovanillic alcohol sulfate (sulfHVAlc) (19%),

488   and in a minor proportion the homovanillic acid sulfate (sulfHVac). The Caco-2

489   intestinal cell model allowed us obtaining a mixture in which the main metabolites were

490   HTsulf (80%), sulfHVAlc (10%) and in a minor proportion, HT glucuronides and free

491   HT (**Figure 5**). So the obtained mixture of metabolites is very close to the previously

492   observed human plasma profile, with a 90% of resemblance.

493   Results from the TNF-α stimulated HAEC cells indicated that after 24 h of

494   incubation both HT metabolites and free HT at physiological concentrations (1 to 10

495   μM) were effective at reducing E-selectin, P-selectin, VCAM-1 and ICAM-1 molecules

496   involved at the first stages of atherosclerosis. In a previous study the inhibition of

497   endothelial activation by free HT was described in a human umbilical vein endothelial

498   cell (HUVEC) model, observing that HT was able to reduce lipopolysaccharide (LPS)-

499   stimulated expression of VCAM-1 at low micromolar concentrations [15]. Another

500   study observed reductions in E-selectin, VCAM-1 and ICAM-1 after HT exposure on

501   HUVEC at doses of 5 and 25 μM of HT. [39]. Regarding MCP-1, Richard N *et al.* also

502   observed a significant reduction of MCP-1 after HT exposure (25μM) on murine

503   macrophages (RAW264.7 cells) [40]. In the two latter studies, the effect of HT

504   metabolites was not tested, so the physiological significance of the results is

505   questionable.

506   After the HT treatments we also observed that the remaining amounts of free

507   HT in the cells and media did no differ significantly among the tested doses, which was

508   in accordance with the observed effects in the modulation of the cell adhesion

509   molecules that were also maintained regardless of the dose. However, when HT

510   metabolites were incubated with HAEC cells, an increasing concentration of these

511   metabolites was detected in the cells with a parallel dose-dependent inhibition. These

512   data provide evidence of the potential effect of HT phase II metabolites in the reduction

513   of the endothelial dysfunction biomarkers.

514   The residual amount of free HT in the obtained metabolite mixture represents

515   the major difference with the plasma data. Therefore, in order to test the possible

516   activity of the residual amounts of HT present in the metabolite mixtures, lower

517   concentrations were tested. Results indicated that in the case of adhesion molecules

518   (VCAM-1, ICAM-1, E-selectin and P-selectin) the attenuation effect of the metabolite

519   mixtures could be due to a large extent to the residual HT. However, in all cases,

520   reductions of the metabolite mixtures were 10% greater compared to their

521   corresponding amounts of residual HT, indicating that metabolites also contribute in the

522   inhibition. The effect of the metabolites was confirmed when the dose of 2 uM HT

523   metabolites was tested, observing a significant inhibition of all adhesion molecules

524   secretion, whereas its corresponding percentage of residual HT did not show any effect.

525   From these results it can be concluded that metabolites might be in part responsible for

526   the attenuation of the inflammation-induced expression of adhesion molecules.

527   In the case of the chemokine MCP-1, HT did not show any effect at any tested

528   dose, whereas the highest doses (5 and 10 μM) of HT metabolites presented a

529   significant effect, indicating in this case that metabolites clearly seem to be active on the

530   inhibition of the secretion of this chemokine. The specific effect of HT metabolites on

531   reducing MCP-1 could also indicate that free HT and HT metabolites could act reducing

532   the endothelial dysfunction through different endpoints and having complementary

533   activities.

22

534   Unexpectedly, after the incubation assays with HT, an important amount of HT

535   metabolites were detected in the cells and media, most of them sulfo and methylsulfo-

536   conjugated. Catechol-*O*-methyltransferase (COMT) have been previously described in

537   endothelial cells after incubating HUVEC with ()-epicatechin, identifying the

538   endothelial NADPH oxidase as a specific candidate target for the methylated forms

539   [41]. SULT1A1, the main sulfotransferase enzyme responsible for the detoxification of

540   xenobiotics, has been found in many different human tissues, with the highest

541   abundance found in the liver, but it has never been detected in human aortic endothelial

542   cells. Thus, the data provided in the present work add value to the study suggesting that

543   aortic sulfotransferases may play an important role in reducing local cellular

544   concentrations of native forms of phenolic compounds.

545   Although phase II phenolic metabolites have been generally considered to be

546   pharmacologically inactive and targets for excretion [42], our results indicate that the

547   inhibition of the endothelial dysfunction could be partly due to the HT metabolites. The

548   effect of flavonoids (quercetin and (+)-catechin) and their different conjugates on cell

549   adhesion has been recently reviewed [43] and it was established that conjugation can

550   significantly affect the activity of aglycones both reducing and increasing the effects.

551   So, given that the effects of conjugation can differ greatly on cell adhesion, research on

552   the biological activity has to be done on a case-by-case basis.

553   Our results also demonstrate that effects other than the reduction of ox-LDL and

554   cholesterol, which have been demonstrated in humans [1,44–46], may explain the anti-

555   atherogenic action of VOO phenolic compounds. Two additional mechanisms involved

556   in the vascular damage, platelet aggregation and proliferation of smooth muscle cells,

557   are also antagonized by the VOO phenolic compounds, it was particularly observed that

558   HT inhibits *in vitro* platelet aggregation induced by thromboxane B2 production and

23

559   collagen [16].

560   **5.   Conclusions**

561   This is the first time that HT metabolites have been synthetized by using a

562   differentiated Caco-2 cell line, obtaining a purified extract that resembles the human

563   plasma HT metabolite profile after VOO intake. Results indicate that both free HT and

564   HT metabolites are effective in the reduction of the endothelial dysfunction biomarkers

565   but only HT metabolites reduce MCP-1 at physiological doses. Novel data is presented

566   regarding the presence of xenobiotic detoxification enzymes in HAEC cells, which add

567   value to the study suggesting that catechol-*O*-methyltransferase and sulfotransferases

568   may play an important role in reducing local cellular concentrations of native forms of

569   phenolic compounds in aortic endothelial cells. Our data suggest that HT metabolites

570   might be the responsible in part for the protection against endothelial dysfunction, and

571   thus they are likely to contribute to the reduced risk in the early stages of

572   atherosclerosis, which provide a clear future direction in olive oil phenols research.

573   **Acknowledgements**

577   **Figure captions**

578   **Figure 1.** Caco-2 cell culture treatments for HT metabolites production and the

579   methodology used for their extraction and purification. **A:** Caco-2 cell culture

580   treatments tested to obtain HT metabolites. T75 flask and transwell inserts were used as

581   two different culture conditions to obtain HT metabolites with different differentiation

582   states and at different times of HT exposure (6, 12 and 24h). **B:** procedure for the

583   extraction and purification of HT metabolites from Caco-2 cells supernatants.

584   **Figure 2**. Effect of HT and HT metabolites on E-selectin, P-selectin, VCAM-1, ICAM-

585   1 and MCP-1 protein secretion in HAEC stimulated by TNF-α after 24h.

586   HAEC were co-incubated with HT or HT metabolites at 1, 2, 5 and 10 µM and TNF-α

587   (10 ng/mL) for 24h. **A)** Effect of HT or HT metabolites on E-selectin protein secretion.

588   **B)** Effect of HT or HT metabolites on P-selectin protein secretion. **C)** Effect of HT or

589   HT metabolites on VCAM-1 protein secretion. **D)** Effect of HT or HT metabolites on

590   ICAM-1 protein secretion. **E)** Effect of HT or HT metabolites on MCP-1 protein

591   secretion. Results are expressed as the percentage of soluble cellular adhesion molecules

592   or chemokine protein secretion adjusted by total cellular protein and standard error of

593   the mean (SEM; error bars). * $p < 0.05$ *versu*s TNF-α alone. † $p < 0.05$ compared

594   between HT and HT metabolites at the same concentration.

595   **Figure 3.** Effect of HT at lower concentrations (<1 µM) on E-selectin, P-selectin,

596   VCAM-1, ICAM-1 and MCP-1 protein secretion in HAEC stimulated by TNF-α after

597   24h.The tested concnetrations (0.9, 0.45, 0.18 and 0.09 µM) were equivalent to the 9%

598   of the tested doses of HT metabolites (10, 5, 2 and 1 µM).

599   **Figure 4.** HT and metabolites in HAEC cells and culture media after co-incubation with

600   HT or HT metabolites at 1, 2, 5 and 10 µM and TNF-α (10 ng/mL) for 24h.

601   **Figure 5.** HT metabolites profile in human plasma *versus* Caco-2 cell production.

602   Extracted ion chromatograms in human plasma after a sustained intake of virgin olive

603   oil and in purified extract obtained from Caco-2 exposed to HT (differentiated cells in

604   flasks / 24 h exposure of HT 100 µM).

605   **Supporting information.**

606   **Figure S1.** Cytotoxicity from HAEC' supernatants after co-incubation with HT or HT

607   metabolites and TNF-α. HAEC were co-incubated with HT (**A**) or HT metabolites (**B**)

608   at 1, 2, 5 and 10 µM and TNF-α (10 ng/mL) for 18 and 24 h. Vehicle control refers to

609   cells incubated with HT vehicle (H_2O) or HT metabolites vehicle (MetOH). Results are

610   expressed as the mean and the SD (error bars) of optical density (OD; 492 nm) from one

611   representative experiment where each set of experimental conditions was run in

612   duplicate. *$P<0.05$ compared to maximum stimulation TNF-α alone.

613   **Figure S2.** Effect of HT and HT metabolites on E-selectin, P-selectin, VCAM-1,

614   ICAM-1 and MCP-1 protein secretion in HAEC stimulated by TNF-α after 18h.

615   HAEC were co-incubated with HT or HT metabolites at 1, 2, 5 and 10 µM and TNF-α

616   (10 ng/mL) for 18h. **A)** Effect of HT or HT metabolites on E-selectin protein secretion.

617   **B)** Effect of HT or HT metabolites on P-selectin protein secretion. **C)** Effect of HT or

618   HT metabolites on VCAM-1 protein secretion. **D)** Effect of HT or HT metabolites on

619   ICAM-1 protein secretion. **E)** Effect of HT or HT metabolites on MCP-1 protein

620   secretion. Results are expressed as the percentage of soluble cellular adhesion molecules

621   or chemokine protein secretion adjusted by total cellular protein and standard error of

622   the mean (SEM; error bars). * $p < 0.05$ *versu*s TNF-α alone. † $p < 0.05$ compared

623   between HT and HT metabolites at the same concentration.

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771   refined olive oil are associated with less oxidation of plasma LDL. *J. Nutr.* 2010,
772   140, 501–8.

Table 1. HT and its metabolites concentration (µM) in culture media after HT exposure among different Caco-2 cell pretreatment models (T75 flask or transwell inserts) at different times of exposure (6, 12 and 24h). A: 3-day cultured cells in T75 flasks; **B**: 21-day cultured cells in T75 flasks; **C**: 21-day cultured cells in the apical side of transwell inserts and **D**: 18-day cultured cells in the apical side plus 3-day co-cultured cells in the basolateral side of transwell inserts (21 days in total). For treatments C and D values were the sum of apical and basolateral sides. The initial concentration of HT in culture media for all the experiments was 100µM. Based on the yield transformation of HT into phase II metabolites, the differentiated cells in flasks (B treatment) after 24 h of HT exposure were selected as the best approach.

| Concentration (µM)	6 h				12 h				24 h			
	T75 FLASK		TRANSWELL		T75 FLASK		TRANSWELL		T75 FLASK		TRANSWELL	
	A	B	C	D	A	B	C	D	A	B	C	D
HT *	76.10 ± 10,60[a]	66.47 ± 10.47[b]	41.87 ± 10.25[c]	23.52 ± 9.81[c]	45.15 ± 15.71[a]	39.20 ± 5.24[b]	9.29 ± 3.04[c]	13.90 ± 1.53[c]	39.91 ± 1.32[a]	6.98 ± 0.20[b]	5.18 ± 3.77[b]	5.25 ± 0.33[b]
sulfHT **	0.11 ± 0.00[a]	10.77 ± 0.88[b]	10.08 ± 3.02[b]	10.37 ± 3.30[b]	0.63 ± 0.77[a]	27.73 ± 3.57[b]	758 ± 2.62[c]	7.27 ± 3.58[c]	2.33 ± 0.83[a]	63.42 ± 9.23[b]	8.00± 2.44[c]	7.99 ± 1.33[c]
4-GlucHT*	n.d.	0.19 ± 0.04	1.03 ± 0.63	1.08 ± 0.72	n.d.	0.48 ± 0.19	0.62 ± 0.40	0.62 ± 0.35	0.13 ± 0.03[a]	0.60 ± 0.13[b]	0.64 ± 0.43[b]	0.76 ± 0.39[b]
3-GlucHT*	n.d.	0.06 ± 0.03	0.03 ± 0.01	0.04 ± 0.02	n.d.	0.10 ± 0.11[a]	0.01 ± 0.01[b]	0.02 ± 0.01[b]	0.02 ± 0.01[a]	0.31 ± 0.06[b]	0.02 ± 0.01[a]	0.03 ± 0.00[b]
sulfHVac*	n.q.	n.q.	0.08 ± 0.02	0.07 ± 0.04	n.q.	n.q.	0.06 ± 0.03	0.07 ± 0.06	n.q.	0.06 ± 0.04	0.03 ± 0.01	0.03 ± 0.01
glucHVac*	n.d.	n.d.	0.02 ± 0.02	0.03 ± 0.04	n.d.	n.d.	0.04 ± 0.02	0.03 ± 0.01	n.d.	0.01 ± 0.01	0.02 ± 0.01	0.02 ± 0.00
sulfHVAlc *	0.03 ± 0.00[a]	0.28 ± 0.02[b]	0.03 ± 0.00[a]	0.05 ±0.02[a]	0.02 ± 0.00	n.q.	0.03 ± 0.02	0.02 ±0.02	0.02 ± 0.02[a]	2.27 ± 0.48[b]	0.01 ± 0.00[a]	0.02 ± 0.02[a]
glucHVAlc*	n.q.	0.11 ± 0.02*	n.q.	n.q.	n.q.	0.35 ± 0.08	n.q.	n.q.	n.q.	0.29 ± 0.16	n.q.	n.q.

HT: hydroxytyrosol; sulfHT: hydroxytyrosol sulfate; 4-GluHT: hydroxytyrosol 4' glucuronide; 3-GlucHT: hydroxytyrosol 3' glucuronide; sulfHVac: homovanillic acid sulfate; glucHVac: homovanillic acid glucuronide; sulfHVAlc: homovanillic alcohol sulfate; glucHVAlc: homovanillic alcohol glucuronide.

n.d.: not detected; n.q.: not quantified

Different superscript letters [(a,b,c)] mean significant differences between treatments (A,B, C and D) in the same time tested for each metabolite.
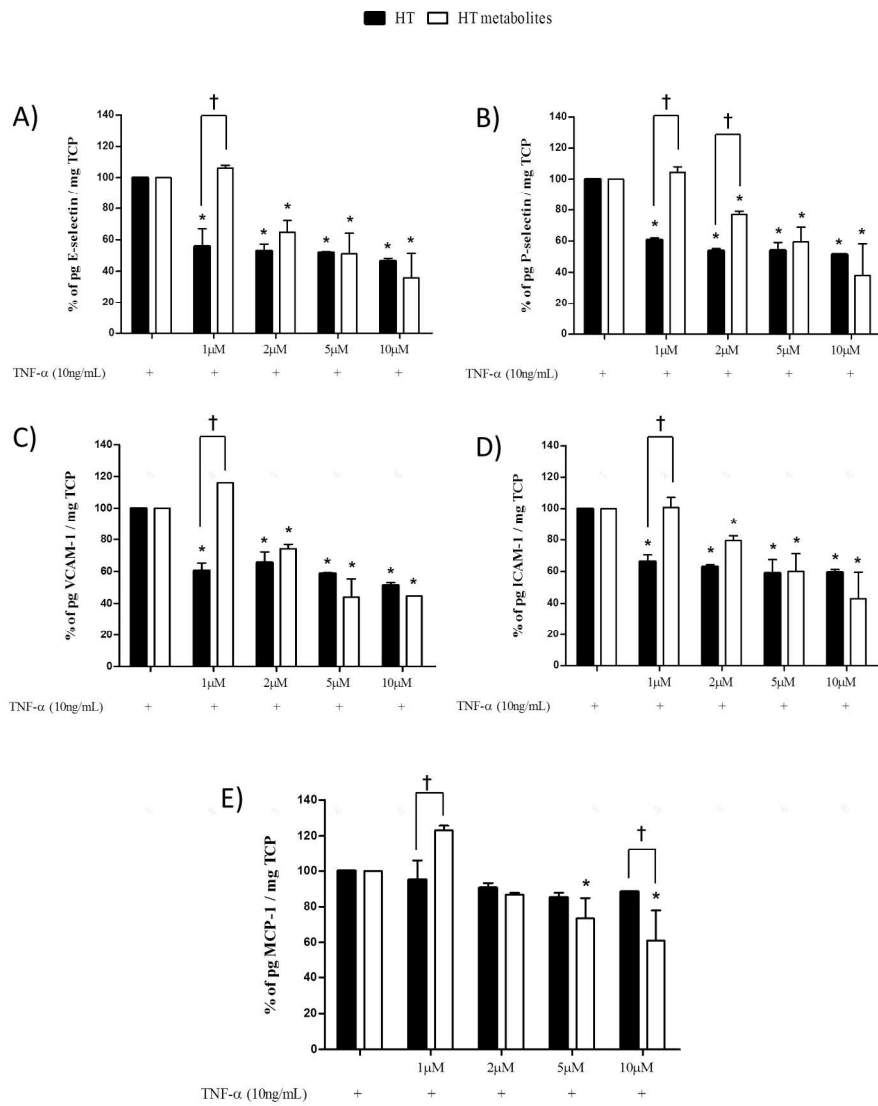
One-way Analysis of variance (ANOVA) followed by the Fisher's least significant difference (LSD) post-hoc test was used for multiple comparisons ($p < 0.05$).

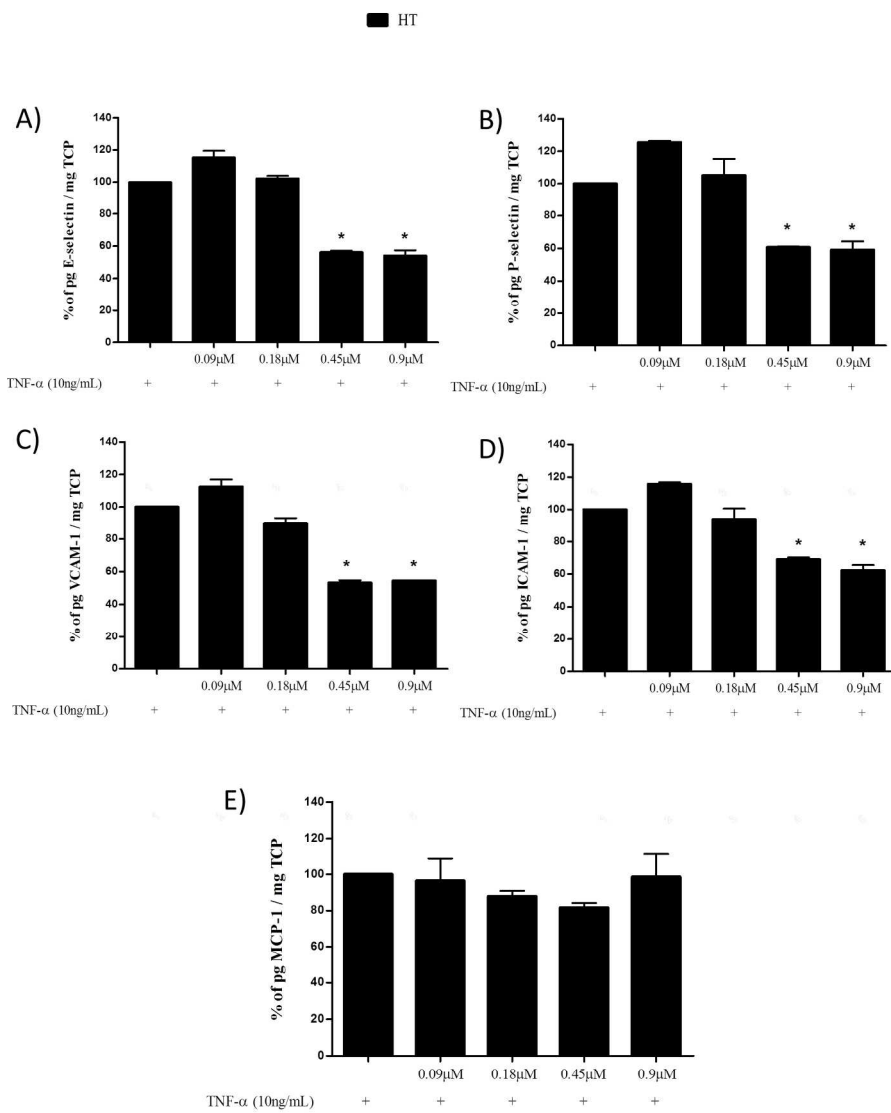* Quantified by using the calibration curve of HT.
** Quantified by using the calibration curve of HT-3-*O*-sulfate.
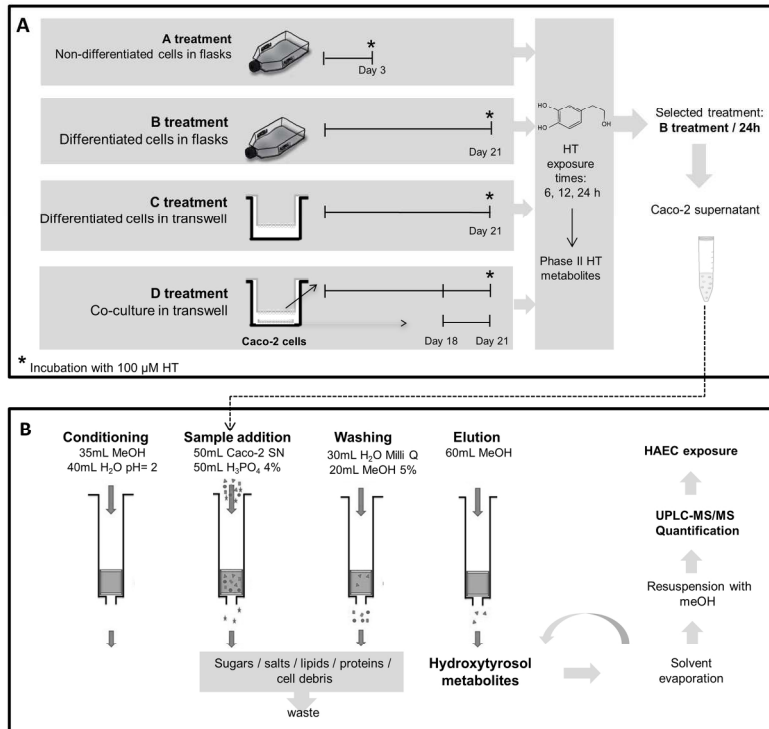
190x142mm (300 x 300 DPI)

254x338mm (300 x 300 DPI)

254x338mm (300 x 300 DPI)
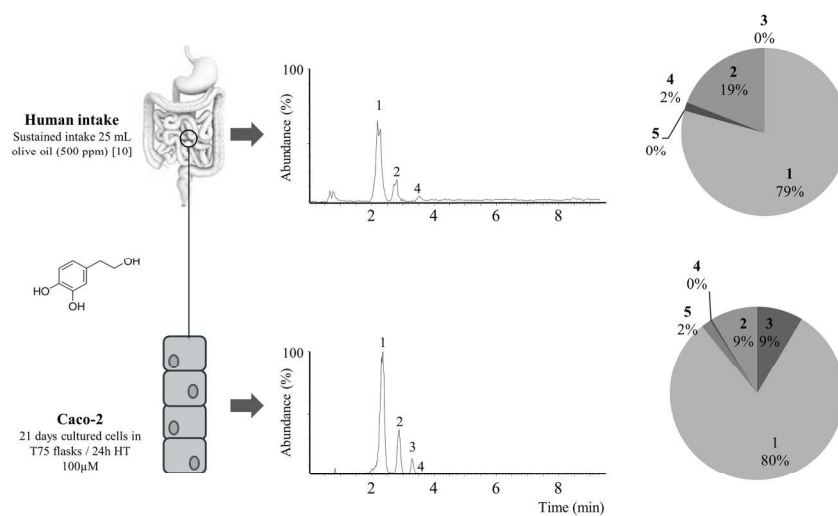
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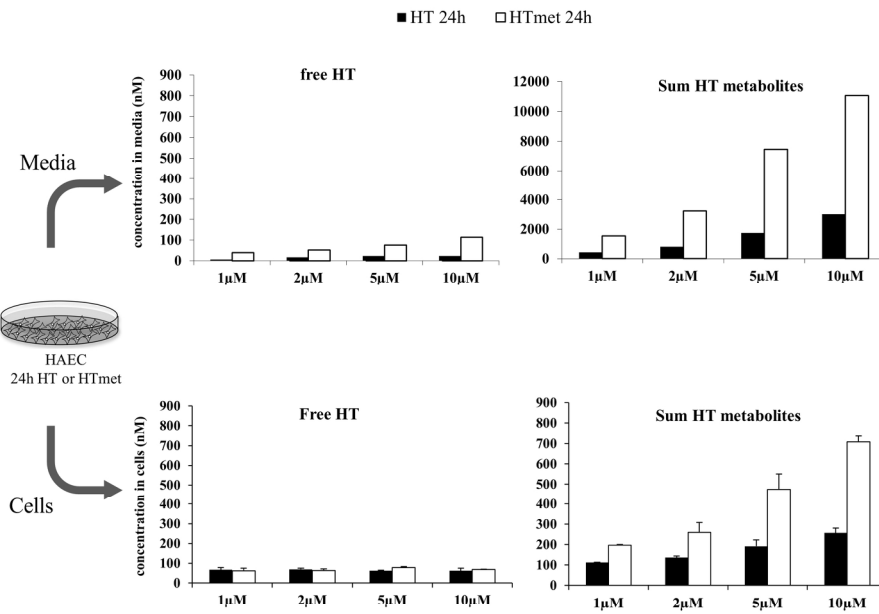
1, hydroxytyrosol sulfate forms; **2**, homovanillic alcohol sulfate; **3**, hydroxytyrosol; **4**, homovanillic acid sulfate; **5**, hydroxytyrosol glucuronides

190x142mm (300 x 300 DPI)