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(Article begins on next page)

1 **ANTIATHEROGENIC EFFECTS OF ELLAGIC ACID AND UROLITHINS *IN VITRO***

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20

21 **Abstract**

22 Atherosclerosis, one of the leading causes of death worldwide, is characterized by impaired endothelial  
23 function and lipid metabolism, among other factors. Ellagitannins are a class of phenolic compound  
24 that may play a role in cardiovascular health. This work aimed to study the potential atheroprotective  
25 effects of urolithins, ellagitannin-derived gut microbiota metabolites, on different key factors in  
26 atherosclerosis development: the ability of monocytes to adhere to endothelial cells and the uptake and  
27 efflux of cholesterol by macrophages. The biotransformations urolithins may undergo in peripheral  
28 cells were also evaluated. Results indicated that some urolithins and ellagic acid, at physiologically  
29 relevant concentrations, were able to reduce the adhesion of THP-1 monocytes to human umbilical vein  
30 endothelial cells (HUVECs) and the secretion of a cellular adhesion molecule (sVCAM-1) and pro-  
31 inflammatory cytokine (IL-6). Urolithin C, a combination of urolithins A and B, and ellagic acid also  
32 decreased the accumulation of cholesterol in THP-1-derived macrophages treated with  
33 hypercholesterolemic serum and acetylated LDL, but they were not able to promote cholesterol efflux.  
34 The analysis of cell media by UHPLC-ESI-MS<sup>n</sup> indicated urolithins and ellagic underwent extensive  
35 metabolism within vascular cells, representing significant sulfate and methyl conjugation. This  
36 evidence indicates atherosclerotic processes may be attenuated by urolithins following ellagitannin  
37 consumption, however, future human intervention trials are required to establish if regular intake of  
38 ellagitannin-rich foodstuffs attenuates atherosclerotic.

39

40 **Keywords:** atherosclerosis, cholesterol transport, endothelial function, ellagitannin metabolites,  
41 urolithins, peripheral metabolism

42 **1. Introduction**

43 Atherosclerosis is a chronic disorder caused by multiple factors that impair vascular function and  
44 damage the artery wall. Dysfunctional endothelium, characterized by increased expression of cell  
45 adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion  
46 molecule-1 (ICAM-1), allows accumulation of monocytes in the subendothelial matrix. Infiltrated  
47 monocytes differentiate into cells with macrophage and/or dendritic cell-like features. Uptake of  
48 oxidized low density lipoprotein (oxLDL) and native LDL by these cell types (via scavenger receptors  
49 or fluid phase pinocytosis, respectively), leads to foam cell formation [1, 2]. The presence of lipid-rich  
50 foam cells contributes to plaque formation and eventually progresses to the clinical presentation of  
51 atherosclerosis [3].

52 Ellagitannins are a subclass of hydrolysable tannins characterized by the presence of ellagic acid (EA)  
53 and glucose [4]. They are mostly found in fruits such as pomegranate, raspberries, strawberries,  
54 blackberries, blueberries, muscadine grapes and persimmon, nuts including walnuts, pistachio, cashew,  
55 and hazelnuts, and oak-aged wines (where tannins are released from the barrels) [5]. For the past  
56 decade, many studies have focused on the *in vitro* atheroprotective effect of ellagitannin-rich extracts.  
57 Ellagitannin extracts have been reported to delay LDL oxidation [6] and to reduce cholesterol  
58 accumulation in macrophages, both inhibiting the uptake and stimulating high density lipoprotein  
59 (HDL) efflux [7]. Ellagitannins have been shown to improve vascular function through inducing the  
60 expression of endothelial nitric oxide synthase (eNOS) in human artery endothelial cells [8, 9],  
61 inhibiting platelet aggregation and monocyte adhesion to endothelial cells, and reducing the expression  
62 of ICAM-1 and VCAM-1 in human endothelial cells [10]. Some of these protective effects were  
63 confirmed *in vivo* in animal and human studies, where consumption of ellagitannins from different  
64 sources was mostly associated with improvement of serum lipid profile and antioxidant activity [11,  
65 12]. However, the number of intervention studies conducted to date is limited and most of the *in vitro*

66 studies have been poorly designed, since cells of the vascular system were exposed to plant extracts  
67 rich in ellagitannins, which are unlikely to reach the systemic circulation after consumption of  
68 ellagitannin-containing foodstuffs [5, 11]. It now established that ellagitannins are hydrolyzed in the  
69 small intestine, releasing EA; the free EA is poorly absorbed and more than 99% of it is metabolized by  
70 the gut microbiota, forming the bioavailable urolithins. Urolithins are molecules characterized by a  
71 common 6*H*-dibenzo[*b,d*]pyran-6-one nucleus and a decreasing number of phenolic hydroxyl groups  
72 (urolithin D → C → A → B) [13, 14]. They can be further metabolized by phase II enzymes  
73 (methylation, glucuronidation and sulfation), mainly by enterocytes and hepatocytes, and appear in  
74 circulation at low micromolar concentrations [15].

75 Despite a limited number of studies attempting to elucidate the anti-inflammatory properties of  
76 urolithins in endothelial cells and macrophages [16, 17], very little is known about the preventive role  
77 of urolithins in atherosclerosis. Therefore, the aim of the present study was to evaluate the potential  
78 atheroprotective effects of urolithins with diverse patterns of hydroxylation, on different key  
79 atherogenic processes, namely monocyte adhesion to endothelial cells and cholesterol transport.  
80 Distinct emphasis was placed on the ability of urolithins to promote cholesterol efflux, a strong  
81 predictor of the extent of atherosclerosis, which is inversely associated to cardiovascular risk [18, 19].  
82 This work also aimed to identify the cellular mechanisms underlying the cardioprotective effects of  
83 ellagitannin-containing foodstuffs. Moreover, considering the extensive biotransformations that  
84 urolithins are likely to undergo in peripheral cells [20], the metabolism of these ellagitannin-derived  
85 molecules in the applied cell models was studied.

86

## 87 **2. Material and methods**

### 88 **2.1. Materials**

89 Fetal calf serum (FCS), bovine serum albumin (BSA), Acyl CoA:cholesterol acyltransferase (ACAT)  
90 inhibitor, DNase, phorbol, 12-myristate, 13-acetate (PMA), and bovine skin-derived gelatin solution  
91 were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI/HEPES culture medium and PBS  
92 were from Lonza (Walkersville, MD, USA). Tumor Necrosis Factor- $\alpha$  (TNF- $\alpha$ ), Medium 200, Low  
93 Serum Growth Supplement Kit, Amplex Red Cholesterol Assay Kit, soluble VCAM-1 (sVCAM-1)  
94 ELISA kit, and IL-6 ELISA kit from Thermo Fisher Scientific (Waltham, MA USA). [1,2-  
95  $^3\text{H}$ ]cholesterol was obtained from PerkinElmer (Boston, USA). Human LDL were kindly provided by  
96 Prof. Calabresi (University of Milan, Italy) and were acetylated (acLDL) according to Basu et al. [21].  
97 Human serum was collected with approved consent from healthy normolipidemic individuals.  
98 Hypercholesterolemic serum was collected with informed consent from patients affected by familial  
99 hypercholesterolemia at the Reference Center for Hereditary Dyslipidaemias (Pisa, Italy) and pooled.  
100 Urolithin A (Uro A) and urolithin B (Uro B) were provided by Prof. O. Dangles (INRA, Avignon,  
101 France), while urolithins C and D (Uro C and Uro D) were purchased from Dalton Pharma Services  
102 (Toronto, ON, Canada). EA was from Sigma-Aldrich. All solvents and reagents for extraction and  
103 UHPLC-MS analysis were purchased from Carlo Erba Reagents (Milan, Italy).

104

## 105 2.2. Cells

106 Human Umbilical Vein Endothelial Cells (HUVECs) were purchased from GIBCO, Life Technologies  
107 (Frederick, MD, USA) and cultured in Medium 200 supplemented with Low Serum Growth  
108 Supplement Kit (as recommended by the manufactory). HUVECs were used for experiments between  
109 II and V passage.

110 Human monocytes-derived macrophages THP-1 were purchased from ATCC (Teddington, UK) and  
111 cultured in RPMI/HEPES supplemented with 1 mM sodium pyruvate, 50  $\mu\text{M}$   $\beta$ -mercaptoethanol, 2.5

112 mg/mL glucose and 10% FCS. Differentiation into macrophages was obtained by treating cells with 50  
113 ng/mL of PMA for 72 h.

114

### 115 **2.3. Monocyte adhesion to endothelial cells**

116 HUVECs were grown on gelatin-coated 96-well plates (30.000 cells/well) until confluence. Cells were  
117 treated with or without urolithins A, B, C, and D, and EA at 10  $\mu$ M for 6 h. An association of Uro A  
118 and Uro B at 10  $\mu$ M each (named Uro A+Uro B, 10  $\mu$ M) was also tested. During the last 2 h of  
119 treatment, cells were incubated with 15 ng/mL TNF- $\alpha$ . THP-1 monocytes were labeled with 5  $\mu$ M  
120 calcein and a cell suspension was added to the HUVECs monolayers (50.000 cells/well). Cells were co-  
121 incubated for 30 min, following rinsing of unbound THP-1 and subsequent determination of adherent  
122 cells fluorometrically. Results were expressed as percentage of adherence of control TNF- $\alpha$  treated  
123 cells.

124

### 125 **2.4. IL-6 and sVCAM secretion in endothelial cells**

126 HUVECs were seeded on gelatin-coated 24-well plates (180.000 cells/well) until confluence. Cells  
127 were pre-treated with or without Uro C, Uro D, association of Uro A and Uro B (Uro A+Uro B), and  
128 EA at 10 $\mu$ M for 6 h. After 18 h incubation with the treatments and 15 ng/mL TNF- $\alpha$ , cell supernatants  
129 were collected and immediately frozen at -80  $^{\circ}$ C. IL-6 and sVCAM protein levels were quantified in  
130 HUVEC cell surnatants by using commercial enzyme-linked immunosorbent assays, according to the  
131 manufacturer's instructions. Results of IL-6 and sVCAM were expressed as pg/mL and ng/mL,  
132 respectively.

133

### 134 **2.5. Macrophage cholesterol loading**

135 THP-1-derived macrophages were cultured in the presence of human hypercholesterolemic serum  
136 (HCS) (5%, v/v) or acetylated LDL (acLDL) (50 µg/mL) to induce foam cell formation, with or  
137 without urolithins A, B, C, and D, combination of Uro A and Uro B (Uro A+Uro B), and EA at 10 µM.  
138 After 24 h cells were lysed using 1% sodium cholate and DNase (50 U/mL). Cholesterol was  
139 measured by fluorimetric analysis with the Amplex Red Cholesterol Assay Kit, according to  
140 manufactures instructions. The amount of cholesterol in each well was corrected for the protein content  
141 of the well and successively expressed as percentage of cholesterol content of control cholesterol-  
142 loaded cells. Protein content in the lysate was measured by the use of bicinchoninic acid assay [22].  
143 The same protocol was used to evaluate the effect of the treatments on cholesterol content in THP-1-  
144 derived macrophages before exposure to HCS or acLDL.

145

## 146 **2.6. Macrophage cholesterol efflux**

147 Cholesterol efflux from THP-1-derived macrophages was evaluated using a radioisotope assay [23].  
148 Briefly, cells were radiolabeled with 2 µCi/mL [1,2-<sup>3</sup>H]cholesterol in presence of ACAT inhibitor (2  
149 µg/mL) for 24 h. Successively, cells were incubated for 18 h in culture medium supplemented with  
150 0,2% (w/v) of BSA and ACAT inhibitor (2µg/ml) in presence or absence of human HCS (5%, v/v) or  
151 acLDL (50 µg/mL), and Uro C, Uro D, combination of Uro A and Uro B (Uro A+Uro B), and EA at 10  
152 µM. Cholesterol efflux was promoted to human normocholesterolemic serum (NCS) (2%, v/v) for 4 h.  
153 Efflux was expressed as a percentage of <sup>3</sup>H-cholesterol released in the medium relative to the total  
154 amount incorporated by cells.

155

## 156 **2.7. Ultra-high performance liquid chromatography coupled to mass spectrometry (UHPLC- 157 MS<sup>n</sup>) analysis of cell media**



158 Cell culture supernatants were collected at the end of the experiments and analyzed by UHPLC-MS<sup>n</sup> to  
159 determine the stability and peripheral metabolism of the urolithins in cell media. Cell media was  
160 extracted according to Sala et al. [20] and analyzed according to Sala et al. [20]; with minor  
161 modifications. Briefly, samples were analysed using an Accela UHPLC 1250 equipped with a linear  
162 ion trap-mass spectrometer (LTQ XL, Thermo Fisher Scientific Inc., San Jose, CA, USA) fitted with a  
163 heated-electrospray ionization probe (H-ESI-II; Thermo Fisher Scientific Inc.). Separations were  
164 performed using a XSELECTED HSS T3 (50x2.1 mm), 2.5 µm particle size (Waters, Milford, MA,  
165 USA), with an injection volume of 5 µL, column oven temperature of 30°C and elution flow rate of 0.2  
166 mL/min. The initial gradient was 75% of 0.1% aqueous formic acid and 25% acetonitrile (in 0.1%  
167 formic acid), reaching 80% acetonitrile at 6 min. The MS conditions included: capillary temperature of  
168 275 °C and source heater temperature of 250 °C, sheath gas flow of 40 units, auxiliary and sweep gas  
169 of 5 units, source voltage of 3 kV and capillary voltage and tube lens of -5 and -68 V, respectively.  
170 Analyses were carried out using full scan, data-dependent MS<sup>3</sup> scanning from *m/z* 100 to 600, with  
171 collision induced dissociation (CID) equal to 35 (arbitrary units). Pure helium gas was used for CID.  
172 Data processing was performed using Xcalibur software from Thermo Scientific.

173

## 174 **2.8. Statistical analyses**

175 Statistical analysis was assessed using Prism 5.0 (GraphPad Inc., San Diego, CA, USA). Comparisons  
176 among means were performed with one-way ANOVA followed by Newman-Keuls Multiple  
177 Comparison Test. Significant differences were defined as  $p < 0.05$ .

178

## 179 **3. Results**

### 180 **3.1. Effect of ellagitannin metabolites on human endothelial cells**

181 To determine whether urolithins or EA could exert anti-inflammatory effects on the human  
182 endothelium, their impact on the capacity of monocytes to adhere to endothelial cells was tested.  
183 HUVECs were treated with non-cytotoxic concentrations of ellagitannin metabolites (10  $\mu$ M), as  
184 demonstrated by protein content of cellular lysates, which did not show significant differences among  
185 treatments (Table 1). The effect of ellagitannin metabolites on the adhesion of THP-1 monocytes to  
186 HUVEC is showed in Fig. 1. Exposure of HUVEC monolayers to 15 ng/mL of TNF- $\alpha$  for 2 h caused a  
187 4-fold increase in monocyte adhesiveness compared to untreated cells ( $p < 0.001$ ). Treatment with  
188 single urolithins at 10  $\mu$ M for 6 h slightly reduced the adhesion of THP-1 monocytes to HUVECs (Uro  
189 D, -23.2%; Uro C, -24.7%; Uro A, -26.7%; Uro B, -10.7%; not significant). The effect co-treatment  
190 with 10  $\mu$ M of both Uro A and Uro B significantly limited monocyte adhesiveness compared with cells  
191 treated only with TNF- $\alpha$  (-39.8%,  $p < 0.05$ ). However, this anti-adhesive effect was lost at the dose of 5  
192  $\mu$ M each one. A similar effect was observed for EA: the treatment with 10  $\mu$ M significantly reduced  
193 monocyte adhesion to HUVECs (-29.3%,  $p < 0.05$ ), while at 5  $\mu$ M there was no effect.

194 Since the interaction between endothelium and monocytes is dependent on the expression of adhesion  
195 molecules on cell surface [1], the effect of urolithins and EA on VCAM-1 expression in HUVEC was  
196 investigated. Assay optimization involved a preliminary experiment establishing the time-course of  
197 TNF- $\alpha$ -induced sVCAM-1 secretion (data not shown); where 18h stimulation with TNF- $\alpha$  was  
198 established as optimal (i.e., maximum sVCAM-1 protein response). Subsequently, EA, Uro D, and Uro  
199 C were tested as distinct molecules at 10  $\mu$ M, whereas Uro A and Uro B were in mixture, but not  
200 individually, as the combination influenced monocyte adhesion in previous experiments (Fig. 1). In  
201 these experimental conditions (Fig. 2A), incubation with TNF- $\alpha$  caused a significant increase in  
202 sVCAM-1 secretion (250-fold higher than non-treated,  $p < 0.001$ ) that was partially reduced by EA (-  
203 25,6%,  $p < 0.05$ ) and, to a lesser extent, by Uro C (-17,5%,  $p = 0.08$ ).

204 To evaluate whether the anti-adhesive effect of some ellagitannin metabolites was associated with a  
205 reduction in the secretion of inflammatory cytokines, IL-6 was quantified (Fig. 2B). Interestingly, both  
206 Uro C and EA significantly reduced the secretion of IL-6 (-36.2% and -39.7%, respectively;  $p < 0.05$ ).  
207 in comparison to the cells treated with TNF- $\alpha$  alone.

208

### 209 **3.2. Effect of ellagitannin metabolites on macrophage cholesterol accumulation**

210 The role of urolithins in the uptake and efflux of cholesterol from macrophages was investigated as a  
211 key factor in plaque development [18]. Uptake experiments (Fig. 3) involved THP-1 macrophages  
212 treated with non-cytotoxic concentrations of ellagitannin metabolites, as demonstrated by the protein  
213 content of cellular lysates treated at 10  $\mu$ M. No significant differences among the treatments were  
214 observed (Table 2). Incubation of cells with hypercholesterolemic serum (HCS) for 24 h increased  
215 cholesterol content 1.5 fold ( $p < 0.001$ , Fig. 3A). Co-incubation of HCS and Uro C at 10  $\mu$ M and 5  $\mu$ M  
216 reduced cholesterol accumulation to a similar extent (21.3% and 18.6%, respectively;  $p < 0.01$ ),  
217 whereas the lowest dose (1  $\mu$ M) was ineffective. The EA dosage reduced cholesterol accumulation in a  
218 concentration dependent manner: -16.9% ( $p < 0.01$ ), -9.7% ( $p < 0.05$ ), and -4.2% ( $p = 0.45$ ) at 10, 5,  
219 and 1  $\mu$ M, respectively (Fig. 3A). Conversely, the other urolithin treatments had no impact on  
220 cholesterol loading induced by HCS. Alternatively, incubation of THP-1 macrophages with acetylated  
221 LDL (acLDL) increased cell cholesterol content 1.9-fold, with respect to untreated cells ( $p < 0.001$ ),  
222 however, neither Uro C nor EA at 10  $\mu$ M were able to attenuate cholesterol accumulation (Fig. 3B).  
223 Nevertheless, the simultaneous treatment with Uro A and Uro B significantly limited cholesterol  
224 accumulation at the highest concentration, 10  $\mu$ M (-29.3%,  $p < 0.001$ ; Fig. 3B).

225 As cellular cholesterol content is the net result of a bidirectional flux of cholesterol mediated by  
226 lipoproteins [24], the observed effect on cellular cholesterol content could be related either to a  
227 reduction in cholesterol uptake or to a promotion of cholesterol efflux. Therefore, the effect of

228 metabolites on reducing cellular cholesterol content by improving cholesterol efflux was assessed (Fig.  
229 4). THP-1 macrophages were treated for 18 h with cholesterol donors (HCS or acLDL) in order to  
230 induce foam cell formation in either the presence or absence of urolithins or EA. Subsequent incubation  
231 with normocholesterolemic serum (NCS)-containing medium promoted cholesterol efflux (3.3-fold  
232 increase) in HCS pre-treated cells ( $p < 0.001$ , Fig. 4A) and 6-fold increase in acLDL pre-treated cells ( $p$   
233  $< 0.001$ , Fig. 4B), compared to cells incubated with serum-free medium during the efflux period (Basal  
234 treatment). However, none of the treatments with urolithins significantly affected cholesterol efflux  
235 (Fig. 4).

236 As all mammalian cells synthesize cholesterol [25], in addition to uptake and efflux experiments, the  
237 effect of the tested metabolites was also evaluated on basal cellular cholesterol levels (i.e., content  
238 before the addition of cholesterol). There was no effect of urolithins or EA at 10  $\mu$ M on cellular  
239 cholesterol content (Table 3).

240

### 241 **3.3. *In vitro* metabolism of urolithins in cell cultures**

242 The stability of EA and urolithins and appearance of newly-formed metabolites in cell media was  
243 evaluated at the beginning and end of each experiment. In order to establish the role of cell metabolism  
244 on the production of ellagitannin-derived metabolites, culture media without cells were used as controls  
245 to assess chemical degradation. Different metabolic reactions, including (de)hydroxylation, conjugation  
246 with methyl, glucuronide, sulfate, cysteine, and glutathione moieties, and formation of quinones, were  
247 monitored. All the tested compounds underwent extensive biotransformations in HUVECs and THP-1-  
248 derived macrophages (Fig. 5) and fourteen newly formed metabolites were identified (Table 4). The  
249 newly-formed metabolites were detected in both cell types and the metabolic reactions were limited to  
250 sulfation and methylation (Table 4). These metabolites were not quantified due to the lack of

251 commercially available standard compounds, but peak area values were taken into account to establish  
252 comparisons among experiments.

253 In HUVECs, the stability and metabolism of EA and urolithins were studied at two different incubation  
254 times, corresponding with the study of monocyte adhesion to endothelial cells (6 h) and the secretion of  
255 sVCAM-1 and IL-6 (18 h). The molecules were stable under the experimental conditions (cell free  
256 incubations), apart from Uro D, which shown a reduced recovery even immediately after addition to  
257 the culture media. When incubated with cells, MS peak areas of the metabolites increased  
258 proportionately with incubation time. For instance, dimethyl-*O*-Uro C increased from lower limit of  
259 detection at 6 h to prominent areas after 18 h of incubation. When more than one hydroxy group was  
260 available on the molecular scaffold, isomers were often detected reflecting the number of hydroxyls  
261 present. The abundance of sulfated isomers of both Uro A and Uro C were constant, while one isomer  
262 of methyl-*O*-Uro C was produced in much higher quantities than the other. The dimethyl derivative of  
263 Uro D was predominant with respect to the monomethylated form at both 6 and 18 h, whereas the  
264 opposite occurred for EA, as the monomethylated form was higher than the dimethyl at 6 h, but not at  
265 18 h.

266 In the case of THP-1-derived macrophages, a single time point was studied (24 h), but the effect of two  
267 different promoters of cholesterol accumulation (HCS and acLDL) was assessed. Again, Uro D was the  
268 most unstable molecule in cell free incubations. The production of all metabolites was elevated  
269 (between 1- and 16-fold) in the cultures containing acLDL, with respect to the HCS media (based on  
270 peak areas).

271

#### 272 **4. Discussion**

273 The cardioprotective effect of ellagitannin-enriched diets has been related to the modulation of  
274 endothelial function as well as to hypocholesterolaemic effects [11, 12]. However, some of the *in vitro*

275 studies feeding ellagitannin-rich extracts have revealed that ellagitannins do not make it into the  
276 systemic circulation and therefore never reach vascular cells [7-9]. In this study, the effect of  
277 physiologically relevant ellagitannin metabolites Uro A, Uro B, Uro C, and Uro D, as well as their  
278 precursor EA, were investigated at low  $\mu\text{M}$  concentrations (5–10  $\mu\text{M}$ ) on two key events in the  
279 formation of the atherosclerotic plaques: i) endothelium activation and resulting monocyte recruitment  
280 and ii) cholesterol transport and foam cells formation. Among the tested metabolites, Uro C was the  
281 most effective, with a bioactivity similar to that of EA, while Uro A and Uro B were active in  
282 combination at 10  $\mu\text{M}$  (20  $\mu\text{M}$  cumulative concentration).

283 Endothelium activation that promotes the recruitment of circulating monocytes is one of the earliest  
284 events in the formation of atherosclerotic plaques [1]. The pro-inflammatory cytokine TNF- $\alpha$ , mostly  
285 produced by lymphocytes and activated monocytes/macrophages, has been implicated in the  
286 pathogenesis of a number of cardiovascular diseases, including atherosclerosis. Its production is  
287 directly linked to the activation of the endothelium, where it induces the expression of adhesion  
288 molecules, including VCAM-1, that are essential for monocyte recruitment [26]. Therefore, inhibition  
289 of its activity and, as a consequence, of endothelium activation may have an important role in the  
290 inhibition of plaque formation. Our study demonstrated that exposure of HUVECs to ellagitannin  
291 metabolites could modestly reduce the TNF- $\alpha$ -stimulated adhesion of monocyte to HUVECs (Fig. 1).  
292 However, this reduction reached significance only for EA and for the co-treatment of Uro A and Uro B  
293 at 10  $\mu\text{M}$ .

294 Some effects of ellagitannin metabolites Uro A, Uro B, and their glucuronide forms on endothelial  
295 function were previously described by Giménez-Bastida et al. [17], who demonstrated that only Uro  
296 A-glucuronide has a slight effect on monocyte adhesion to endothelial cells. Their results appeared to  
297 be in accordance with the results reported herein, since neither Uro A nor Uro B as aglycone forms  
298 could significantly affect monocyte adhesion to endothelial cells at higher concentrations (18.5  $\mu\text{M}$  and

299 14.9  $\mu\text{M}$ , respectively) [17] than the ones used in the present study. As Uro A and Uro B are the two  
300 EA-derived metabolites absorbed at higher concentrations and can co-occur in some urolithin  
301 phenotypes [15, 27, 28], they were tested together. Their association was able to significantly inhibit  
302 monocyte adhesion to HUVEC (Fig. 1). Therefore, it could be hypothesized that Uro A and Uro B may  
303 exert a synergic effect on the inhibition of adhesion, as they were not active in the reduction of  
304 monocyte adhesiveness as single molecules in the present experiments, or even at higher reported  
305 concentrations [17]. However, whether the increased effect is depended on the total cumulative  
306 concentration (20  $\mu\text{M}$ ) of two similar molecules (10  $\mu\text{M}$  Uro A+10  $\mu\text{M}$  Uro B) is something that  
307 cannot be ruled out.

308 The proinflammatory response characterized by TNF- $\alpha$ -stimulated secretion of sVCAM-1 and IL-6  
309 (Fig. 2) was counteracted by EA and Uro C at low  $\mu\text{M}$  concentrations (10  $\mu\text{M}$ ). This effect is crucial to  
310 demonstrate the atheroprotective activities of ellagitannin metabolites, since it is well established that,  
311 within the different adhesion molecules, VCAM-1 plays a major role in the early stages of  
312 atherosclerosis [29] and that sVCAM-1 secretion directly correlates with the expression of its non-  
313 soluble form on the cell surface [30, 31]. The atheroprotective effects of EA have also been confirmed  
314 in previous studies demonstrating that EA reduces endothelial activation by inhibiting monocyte  
315 adhesion to endothelial cells and the expression of ICAM-1, VCAM-1, E-selectin, and IL-6 [10, 32,  
316 33]. The inhibition of ROS production and modulation of NF- $\kappa\text{B}$  activity have also be related to the  
317 anti-inflammatory effects of EA [33]. Similarly, Piwowarski et al. [16] showed that the anti-  
318 inflammatory activity of urolithins A, B, and C in RAW 264.7 murine macrophages was associated  
319 with the inhibition of NF- $\kappa\text{B}$  translocation into the nucleus.

320 Interestingly, the same molecules exerting atheroprotective effects on endothelium displayed an  
321 activity in another critical step for atherosclerotic plaque formation; specifically macrophage  
322 cholesterol accumulation. EA, Uro C, and the mixture of Uro A and Uro B reduced net cholesterol

323 content in human macrophages exposed to cholesterol sources, such as acLDL or HCS (Fig. 3). It was  
324 observed that these treatments caused an impairment of cholesterol uptake, without affecting  
325 cholesterol efflux (Fig. 4), despite previous data having demonstrated an effect of EA in ABCA1  
326 expression and ABCA1 dependent-cholesterol efflux [34]. The same study reported that EA is able to  
327 block the uptake of cholesterol by macrophages exposed to oxLDL [34], which enters macrophages  
328 through scavenger receptors [1]. Nevertheless, in the present model, EA at 10  $\mu$ M (Fig. 3B) did not  
329 inhibit cholesterol accumulation induced by exposition to acLDL, which represents a well-accepted  
330 experimental model of oxLDL which exploits the same scavenger receptors. In these conditions, only  
331 the association of Uro A and Uro B at 10  $\mu$ M significantly reduced cholesterol accumulation.  
332 Conversely, the capacity of EA (5-10  $\mu$ M) to block macrophage uptake of cholesterol derived from  
333 HCS, that is rich in non-modified LDL (native LDL) was also apparent (Fig. 3A). For the first time, it  
334 has been demonstrated that not only EA but also its metabolite Uro C is able to significantly reduce  
335 cholesterol accumulation in macrophages exposed to HCS at low  $\mu$ M concentration (5-10  $\mu$ M). Uptake  
336 of native LDL by macrophages occurs through the LDL receptor (LDLr) or through macropinocytosis,  
337 which has been shown to induce foam cell formation and has been proposed as a new target to reduce  
338 cholesterol accumulation in atherosclerotic plaque [2, 35]. The possibility that they act by a reduction  
339 of LDLr expression or activity is unlikely because LDLr undergoes down-regulation in response to  
340 macrophage cholesterol loading [36]. Thus, it can be speculated that the mechanism underlying EA and  
341 Uro C activity is an impairment in micropinocytosis. Moreover, since these ellagitannin-derived  
342 molecules did not affect cholesterol accumulation and efflux induced by acLDL, it can be ruled out that  
343 the activity is through the scavenger receptors.

344 As every mammalian cell synthesizes cholesterol [25], changes in cholesterol content can also be  
345 dependent on cell specific modulation of cholesterol synthesis. An impact of urolithins on cholesterol  
346 synthesis can be excluded, as exposure to urolithins without the presence of a source of cholesterol did



347 not affect cell cholesterol content. However, EA caused a slightly but not statistically significant  
348 reduction in cholesterol content before addition of serum and, thus, we cannot totally exclude a  
349 possible effect of EA in cholesterol synthesis since an inhibitory activity on squalene epoxidase, a rate-  
350 limiting enzyme of cholesterol biosynthesis, has been previously described [37].

351 A point worth mentioning is the role the hydroxylation pattern of urolithins may have on their  
352 physiological activity [17, 38-40]. It was not possible to associate the extent of urolithin and EA  
353 hydroxylation with the anti-atherosclerotic effects observed in the present study, and this may be partly  
354 due to the extensive biotransformations undergone by the tested molecules in our cell models (Table 4,  
355 Fig. 5). It has been demonstrated that different phase II conjugates of the same urolithin may exert  
356 different biological effects [39, 41]. On the other hand, it should be noted that the metabolism of  
357 urolithins and EA by human endothelial cells and macrophages was limited to methylation and  
358 sulfation, while glucuronidation, which had previously been pointed out as one of the main reaction  
359 occurring *in vitro* and *in vivo* [20, 28], was not observed. Finally, the marked instability of Uro D in  
360 different cell cultures is something that should be better clarified in the near future, even if its  
361 bioactivity may not be affected [42].

362

## 363 **5. Conclusions**

364 Taken together, these results indicate that some urolithins, singularly or as mixtures, may impact key  
365 process in the development and progression of atherosclerosis. Some cellular mechanisms behind the  
366 cardioprotective features of these ellagitannin-derived metabolites have been hypothesized, but further  
367 work is needed targeting the molecular pathways involved. At the same time, *in vivo* studies aimed at  
368 unravelling the contribution of ellagitannin-containing foods to cardiovascular prevention and, in  
369 particular, to the atherosclerotic scenario, should consider clinical endpoints related to not only the anti-  
370 inflammatory response but also the lipid transport and metabolism.

371 From a methodological point of view, the drastic biotransformations of phenolic metabolites taking  
372 place in cell cultures should be carefully taken into account to fully elucidate the real compounds  
373 exerting the bioactivity observed. This would also help in understanding the impact of peripheral  
374 metabolism of plant-derived bioactives on human health.

375

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380

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487 **Figure captions**

488 **Figure 1:** Effect of EA and urolithins (5-10  $\mu\text{M}$ ) on monocyte adhesion to endothelial cells. Mean  
489  $(n=9)\pm\text{SD}$ .  $\text{\}\text{\}\text{\}\text{\} = } p < 0.001$  vs Control;  $\text{\}\text{\} = } p < 0.05$  vs  $\text{TNF}\alpha$ .

490 **Figure 2:** Effect of EA and urolithins (10  $\mu\text{M}$ ) on endothelial cells secretion of sVCAM-1(A) and IL-6  
491 (B). Mean  $(n=3)\pm\text{SD}$ .  $\text{\}\text{\}\text{\}\text{\} = } p < 0.001$  vs Control;  $\text{\}\text{\} = } p < 0.05$  vs  $\text{TNF}\alpha$ ,  $\text{\}\text{\}\text{\} = } p < 0.01$  vs  $\text{TNF}\alpha$ .

492 **Figure 3:** Effect of EA and urolithins (1-10  $\mu\text{M}$ ) on macrophage cholesterol loading. Mean  $(n=9)\pm\text{SD}$ .  
493 (A), HCS: Hypercholesterolemic serum; HCS cell cholesterol content ( $\mu\text{g}$  cholesterol/mg protein) =  
494  $20.16 \pm 1.54$ .  $\text{\}\text{\}\text{\}\text{\} = } p < 0.001$  vs Control,  $\text{\}\text{\} = } p < 0.05$  vs HCS,  $\text{\}\text{\}\text{\} = } p < 0.01$  vs HCS. (B), acLDL:  
495 acetylated LDL; acLDL cell cholesterol content ( $\mu\text{g}$  cholesterol/mg protein) =  $27.87 \pm 0.71$ .  $\text{\}\text{\}\text{\}\text{\} = } p <$   
496  $0.001$  vs Control,  $\text{\}\text{\} = } p < 0.05$  vs acLDL  $\text{\}\text{\}\text{\}\text{\} = } p < 0.001$  vs acLDL.

497 **Figure 4:** Effect of EA and urolithins (10  $\mu\text{M}$ ) on macrophage cholesterol efflux. HCS:  
498 Hypercholesterolemic serum; acLDL: acetylated LDL; NCS: normocholesterolemic serum. Mean  
499  $(n=9)\pm\text{SD}$ . No significant differences were detected among treatments (ANOVA  $p > 0.05$ ).

500 **Figure 5:** Metabolic biotransformations occurring in endothelial cells and macrophages

501

502 **Table 1.-** Effect of EA and urolithins (10 $\mu$ M) on macrophage cholesterol content ( $\mu$ g cholesterol/mg  
503 protein) (10  $\mu$ M).

<b>Treatment</b>	<b>average <math>\pm</math> SD</b>
<b>Control</b>	12.94 $\pm$ 0.17
<b>EA</b>	11.38 $\pm$ 0.87
<b>Uro D</b>	13.15 $\pm$ 0.97
<b>Uro C</b>	15.09 $\pm$ 1.64
<b>Uro A</b>	12.96 $\pm$ 0.78
<b>Uro B</b>	13.42 $\pm$ 0.84
<b>Uro A+Uro B</b>	14.75 $\pm$ 1.08

504  
505 Mean (n=3) $\pm$ SD. No significant differences were  
506 detected among treatments (ANOVA  $p > 0.05$ ).  
507



508 **Table 2.- Ellagic acid and urolithin metabolites detected in endothelial cells and/or macrophages.**

Compound	Derived Metabolite	Retention time (min)	[M-H] <sup>-</sup> (m/z)	MS <sup>2</sup> ion fragments (m/z)	MS <sup>3</sup> ion fragments (m/z)	
<i>Urolithin B</i>	Uro B	4.61	211	167, 182		
	Uro B-sulfate	3.75	291	211	167	
<i>Urolithin A</i>	Uro A	3.28	227	183, 159, 199		
	Uro A-sulfate isomer 1	2.22	307	227	183	
	Uro A-sulfate isomer 2	2.50	307	227	183	
<i>Urolithin C</i>	Uro C	2.35	243	215, 199, 226		
	Uro C-sulfate isomer 1	2.13	323	243	215	
	Uro C-sulfate isomer 2	2.70	323	243	215	
	Methyl- <i>O</i> -Uro C isomer 1	3.39	257	242, 211, 239		
	Methyl- <i>O</i> -Uro C isomer 2	3.58	257	242, 211		
	Dimethyl- <i>O</i> -Uro C	4.43	271	256, 257	187	
	Methyl- <i>O</i> -Uro C-sulfate isomer 1	2.56	337	257	214	
	Methyl- <i>O</i> -Uro C-sulfate isomer 2	3.03	337	257	214	
	<i>Urolithin D</i>	Uro D	1.89	259	241, 213, 231	
		Methyl- <i>O</i> -Uro D	3.19	273	258	
Dimethyl- <i>O</i> -Uro D		3.68	287	272, 241, 219		
<i>Ellagic acid</i>	Ellagic acid	1.87	301	257, 229		
	Methyl- <i>O</i> -ellagic acid	2.94	315	300		
	Dimethyl- <i>O</i> -ellagic acid	3.98	329	314, 215		

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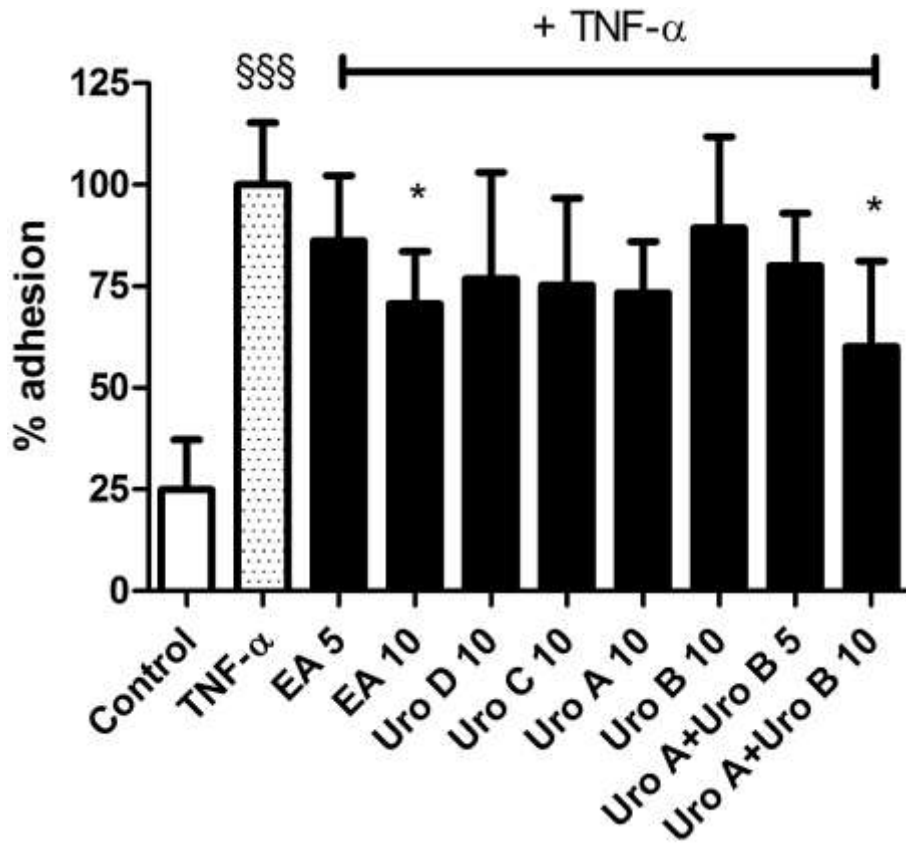
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523 Figure 1



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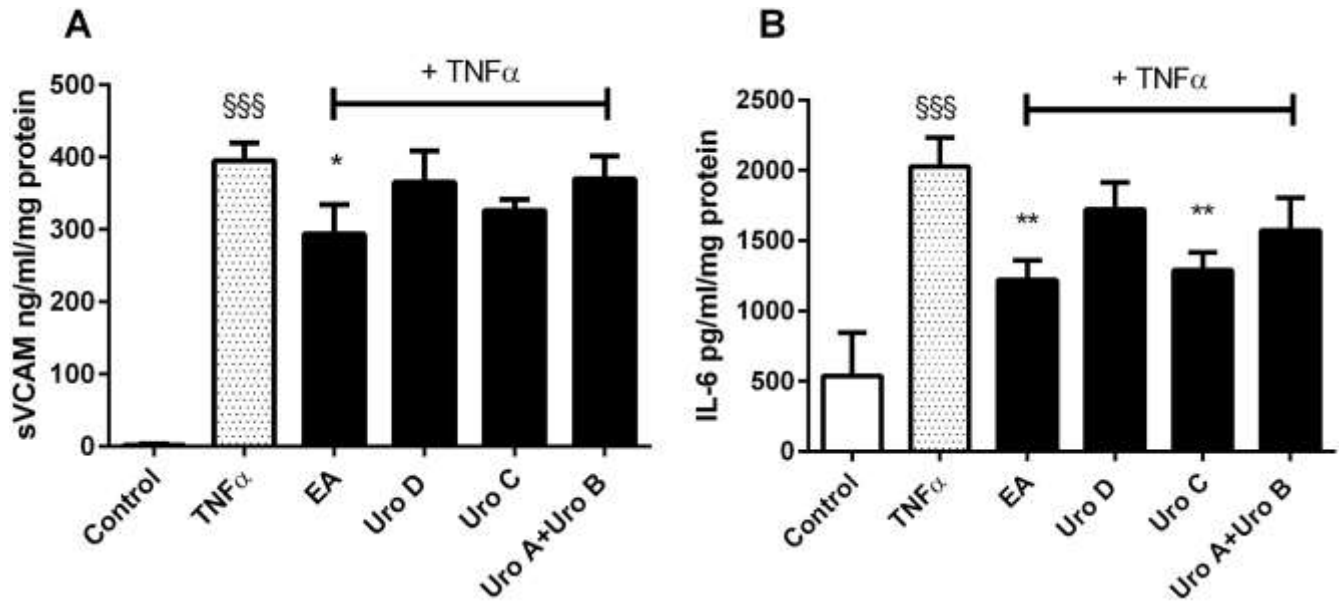
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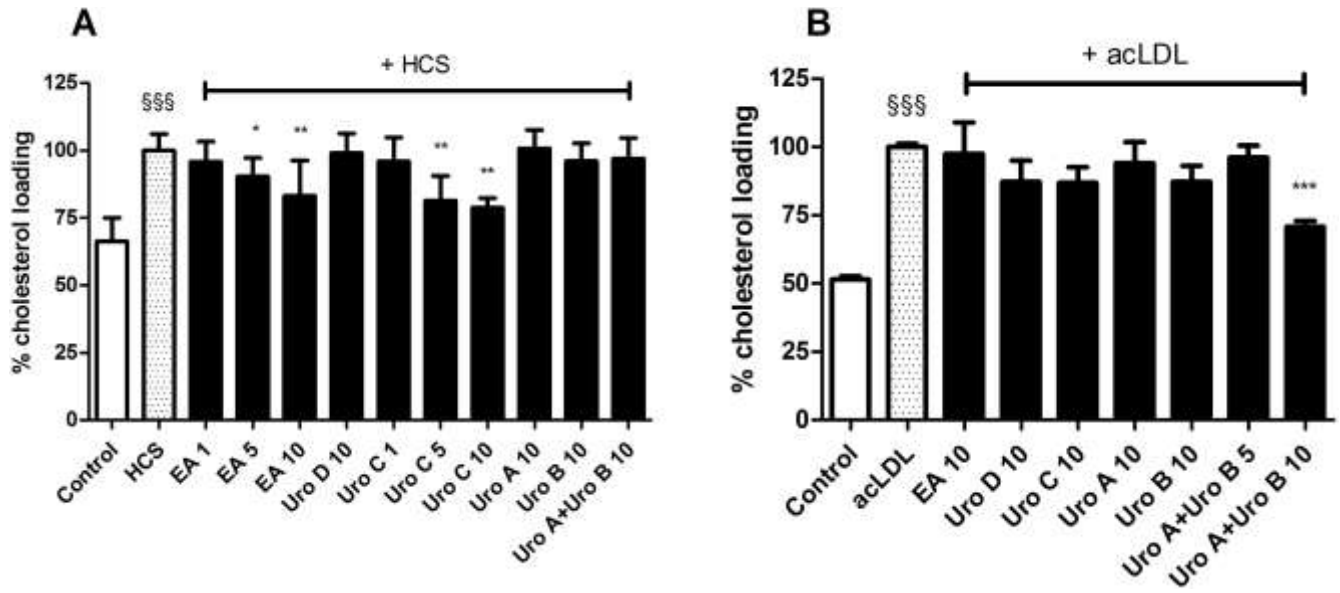
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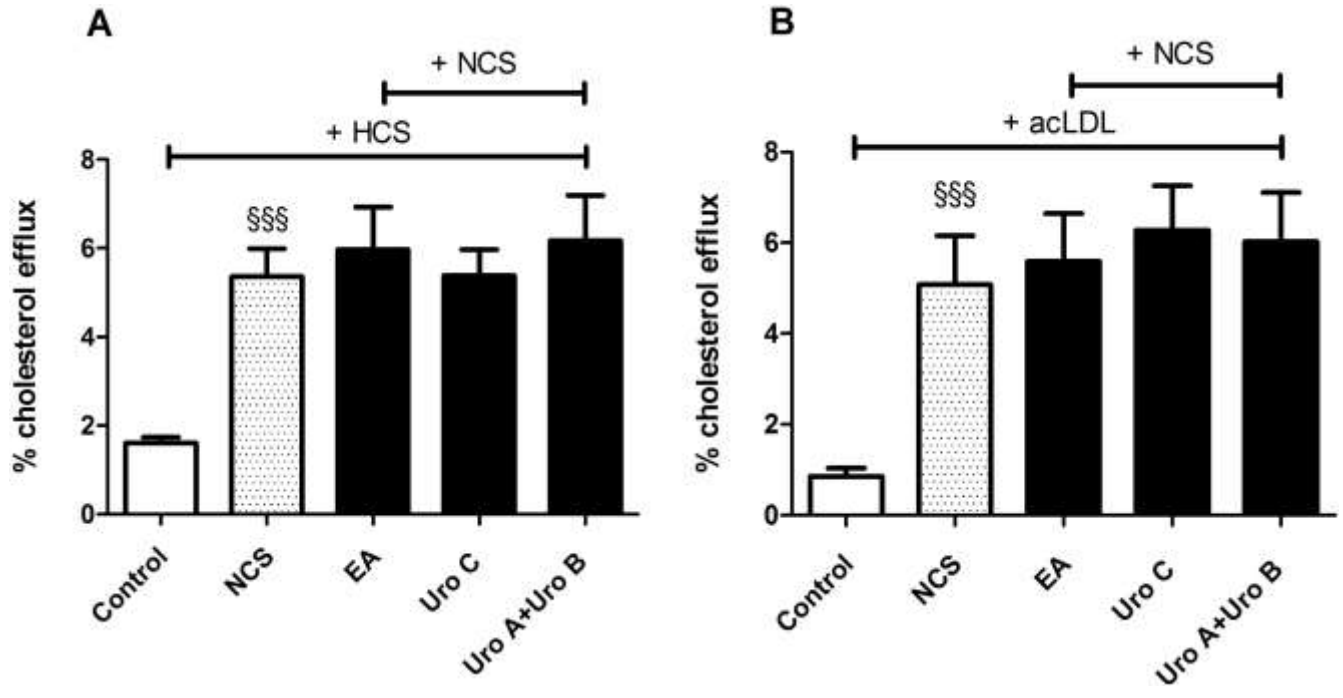
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573 Figure 4



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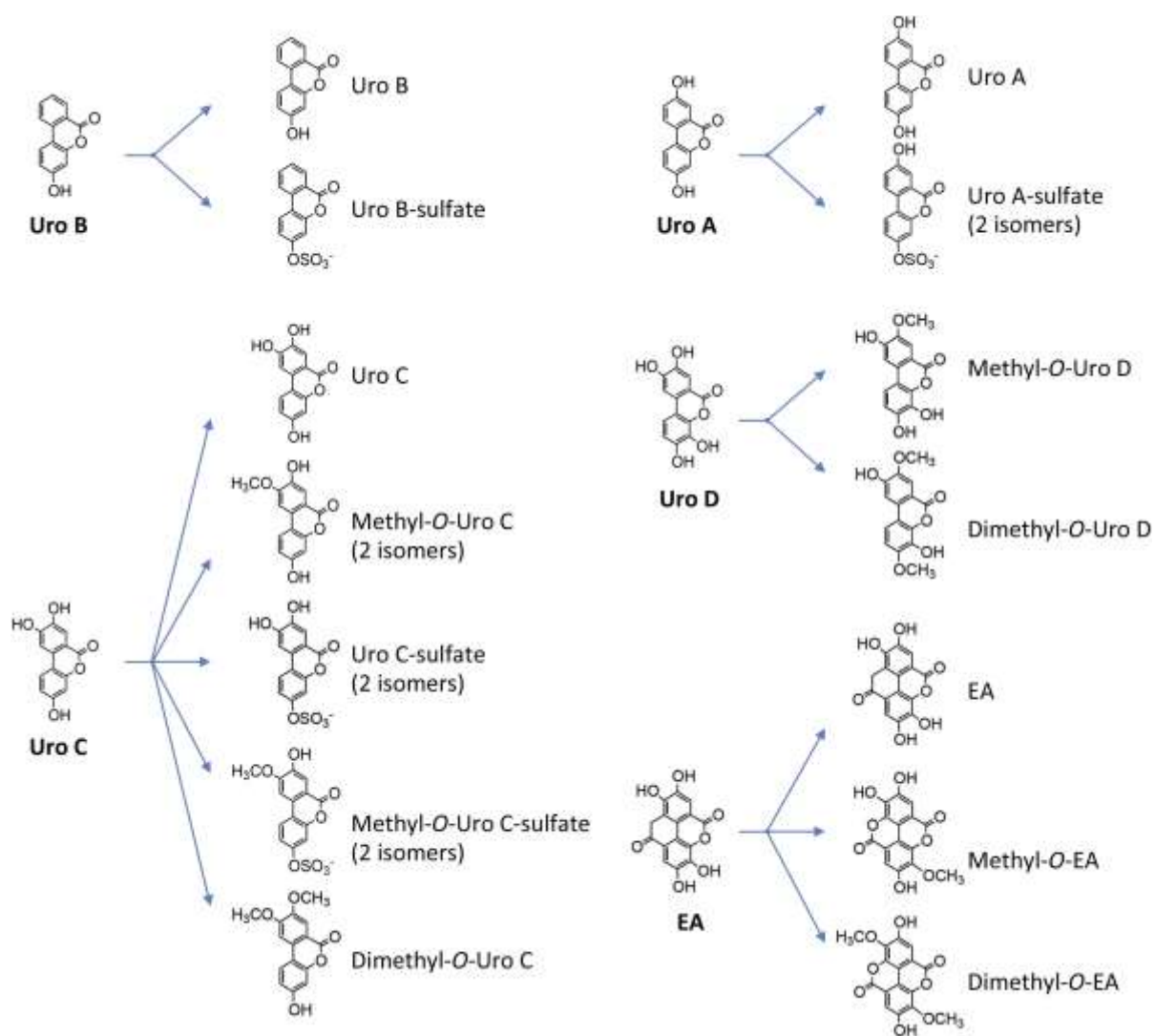
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Figure 5

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