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1 **Potential anti-inflammatory, anti-adhesive, anti/estrogenic, and angiotensin-**
2 **converting enzyme inhibitory activities of anthocyanins and their gut metabolites**

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21 **ABSTRACT**

22 Epidemiological studies have indicated a positive association between the intake of foods
23 rich in anthocyanins and the protection against cardiovascular diseases. Some authors have
24 shown that anthocyanins are degraded by the gut microflora giving rise to the formation of
25 other breakdown metabolites, which could also contribute to anthocyanin health effects.
26 The objective of this study was to evaluate the effects of anthocyanins and their breakdown
27 metabolites, protocatechuic, syringic, gallic, and vanillic acids, on different parameters
28 involved in atherosclerosis, including inflammation, cell adhesion, chemotaxis, endothelial
29 function, estrogenic/antiestrogenic activity and angiotensin-converting enzyme (ACE)
30 inhibitory activity. From the assayed metabolites, only protocatechuic acid exhibited a
31 slight inhibitory effect on NO production and TNF-α secretion in LPS-INF-γ-induced
32 macrophages. Gallic acid caused a decrease in the secretion of MCP-1, ICAM-1 and
33 VCAM-1 in endothelial cells. All anthocyanins showed an ACE inhibitory activity.
34 Delphinidin-3-glucoside, pelargonidin-3-glucoside and gallic acid showed affinity for ERβ
35 and pelargonidin and peonidin-3-glucosides for ERα. The current data suggest that
36 anthocyanins and their breakdown metabolites may partly provide a protective effect
37 against atherosclerosis that is multi-causal and involves different biochemical pathways.
38 However, the concentrations of anthocyanins and their metabolites, as used in the present
39 cell culture and in vitro assays mediating anti-inflammatory, anti-adhesive, anti- anti-
40 estrogenic, and angiotensin-converting enyzme inhibitory activities, were often manifold
41 higher than those physiologically achievable.

42

43 **INTRODUCTION**

44 Polyphenolic substances, such as anthocyanins, exert a great variety of physiological
45 activities responsible for the health effects attributed to some foods, including a reduced
46 risk of cardiovascular diseases. This is partly due to their anti-inflammatory properties
47 (González-Gallego 2010; Landberg 2011; Rotelli 2003), antioxidant and free radical
48 scavenging activities (García-Alonso 2009; Gray 1999; Kahkonen 2003; Matsumoto 2002;
49 Tsuda 1994; Wang 1997), peroxidation inhibition (Tsuda 1996) and
50 estrogenic/antiestrogenic activity (Cassidy 2003). It has been largely proven that the
51 beneficial potential of polyphenols as part of a healthy diet can not be only explained by
52 their antioxidant characteristics (Virgili 2008).

53 One of the biological mechanisms by which flavonoids exhibit anti-inflammatory effects
54 appears to be associated with the inhibition of nitric oxide (NO) production (Vallance
55 2002). A critical step in both inflammation and atherosclerosis is the adhesion of circulating
56 monocytes to vascular endothelial cells which involves vascular cell adhesion molecule-1
57 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1). Studies *in vitro* suggest that
58 flavonoids participate in the prevention and attenuation of inflammatory diseases by
59 decreasing ICAM-1 and VCAM-1 levels (Kwon 2005; Lotito 2006). In addition, dietary
60 anthocyanins and hydroxycinnamic acids have been reported to reduce TNF-α-induced up-
61 regulation of various inflammatory mediators such as ICAM-1 or MCP-1. Therefore, the
62 ability of polyphenols to mediate inflammatory processes is likely to contribute to their
63 antiatherogenic properties.

64 The effect of flavonoids on the arterial wall due to their estrogenic activity is well known.
65 Flavonoids reduce the risk of cardiovascular diseases (Kris-Etherton 2002), decrease serum
66 cholesterol, low-density lipoproteins (LDL) and triglyceride levels (Ricketts 2005), prevent

osteoporosis (Dang 2005) and improve menopausal symptoms (McCann 2005). Notably, work has already shown that the estrogenic properties of wine flavonoids inlcuding anthocyanins are due, in part, to their ability to bind the estrogen receptor (Chalopin 2010; Schmitt 1996).

In addition, flavonoids have been suggested to decrease cardiovascular risk by reducing levels of angiotensin II, a well-known proinflammatory mediator (Naruszewicz 2007). It has already been reported that some anthocyanin containing foods as well as delphinidin, inhibit ACE activity (Actis-Goretta 2006; Lacaille-Dubois 2001; Persson 2009).

Anthocyanins intake in humans has been estimated to be between 3 and 215 mg/day (Chun 2007; Frankel 1995; Pérez-Jimenez 2011; Wu 2006). Most studies have shown very low bioavailability of anthocyanins based only on the measurement in plasma or urine of the original anthocyanins and their conjugated metabolites, glucuronidated and sulphated anthocyanins (Manach 2005). More recently, it has been established that the intestinal microflora plays a key role in the metabolism of anthocyanins. After ingestion, anthocyanins can be hydrolysed by intestinal glucosidases, and the resulting aglycones are further metabolised in the large intestine to other breakdown metabolites such as protocatechuic, gallic, syringic and vanillic acids (Avila 2009; Forester 2008, Keppler 2005; Vitaglione 2007). Moreover gallic acid has been determined in plasma after its ingestion at levels as high as 1.8 μmo/L in its original form and at 2.2 μmo/L as its derivative 4-O-methylgallic acid (Shahrzad 1998, 2001). Therefore, metabolites produced by the intestinal microflora could account partly for the health benefits associated with anthocyanin consumption in humans.

The aim of this study was to further elucidate the potential mechanisms by which anthocyanins and their metabolites reduce the initial stages of atherosclerosis. Specifically,

91 we have studied the effect of anthocyanins and their metabolites on 1) NO production and
92 TNF-α secretion in macrophages, and 2) ICAM-1, VCAM-1 and MCP-1 secretion in
93 endothelial cells. The ACE inhibitory activity of anthocyanins and their metabolites was
94 also measured. Finally, their ERα and ERβ binding ability was measured. Docking studies
95 helped to rationalize selectivity on ERs.
96
97 **MATERIALS AND METHODS**
98
99 **Chemicals**
100 Cyanidin-3-O-glucoside, pelargonidin-3-O-glucoside, malvidin-3-O-glucoside,
101 delphinidin-3-O-glucoside and peonidin-3-O-glucoside were purchased from Extrasynthese
102 (Lyon, France). Acetate buffer saline, neutral red, griess reagent, crystal violet, LPS
103 (lipopolisaccharide), human recombinant TNF-α, mouse recombinant IFN-γ, 17-β-
104 estradiol, PBS, Tween-20, BSA, ACE (peptidyl-dipeptidase A, EC 3.4.15.1) and the
105 phenolic acids: gallic, syringic, protocatechuic, vanillic, sinapic, homogentisic, 4-
106 hidroxybenzoic, phloroglucinol, 3-(2´,5´-dimethoxybenzoil) propionic (DMB propionic),
107 coumaric and caffeic acids were purchased from Sigma-Aldrich Química S.A. (Madrid,
108 Spain). Ethanol 99%, glacial acetic acid and dimethyl sulfoxide (DMSO) were obtained
109 from Panreac (Barcelona, Spain). Sodium dodecyl sulphate (SDS) was acquired from
110 Fisher (Madrid, Spain). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum
111 (FBS), penicillin/streptomycin, trypsin, L-glutamine and nonessential amino acids were
112 purchased from Lonza (Barcelona, Spain). Estradiol [2,4,6,7,16,17-^{3}H(N)] and scintillation
113 counting liquid (Optifase HiSafe2) were obtained from Perkin-Elmer (Salem, MA).
114 Estrogen receptors α and β (human recombinant produced in insect cells) were purchased

115 from Invitrogen (Barcelona, Spain).

116

117 **Macrophages**

118 RAW 264.7 cells, a murine monocyte macrophage cell line, were maintained at 37 °C in 5
119 % CO_2 according to standard protocols (Rimbach 2000). The medium consisted of DMEM
120 with 4.5 g/L glucose and L-glutamine supplemented with 10% FBS and 1%
121 penicillin/streptomycin (5000 U/mL). For experiments, cells were harvested with trypsin-
122 EDTA and macrophages were plated in 24-well plates at a density of $5x10^4$ in 0.5 mL of
123 medium for the cytotoxicity test or in 6-well plates at a density of $2x10^5$ in 2 mL of
124 medium, for nitrite and TNF-α measurement. Finally, cells were cultured for 72 h until they
125 reached 80% confluence. Cells in 6-well plates were treated with gallic, vanillic,
126 protocatechuic and syringic acids in a range of concentrations between 0.01-500 μM or
127 DMSO (< 0.1%) as follows; four different treatments were performed. (A) Cells were pre-
128 treated for 3 h with the different compounds, washed twice with PBS and then stimulated
129 with 1 μg/ml LPS for 24 h. (B) Cells were pre-treated with the different compounds for 3 h,
130 washed twice with PBS and then stimulated with 1μg/mL LPS plus 1000 U/mL IFN-γ for
131 24 h. (C) Cells were co-incubated with the different compounds at 200 μM together with 1
132 μg/ml LPS for 24 h. (D) Cells were co-incubated with the different compounds at 200 μM
133 and with 1 μg/ml LPS plus 1000 U/ml IFN-γ simultaneously for 24 h. For all these
134 experiments, control cells were grown under identical conditions but were not exposed to
135 the test compound or LPS/IFN-γ. For the cytotoxic assay, cells were treated with all
136 compounds at the maximal concentration used in the NO production assay.

137

138 **Endothelial cells**

139 EA.hy 926 cells, a cell line derived from human umbilical vein endothelial cells (HUVEC),
140 were a generous gift from Prof. C-J. S. Edgell, University of North Carolina at Chapel Hill.
141 EA·hy 926 cells were generated by fusion of human umbilical vein endothelial cells
142 (HUVEC) with the human lung carcinoma cell line A549 and have been extensively used
143 as a cell model for endothelial function (Fuchs 2005) Cells were grown in DMEM medium
144 containing 4.5 g/L glucose and L-glutamine and supplemented with 10% FBS and 1%
145 penicillin/streptomycin (5000 U/mL). Cells were harvested with trypsin-EDTA and seeded
146 in 0.5 mL of medium in 24-well plates or 2 mL of medium in 6-well plates and cultured
147 until they reached 80% confluence. After pre-treatment in 6-well plates with 10 and 100
148 μM of gallic, vanillic, protocatechuic and syringic acids for 16 h, cells were stimulated with
149 10 ng/mL TNF-α for 6 h.
150

151 **Estrogen receptor (ER) competitive binding assay**

152 The binding activity of anthocyanins and acids to human ER was determined using a
153 radioactivity assay based on the ability of the different compounds to compete with 3H-
154 labeled estradiol for the estrogen receptor. For the ERβ- and ERα-binding assay, the
155 corresponding pure estrogen receptor was used at 2.4 nM together with 5nM tritium-
156 labelled estradiol ([2,4,6,7,16,17-3H]estradiol). Unlabeled estradiol, anthocyanins and acids
157 were prepared in DMSO (<0.5%) and diluted in PBS-T (PBS + 0.15% Tween20) including
158 receptors and Estradiol-H^3*. Briefly, the same volume of each compound, Estradiol-H^3*
159 and receptor were mixed in a final volume of 150 μL, thus the different compounds were
160 tested at a concentration ranging from 1 to 200 μM. The mixture was incubated for 4 h at
161 23 ºC to allow receptor binding. Afterwards, 50 μL of charcoal (charcoal 0.1 g/ml and BSA
162 0.02 g/mL) was added and the samples kept on ice for 15 minutes before being centrifuged

163 at 6000 x *g* for 5 min to remove the non-bound Estradiol-H^{3}*. An aliquot of this
164 supernatant (150 µL) was added to 4 mL of scintillation counting liquid. The bound [3H]-
165 estradiol was measured in a WinSpectral 1414 Liquid Scintillation Counter (Beckman, LS
166 6500). Three independent experiments containing three replicates were performed for each
167 compound tested. Results are expressed as the percentage of specific binding of [3H]
168 estradiol to ER versus log of competitor concentration. IC_{50} values represent the
169 concentration of test compound required to displace 50% [3H] estradiol from the receptor.
170 IC_{50} values were determined by nonlinear regression fitting of experimental data to a
171 sigmoid equation.
172

173 **Docking studies**

174 Geometries of compounds X-Y were first optimized using the *ab initio* quantum chemistry
175 program Gaussian 03 (2004) and the B3LYP/3-21G* basis set. As macromolecules, the X-
176 ray structures of estrogen receptor complexes with genistein were chosen (PDB codes: 1x7r
177 for ERα and 1x7j for ERβ). Crystallographic water molecule close to Arg394 (ERβ
178 Arg346) and Glu353 (ERβ Glu305) were kept as they were considered to be part of the
179 binding site. Different conformers of the ligands were docked using the Lamarckian genetic
180 algorithm implemented in AutoDock 3.1 (Morris 1998) by randomly changing the torsion
181 angles and overall orientation of the molecule. A volume for exploration was defined in the
182 shape of a three-dimensional grid ($80 \times 80 \times 90$ Å^{3}) with a spacing of 0.375 Å that enclosed
183 the binding site, and included the residues that are known to be crucial for activity. At each
184 grid point, the receptor's atomic affinity potentials for carbon, aromatic carbon, oxygen,
185 nitrogen, sulphur, and hydrogen atoms were precalculated for rapid intra- and
186 intermolecular energy evaluation of the docking solutions for each ligand. The original

187 Lennard-Jonnes and hydrogen-bonding potentials provided by the program were used. The
188 parameters for the docking using the LGA were identical for all docking jobs. After
189 docking, the 100 solutions were clustered in groups with root mean square deviations less
190 than 1.0 Å. The clusters were ranked by the lowest energy representative of each cluster.
191

192 **Cell viability**

193 The uptake of neutral red dye was used to assess cell viability as described previously
194 (Valacchi 2001). Macrophages and Ea.hy 926 cells were pre-treated in 24-well plates with
195 the different test compounds for 24 h. After incubation, the culture medium was removed
196 and replaced with fresh medium containing 50 µg/mL of neutral red. Following incubation
197 for 2 h at 37 ºC, the medium was removed and the cells extracted using a solution
198 comprising 50:49:1 (v/v/v) ethanol, water, and glacial acetic acid. Absorbance at 540 nm
199 was recorded using a microplate reader (Power Wave XS, BIOTEK). For all cell culture
200 experiments, compounds were dissolved in DMSO. The final DMSO concentration in the
201 cell culture medium was 0.1% (v/v) or less. Pre-treatment of RAW 264·7 macrophages
202 with up to 500 µM and treatment of Ea.hy 926 with up to 100 µM of any of the assayed
203 compounds did not affect cell viability.
204

205 **NO Production**

206 NO production was assessed by measurement of nitrite concentration (NO_2-) in the medium
207 using the Griess reaction (Wang 2002b). Supernatants of cultured macrophages were
208 collected and deproteinized with 0.3 M NaOH and 0.3 M $ZnSO_4$. An equal volume of the
209 Griess reagent (1% sulfanilamide/0.1% *N*-(1-naphthyl)ethylenediamine
210 dihydrochloride/2.5% H_3PO_4) and the deproteinized samples were incubated for 10 min at

211 room temperature protected from light. The nitrite concentration was determined by
212 measuring the absorbance at 548 nm against a standard curve for sodium nitrite (Park
213 2000).
214
215 **TNF-α Secretion in RAW 264.7 macrophages**
216 Supernatants from RAW 264.7 macrophages were collected for TNF-α secretion
217 measurements as described above for NO production measurements. Upon collection,
218 samples were centrifuged at 15700 x g for 10 min and the supernatants kept at -80 ºC until
219 analysis. TNF-α secretion was measured using a commercially available enzyme-linked
220 immunosorbent assay ELISA kit (Mouse TNF-α immunoassay, eBioscence).
221
222 **Secretion of monocyte chemoattractant protein 1 (MCP-1), intercellular adhesion**
223 **molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) by EA.hy 926**
224 **cells**
225 Upon collection of the supernatant from Ea.hy 926 cells, samples were centrifuged at
226 15700 x g for 10 min and the supernatants were kept at -80 °C until analysis. The secretion
227 of MCP-1, ICAM-1 and VCAM-1 by Ea.hy 926 cells were measured using commercially
228 available ELISA kits (Diaclone, Bionova scientific).
229
230 **Determination of ACE-Inhibitory Activity**
231 ACE-inhibitory activity was measured by fluorescence using the method of Sentandreu and
232 Toldrá (2006) with some modifications. Briefly, ACE (peptidyl-dipeptidase A, EC
233 3.4.15.1) working solution was diluted with 0.15 M Tris buffer (pH 8.3) containing 0.1 μM
234 $ZnCl_2$ with 0.04 U/mL of enzyme in the final reaction solution. A total of 40 μL of this

235 working solution (or distilled water for the blank) was added to each microtiter-plate well,
236 with another 40 μL of distilled water for the blank (B) or 40 μL control (C) or 40 μL
237 samples (S). The enzyme reaction was started by adding 160 μL of 0.45 mM o-Abz-Gly-p-
238 Phe(NO2)-Pro-OH (Bachem Feinchemikalien, Bubendorf, Switzerland) dissolved in 150
239 mM Tris-base buffer (pH 8.3), containing 1.125 M NaCl, and the mixture was incubated at
240 37 ºC. The fluorescence generated was measured at 30 min using a multiscan microplate
241 fluorimeter (FLUOstar optima, BMG Labtech, Offeuburg, Germany). 96-well microplates
242 (Porvair, Leatherhead, UK) were used. Excitation and emission wavelengths were 350 and
243 420 nm, respectively. The software used to process the data was FLUOstar control (version
244 1.32 R2, BMG Labtech).
245 The ACE-inhibitory activity was evaluated in 11 phenolic acids: gallic, protocatechuic,
246 syringic, vanillic, synapic, homogentisic, hydroxybenzoic, phloroglucinol, coumaric,
247 caffeic and DMB propionic acid; and five anthocyanins: cyanidin-3-O-glucoside, malvidin-
248 3-O-glucoside, delphinidin-3-O-glucoside, peonidin-3-O-glucoside and pelargonidin-3-O-
249 glucoside. All samples were diluted in distilled water or ethanol-water 30/70 (v/v).
250 The activity of each sample was tested in triplicate. Inhibitory activity was expressed as the
251 concentration required to inhibit the original ACE activity by 50% (IC_{50}). The formula
252 applied to calculate the percentage of ACE-inhibitory activity was: 100 _ (C _ S)/(C _ B),
253 where C is the fluorescence of ACE with o-Abz-Gly-p-Phe(NO2)-Pro-OH (fluorescence
254 substrate) and without inhibitor, S is the fluorescence of ACE with o-Abz-Gly-p-
255 Phe(NO2)-Pro-OH and with sample as inhibitor, and B is the fluorescence of the
256 fluorescent substrate o-Abz-Gly-p-Phe(NO2)-Pro-OH. This parameter was plotted vs.
257 sample concentration and non-linear logarithmic adjustment was performed as indicated by
258 Quirós et al. (2007) to estimate IC_{50}.

259

260 **Statistical analysis**

261 The results were reported as means ± standard deviation (SD) of at least three
262 measurements or two in the case of ICAM-1, VCAM-1 and MCP-1, each performed in
263 triplicate. One way analysis of variance (ANOVA) was used to compare the means, and the
264 least significant difference (LSD) test showed the values statistically different. Differences
265 were considered significant at $P < 0.05$. All statistical analyses were performed with
266 Statgraphics Plus 5.1 (Statistical Graphics Corporation, Inc., Rockville, MD, USA).

267

268

269 **RESULTS AND DISCUSSION**

270

271 **Effect of phenolic acids on NO production and TNF-α secretion in LPS and LPS/IFN-**
272 **γ-stimulated macrophages**

273 Untreated macrophages did not produce detectable amounts of NO after the 24 hours
274 incubation, but stimulation of macrophages with LPS increased NO production
275 significantly (62.9 ± 20.2 NO nmol/mg protein). A synergistic effect was observed when
276 IFN-γ was added simultaneously with LPS such that NO production was further increased
277 (100.1 ± 9.0 nmol/mg protein). In the present study, gallic, vanillic and syringic acids did
278 not inhibit NO production in activated macrophages. Protocatechuic acid slightly but
279 significantly inhibited NO production in a dose-dependent manner between 0 and 100 μM,
280 reaching an inhibition higher than 25% at 100 μM compared to LPS-treated control and by
281 13% at 100 μM compared to LPS + IFN-γ-treated control (Table 1).

282 When we co-treated macrophages with protocatechuic acid (10 μM) and LPS
283 simultaneously, we found a 20% reduction in the NO content of the media despite
284 observing no significant reduction when cells were pre-treated with 10 μM protocatechuic
285 acid 3 hours prior to LPS stimulation. In addition, co-incubation of macrophages with 10
286 μM protocatechuic acid together with LPS+IFN-γ, caused a larger inhibition of NO
287 production (29%) compared to pre-treatment with 10 μM protocatechuic acid before
288 cytokine stimulation (Table 2). This enhanced inhibition of NO production seen with co-
289 incubation compared to with pre-treatment might be a result of a direct chemical interaction
290 between the protocatechuic acid and the stimuli used.

291 Earlier studies have shown that the anti-inflammatory action of flavonoids is mediated via
292 their inhibition of iNOS protein and mRNA expression as well as their inhibition of nuclear

293 factor kB (NF-kB) and STAT-1 activation which are involved in the expression of several
294 inflammatory genes (Chen 2005; Hämäläinen 1999).
295 We next assessed the effect of anthocyanin metabolites on TNF-α secretion. We found that
296 exposure of macrophages to LPS led to TNF-α secretion (3.0 ± 0.1 ng TNF-α/mg protein),
297 and that exposure to both LPS and IFN-γ together induced a synergistic effect on TNF-α
298 secretion (11.4 ± 1.1 ng TNF-α/mg protein). Similar to the results obtained for NO
299 production, we found that pre-treatment of LPS-stimulated cells with protocatechuic acid at
300 concentrations higher than 10 μM caused a reduction in TNF-α secretion, with a significant
301 inhibition at 100 μM protocatechuic acid of 39 %. For cells treated with LPS+IFN-γ
302 together, a low but significant inhibition of TNF-α secretion was observed only by
303 protocatechuic acid at 50 μM and 100 μM (11.1% and 21.5%, respectively). Co-incubation
304 with the other phenolic acids and stimulus did not affect TNF-α secretion. No effect on
305 TNF-α was shown for any of the other polyphenols assayed, vanillic, protocatechuic and
306 syringic acids in our experimental conditions.
307 Monocyte-derived macrophages are the principal inflammatory cells in atheromas. Their
308 activation is crucial to the progression of multiple inflammatory diseases such as septic
309 shock, chronic inflammation and atherosclerosis, via the release of inflammatory and
310 cytotoxic mediators like cytokines or NO (Tamir 1996). In the present study, we have
311 shown that exposing macrophages to LPS and IFN-γ simultaneously induces a synergistic
312 effect in terms of NO production as well as TNF- α secretion, in accordance with other
313 authors (Orlicek 1996).
314 Studies in the literature investigating the effects of anthocyanins on NO production and
315 TNF-α secretion are controversial. García-Alonso et al. (2004) did not find an effect of
316 anthocyanins on NO production or TNF-α secretion when used to pre-treat RAW 264.7

317 macrophages 24 hours prior incubation with LPS. In contrast, Hämäläinen et al. (1999)
318 observed an inhibition of NO production as well as of iNOS protein and mRNA expression
319 with pelargonidin treatment of macrophages exposed to an inflammatory stimulus (LPS).
320 Also, Wang et al. (2002a) demonstrated an inhibitory effect of anthocyanins on LPS-
321 induced NO production in macrophages.

322 Recently Long et al. (2010) have shown that some flavonoids may increase the levels of hydrogen
323 peroxide in the cell culture medium, thereby possibly affecting also some of the parameters that
324 have been measured within the present. This increase in H_2O_2 in response to the flavonoid treatment
325 may be due to a rapid degradation of some flavonoids at neutral pH and 37°C. This has been
326 reported for anthocyanins such as delphinidin chloride, an extremely unstable compound, and may
327 have also occurred in terms of its 3-glucoside (Avila 2009). Furthermore Long et al. (2010)
328 demonstrated that different cell culture media may have different effects on H_2O_2 production for the
329 same polyphenol test compound. Thus compound instability and generation of H_2O_2 should be
330 taken into account in interpreting effects of anthocyandins in cultured cells (Long et al 2010).

331 Related to our findings, Yan *et al.* (2004) demonstrated that protocatechuic acid isopropyl
332 ester reduced plasma TNF-α, NO and hepatic malondialdehyde levels in a mouse model of
333 septic shock induced by LPS and D-galactosamine. In our study, protocatechuic acid
334 exhibited a protective effect in LPS/INF-γ-induced macrophages by inhibiting the
335 overproduction of inflammatory mediators, namely NO and TNF-α.

336 In our work, and in accordance with other studies (Terra 2007), we compared the ability of
337 anthocyanin metabolites to inhibit NO production in macrophages using four different
338 treatment protocols (preincubation with polyphenols and activation with LPS alone or LPS
339 plus INF-γ, and co-incubation with polyphenols and LPS alone or LPS plus INF-γ). When
340 macrophages were co-incubated simultaneously with the test compound and stimulus,

341 protocatechuic acid was found to exhibit the strongest inhibition of NO secretion. In this
342 case, we propose that protocatechuic acid acts by scavenging NO radicals or by a direct
343 interaction with LPS or IFN-γ. However, the high level of inhibition of NO production and
344 TNF-α secretion observed in cells pre-incubated with protocatechuic acid before LPS
345 activation may be due to different mechanisms of action of flavonoids, as described above.
346 It needs to be considered that that the concentrations of protocatechuic acid which inhibited NO
347 secretion in our cell culture experiments were very high and rather not in the physiological range.
348 Protocatechuic acid has been detected in plasma (human and rat) at concentrations that are around
349 200 nmol/L, which is 100 fold higher than the original anthocyanins concentration in plasma
350 (between 1 and 10 nmol/L) (Caccetta 2000) but still very much lower than the concentrations used
351 in our cell culture experiments.
352 Our results suggest that foods rich in polyphenols, which may lead to elevated levels of
353 protocatechuic acid in plasma, could be beneficial in he prevention of inflammatory
354 diseases since they reduce the production of the cytotoxic oxidative stress mediator NO as
355 well as the production of TNF-α, a crucial cytokine for the synergistic induction of NO
356 synthesis.
357

358 **Effect of phenolic acids on MCP-1, ICAM-1 and VCAM-1 secretion in endothelial**
359 **cells**

360 Untreated EA.hy 926cells released very low levels of MCP-1, ICAM-1 and VCAM-1 into
361 the media. However, when treated with TNF-α (10 ng/ml) to mimic pro-inflammatory
362 conditions, there was a marked increase in the secretion of MCP-1 (2.2 ng/ml ± 0.3),
363 ICAM-1 (0.9 ng/ml ± 0.1) and VCAM-1 (4.9 ng/ml ± 3.6). Pre-treatment with gallic acid
364 elicited a statistically significant dose-dependent decrease in the secretion of MCP-1,

365 ICAM-1 and VCAM-1 at concentrations ≥ 10 μM compared with activated control cells
366 (Table 3). None of the other polyphenols assayed, vanillic, protocatechuic and syringic
367 acids, showed any effect on the secreted levels of MCP-1, ICAM-1 and VCAM-1 in our
368 experimental conditions.
369 Many epidemiological studies have reported that moderate wine consumption exerts a
370 protective effect against cardiovascular diseases (Estruch 2000; Gronbaek 2000). In
371 addition, clinical studies have demonstrated that daily intake of wine reduces monocyte
372 adhesion and circulating markers of inflammation (Badia 2004; Estruch 2004). Moreover,
373 Sacanella et al. (2007), showed a more potent effect of red wine versus white wine,
374 possibly due to its higher anthocyanin content. A suppression of NF-kB in white blood cells
375 by red wine was suggested to play a key role in its anti-inflammatory effects (Blanco-Colio
376 2000). In accordance with these studies, we observed a significant reduction in MCP-1,
377 ICAM-1 and VCAM-1 levels when endothelial cells were pre-treated with gallic acid. This
378 reduction was especially marked in the case of VCAM-1. Previous studies have reported
379 that endothelial cells in human atherosclerotic lesions increase cell adhesion molecules
380 (CAMs) and MCP-1. Therefore, gallic acid might be an effective protector against
381 monocyte recruitment in inflammatory vessels and may prove useful in the prevention of
382 atherosclerotic lesion development due, in part, to a decrease in MCP-1 which promotes
383 monocytes infiltration into the arterial wall.
384

385 **Determination of ACE inhibitory activity**

386 We assessed the ACE inhibitory activity of the potentially bioactive phenolic acids and
387 anthocyanins by measuring their IC_{50} values (the concentration required to inhibit the
388 original ACE activity by 50%). The ACE inhibitor activity was first determined for all the test

389 compounds at a concentration of 500 μM and only those ones showing a percentage of inhibition
390 higher than 50% where used to calculate their corresponding IC_{50} vaules. The ACE inhibotry
391 activity was then tested at 6 different concentrations between 1 and 500 μM in order to obtain a
392 dose-response curve. Data are summarized in Table 4. The anthocyanins inhibited ACE
393 activity in a dose-dependent manner, with delphinidin-3-glucoside being the most active
394 (IC_{50} = 65.4 μM) followed closely by cyanidin-3-O-glucoside (IC_{50} = 70.8 μM),
395 pelargonidin-3-O-glucoside (IC_{50} = 77.7 μM), malvidin-3-O-glucoside (IC_{50} = 83.9 μM)
396 and peonidin-3-O-glucoside (IC_{50} = 104.6 μM). This observation suggests that the presence
397 of a hydroxyl group, as well as the O-glycosides structure, enhances the inhibitory activity.
398 We found that of all of the phenolic acids, only caffeic, gallic and coumaric acids exhibited
399 a marginal ACE- inhibitory activity with IC_{50} values of 157.3 μM, 332.4 μM and 504.2
400 μM, respectively. All the other assayed compounds, protocatechuic, syringic, vanillic,
401 synapic, homogentisic, hydroxybenzoic, phloroglucinol, and DMB propionic acid showed
402 an inhibition of ACE lower than 50% for a 500 μM concentration.
403 The *in vitro* ACE-inhibitory activity of flavonoids is due to the generation of chelate
404 complexes with the zinc atom within the active centre of ACE (García-Saura 2005). Free
405 hydroxyl groups of phenolic compounds are suggested to be important structural moieties
406 to chelate the zinc ions, thus inactivating the ACE activity. It may therefore be the hydroxyl
407 groups within the anthocyanins and phenolics acids that are responsible for their ACE
408 inhibitory activity. In fact, ACE inhibitory activity has been already demonstrated in some
409 compounds derived from plants such as flavonoids (Wille 2001), terpenoids (Morigiwa
410 1986), peptides (Kinoshita 1993) and procyanidins (Wagner 1992).
411 It is therefore likely that anthocyanins and their breakdown metabolites, phenolic acids,
412 have hypotensive and protective effects on endothelial function due, at least in part, to their

413 ACE inhibitory effect since angiotensin II regulates arterial blood pressure, adhesion
414 molecule expression, cytokines, chemokines and growth factors within the arterial wall.
415

416 **Relative affinity for ERα and ERβ**

417 The first step to determine the estrogenic activity of a given compound is to measure the
418 binding of this potential ligand to the estrogenic receptor. In general, the affinity of
419 flavonoids to bind to ERα and β is lower than that of 17-β-estradiol. However some studies
420 have confirmed that genistein, daidzein and equol have a good affinity for ER, especially
421 for ERβ (Mueller 2004).

422 The ER binding affinity of anthocyanins and their metabolites was determined in a
423 radioactivity assay by measuring their ability to compete with 17-β-estradiol for the ER.
424 Table 5 summarizes the IC_{50} values obtained (concentration required to inhibit binding of
425 [^{3}H]-estradiol to the corresponding ER by 50%). Pelargonidin-3-glucoside showed affinity
426 for both receptors, with a relatively higher affinity for ERα (61.3 μM ± 0.7) than for ERβ
427 (93.0 μM ± 0.8), whereas peonidin-3-glucoside only demonstrated affinity for ERα (64.4
428 μM ± 0.9) and delphinidin-3-glucoside only reasonable affinity for ERß (63.2 μM ± 0.8).
429 Among the phenolic acid metabolites assayed, gallic acid showed affinity for ERß (100.3
430 μM ± 0.9) but did not show affinity for ERα.

431 It is interesting to note that delphinidin-3-glucoside and gallic acid, with similar structural
432 features in the hydroxylation pattern of their B ring, showed affinity for ERβ, but not for
433 ERα. Moreover, delphinidin-3-glucoside demonstrated a binding affinity for ERβ that is
434 approximately 2-fold higher than that of gallic acid. These results are in accordance with
435 those obtained in the molecular modeling studies and could be explained by the hypotheses
436 that two molecules of gallic acid are able to bind to the ER and display

437 estrogenic/antiestrogenic activities.

438 All other test compounds did not show any affinity for the ER at the concentrations tested.

439 Schmitt and Stopper (2001) have reported that anthocyanidins (the aglycons of
440 anthocyanins) have high affinity towards ERα. They showed that pelargonidin (6.8 μM)
441 had the highest affinity among the assayed anthocyanidins followed by delphinidin (10.4
442 μM) and cyanidin (12.2 μM) (Cornwell 2004). In a similar way Chalopin et al (2010)
443 showed that the endothelium-dependent vasorelaxation of delphinidin and a red wine
444 extract is mediated via ERα. With respect to ERα, the presence of up to 2-OH groups in the
445 B-ring of the molecular structure decreased the affinity of the anthocyanins to the ERα
446 (Fang 2001). In contrast, delphinidin-3-glucoside and gallic acid with 3-OH groups
447 demonstrated the highest affinity for ERβ in our study.

448

449 **Study of the binding mode on ERβ and ERα: docking studies**

450 We carried out docking studies for the anthocyanidins listed in Table 5 (delphinidin,
451 pelargonidin, peonidina, malvidin and cyanidin) and their metabolites (gallic and
452 protocatechuic acids; see Figure 1 for chemical structures) in the ligand binding domains
453 (LBD) of both ERα and ERβ. In general, docking predictions were in agreement with
454 affinity data.

455 Taking genistein (binding mode) as a reference, our purpose was to study whether the
456 docking protocol was able to predict both the binding poses for anthocyanidins and any
457 binding preference that could suggest selectivity. Regarding the binding poses, the most
458 favourable poses corresponded to docked orientations where OH bonds between hydroxyl
459 groups and His524 (ERβ His476) and Arg394-Glu353 (ERβ Arg346-Glu305) are the main
460 interactions, anchoring the ligand within the LBD of ER. These interactions are crucial to

461 give rise to a stable ligand-receptor complex that could account for the observed affinity.
462 Regarding selectivity, no solutions were predicted for delphinidin by AutoDock when
463 docked to ERα, while favourable binding orientations were found in ERβ (Figure 2). On the
464 contrary, in the case of peonidin, only favourable poses were located in ERα (Figure 3).
465 These results are in agreement with the experimental affinity values (Table 6), which show
466 ERβ selectivity for delphinidin and ERα selectivity for peonidin. Our results are also in
467 agreement with previous reports (Schmitt 1996). As mentioned above, it should be pointed
468 out that delphinidin has 3-OH groups in the B-ring. However, for pelargonidin, similar
469 binding results were predicted without meaningful differences. Also, affinity assays only
470 exhibited a slight preference towards ERα.
471 No binding poses were found for malvidin and cyanidin, in agreement with the absence of
472 affinity, suggesting that these compounds are not suitable ER-ligands.
473 In the case of phenolic acids, which are smaller molecules, all showed ability to bind to
474 different regions of the LBD. Our computational efforts were then directed towards the
475 study of the putative binding of two molecules within the LBD. AutoDock predicted that
476 two units of both phenolic acids are able to interact simultaneously with the LBD, by
477 adopting several orientations. Thus, we found that this hypothesis was possible from a
478 theoretical perspective, since binding poses were found to reproduce the main interactions
479 present in large ligands, justifying the antiproliferative effect shown in the MCF-7 cell
480 model: hydrogen bonds between hydroxyl groups and His524 (ERβ His476) and Arg394-
481 Glu353 (ERβ Arg346-Glu305) (Figure 4). From the study of interactions and predicted
482 binding energies, it can be concluded that gallic acid binds with higher affinity to both ERs,
483 compared with protocatechuic acid. Regarding selectivity, similar binding energy values
484 were predicted for both ERα/ERβ receptors. With these theoretical results, selectivity

485 cannot be sighted.

486 Our results suggest that gallic acid could potentially be considered as an
487 enterophytoestrogen; a gut microflora-derived metabolite that can exhibit higher
488 estrogenic/antiestrogenic activity than its corresponding precursor.

489 Nevertheless, the fact that these polyphenols demonstrate affinity for the estrogen receptors
490 and display agonist/antagonist effects may suggest that these compounds could act in target
491 genes and in tissues where they could collaborate in the health promoting properties of
492 anthocyanins.

493

494 **Concluding remark**

495 The mechanisms underlying the antiatherogenic effect of anthocyanins consumption is
496 probably multifactorial. Our study suggests that the protective health effects of
497 anthocyanins might not only be due to anthocyanins themselves, but also to their
498 metabolites produced by action of the gut microflora. Both anthocyanins and their phenolic
499 acid metabolites might play a role in decreasing vascular inflammatory markers, such as
500 cytokines and adhesion and chemoattractant molecules. Despite observing a slight
501 modulation of NO production by protocatechuic acid, the concentrations required to
502 produce this effect do not fit with this being a potential mechanism of action for the
503 antiatherogenic properties of anthocyanins. It might be the ability of anthocyanins and three
504 of their metabolites to inhibit ACE activity which could decrease the expression of
505 inflammatory markers and therefore improve endothelial function. On the other hand, some
506 of the assayed metabolites have shown a relatively important affinity for ER α and β which
507 regulate transcription of target genes, such as NF-*k*B. This affinity could also be implicated
508 in atheromatosis. Overall, the concentrations of anthocyanins and their metabolites, as used

509 in the present cell culture and in vitro assays mediating anti-inflammatory, anti-adhesive,
510 anti- anti-estrogenic, and angiotensin-converting enzyme inhibitory activities were often
511 manifold higher than those physiologically achievable (Vitaglione 2007). Further research,
512 preferably in vivo, is necessary to determine if, and to what extent, anthocyanin metabolites
513 play a role in the prevention of atherosclerosis in humans.
514

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520

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706

707 **Figure 1.** Chemical structure of the main anthocyanidins and the phenolic metabolites
708 assayed.

709 **Figure 2.** ERβ residues highlighted alongside docked delphinidin, viewed from the front of
710 the binding pocket.

711 **Figure 3.** Configuration of peonidin docked in the ERα binding site. ERα residues in the
712 ligand binding domain are highlighted.

713 **Figure 4.** Model of two docked molecules of gallic acid inside the ERβ ligand binding
714 domain: hydrogen bonds between hydroxyl groups and His524 (ERβ His476) and Arg394-
715 Glu353 (ERβ Arg346-Glu305) are shown.

716

717

718 **Table 1.** Effect of protocatechuic acid on the inhibition of nitric oxide production in RAW

719 264.7 macrophages.

Protocatechuic (μM)	Inhibition of NO production (%)	
	LPS 1μg/mL	LPS 1μg/mL+IFN-γ 1000U/mL
25	16.2 ± 3.3*	4.3 ± 1.3*
50	21.3 ± 3.4*	6.1 ± 0.3*
100	27.4 ± 5.7*	12.7 ± 3.3*

720

721 Cells were pre-treated with protocatechuic acid for 3 h and then stimulated for 24 h with

722 LPS (1 μg/ml) or LPS (1 μg/ml) + INF-γ (1000 U/ml). Data were compared to activated

723 controls treated with LPS 1μg/mL (62.9 ± 20.2 nmol/mg protein) or LPS 1μg/mL + IFN-γ

724 1000U/mL (101.1 ± 9.0 nmol/mg protein). Data derived from at least three independent

725 experiments performed in triplicate and is expressed as means ± S.D. * indicates statistical

726 significance: $p<0.05$ comparing the value with control.

727

728 **Table 2.** Effect of protocatechuic acid on nitric oxide inhibition in RAW 264.7

729 macrophages using two different experimental conditions.

Protocatechuic (μM)	Inhibition of NO production (%)	
	LPS 1μg/mL	LPS 1μg/mL+IFN-γ 1000U/mL
Pre-treated	2.4 ± 16.9	3.7 ± 3.3
Co-treated	19.7 ± 7.5*	29.3 ± 4.1*

730

731 Cells pre-treated with 10 μM protocatechuic acid for 3 h and then stimulated for 24 h with

732 LPS (1 μg/ml) or LPS (1 μg/ml) + INF-γ (1000 U/ml) and cells co-incubated with

733 protocatechuic acid and LPS (1 μg/ml) or LPS (1 μg/ml) + INF-γ (1000 U/ml) for 24 h.

734 Data were compared to activated control. Experiments performed in triplicate and data are

735 expressed as means ± S.D. * indicates statistical significance: $p<0.05$ comparing the value

736 with control.

737

738 **Table 3.** Effect of gallic acid on MCP-1, ICAM-1 and VCAM-1 secretion in Ea.hy 926.

Gallic acid (µM)	MCP-1 (%)	ICAM-1 (%)	VCAM-1 (%)
0	100	100	100
1	96.2 ± 5.0	94.5 ± 4.4	88.0 ± 38.3
10	85.7 ± 3.1*	91.9 ± 0.6*	76.2 ± 13.7*
50	81.2 ± 2.4*	88.6 ± 5.6	50.8 ± 5.9*
100	78.9 ± 1.8*	79.9 ± 0.6*	41.2 ± 4.9*

739

740 Cells were pre-treated with gallic acid (0, 1, 10, 50 and 100 µM) for 16 h and then activated
741 6 h with TNF-α (10 ng/ml). Data were compared to TNF-α stimulated controls (not treated
742 with gallic acid). Data ~~were~~ derive from three independent experiments performed in
743 duplicated and are expressed as means ± S.D. * indicates statistical significance: $p<0.05$
744 comparing the value with control.

745

746 **Table 4.** ACE-inhibitory activity of anthocyanins and phenolic acids.

Sample	**IC_{50} (μM)**
Gallic acid	332.4 ± 40.1
Caffeic acid	157.3 ± 16.1
Coumaric acid	504.2 ± 31.5
Malvidin-3-O-glucoside	83.9 ± 5.1
Delphinidin-3-O-glucoside	65.4 ± 4.0
Cyanidin-3-O-glucoside	70.8 ± 2.0
Pelargonidin-3-O-glucoside	77.7 ± 2.3
Peonidin-3-O-glucoside	104.6 ± 5.8

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748 IC_{50}: concentration of compound needed to inhibit the original ACE activity by 50%.

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750 **Table 5.** Relative affinity of anthocyanins and phenolic acids for ER α and ER β.

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Compound	ERα IC_{50} (μM)*	ERβ IC_{50} (μM)*
Delphinidin-3-O-glucoside	NA	63.2 ± 0.8
Pelargonidin-3-O-glucoside	61.3 ± 0.7	93.0 ± 0.8
Peonidin-3-O-glucoside	64.4 ± 0.9	NA
Malvidin-3-O-glucoside	NA	NA
Cyanidin-3-O-glucoside	NA	NA
Gallic acid	NA	100.3 ± 0.9
Protocatechuic acid	NA	NA
Syringic acid	NA	NA
Vanillic acid	NA	NA

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753 *IC_{50} is defined as the concentration required to achieve 50% inhibition in the binding of
754 [^{3}H]-estradiol to the corresponding estrogen receptor (ER). NA, not achieve binding at the
755 assayed concentration. IC_{50} values are shown as mean ± SD.

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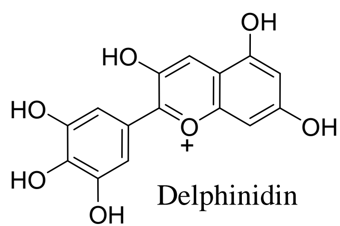

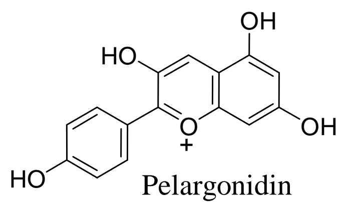

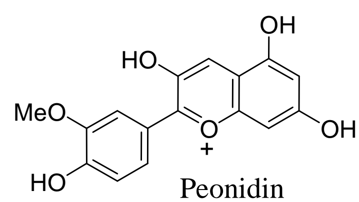

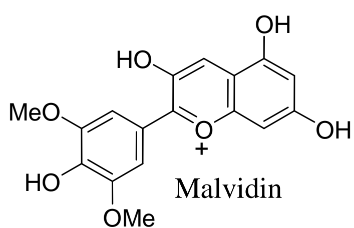

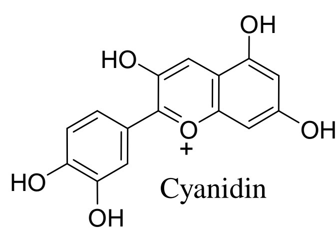

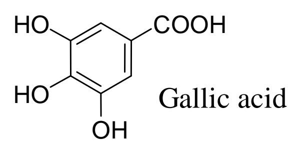

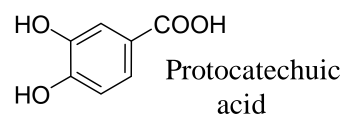

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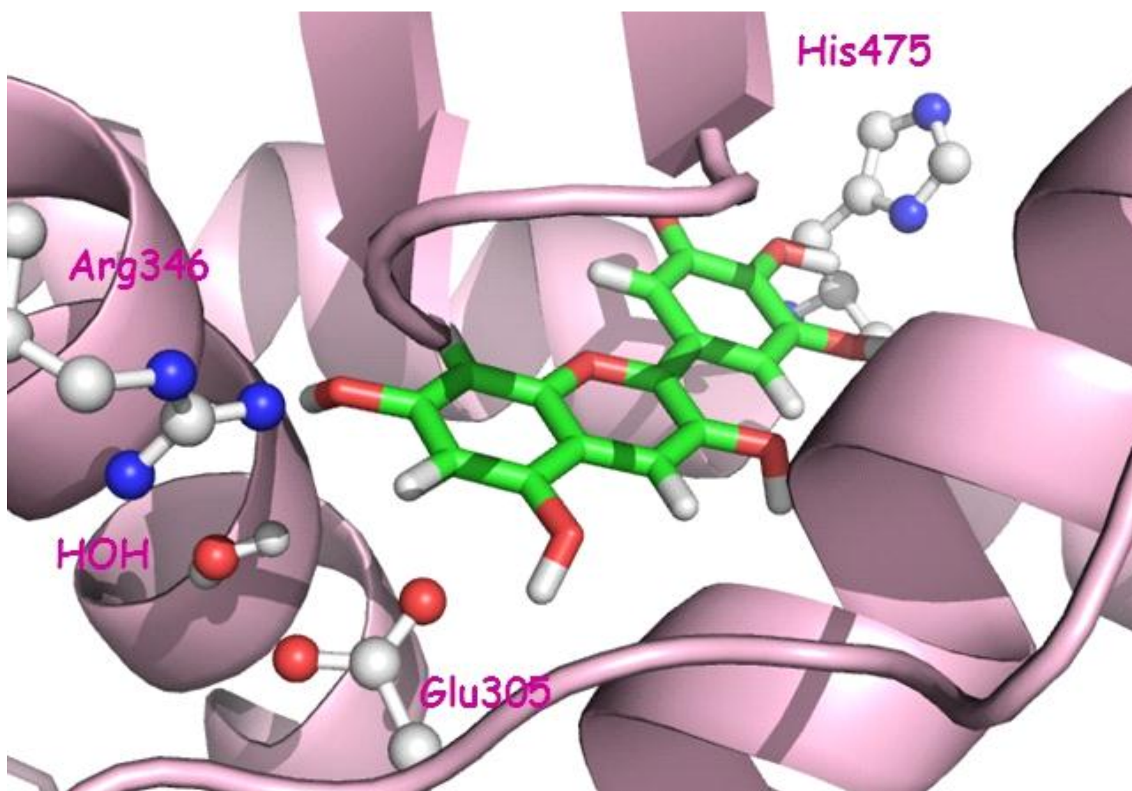

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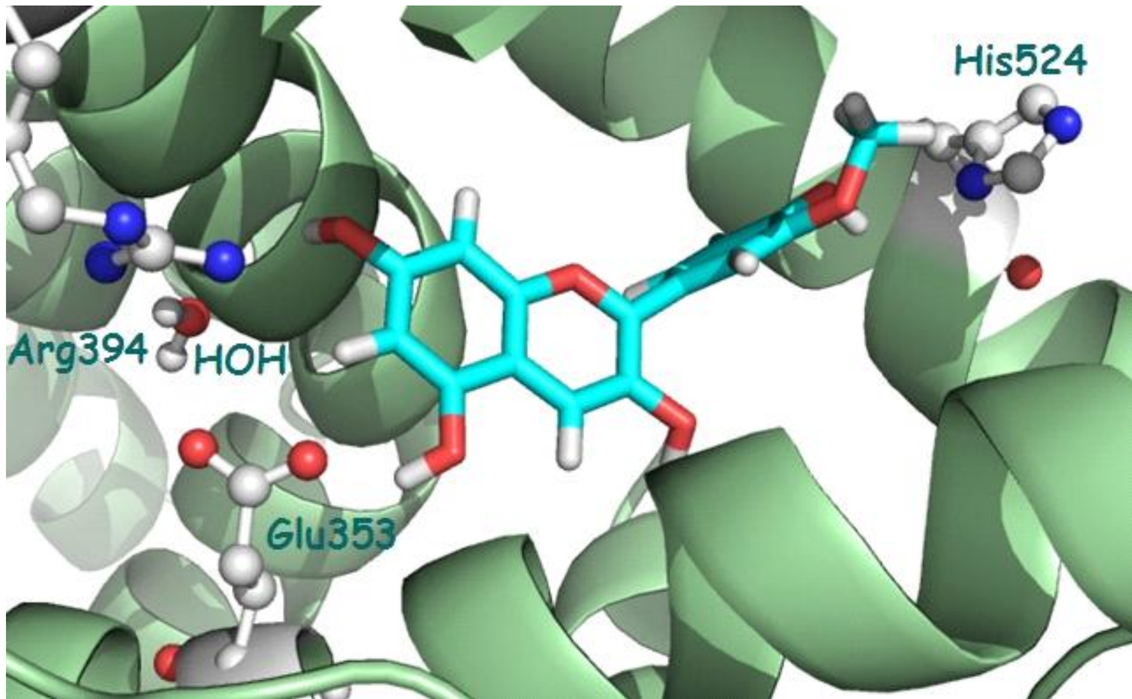

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