Check for updates

View Article Online
View Journal

Food & Function

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: A. López-Jiménez, E. Gallardo, J. L. Espartero, A. Madrona, A. R. Quesada and M. A. Medina, *Food Funct.*, 2018, DOI: 10.1039/C8FO01140K.

This is an Accepted Manuscript, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this Accepted Manuscript with the edited and formatted Advance Article as soon as it is available.

You can find more information about Accepted Manuscripts in the author guidelines.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the ethical guidelines, outlined in our author and reviewer resource centre, still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this Accepted Manuscript or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

rsc.li/food-function

Please do not adjust margins

View Article Online
DOI: 10.1039/C8FO01140K

Food & Function

ARTICLE

Published on 04 July 2018. Downloaded by Universidad de Malaga on 7/4/2018 1:07:04 PM.

Food & Function Accepted Manuscript

Comparison of the anti-angiogenic potential of hydroxytyrosol and five derivatives

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Auxiliadora López-Jiménez[a], Elena Gallardo[b], José L. Espartero[b], Andrés Madrona[b], Ana R. Quesada[c,d] and Miguel Ángel Medina[3]*

Hydroxytyrosol is a phenolic compound present in extra virgin olive oil, either in free form or as derivatives, and related with some of the health benefits described for olive oil intake. We have demonstrated previously that hydroxytyrosol inhibits angiogenesis both *in vitro* and *in vivo*. In the present study, we evaluate the anti-angiogenic potential of five hydroxytyrosol derivatives. Three of these derivatives contain a nitro group and they exhibit a much weaker effect than hydroxytyrosol in the tubule formation assay on Matrigel and therfore were not studied further. In contrast, both hydroxytyrosyl acetate and ethyl hydroxytyrosyl ether show more potent inhibitory effects than hydroxytyrosol in both the *in vitro* tubule formation assay on Matrigel and the *in vivo* chorioallantoic membrane assay. Additionally, these three compounds had slight pro-apoptotic effects and decreased matrix metalloproteinase-2 levels in cell extracts.

Introduction

Addition of antioxidants (AO) to food is often necessary to avoid the oxidation of fatty moieties giving rise to the unpleasant rancid odor and taste, thus ensuring the food quality during processing and commercialization.[1] In virgin olive oil (VOO), this addition is not necessary because of the presence of hydroxytyrosol (3,4-dihydroxyphenylethanol, HT, **1**) (Figure 1) and its derivatives, which are the main responsible of the high oxidative stability of VOO.[2] In the last years, scientific evidence has accumulated pointing to the pleiotropic effects of HT on multiple targets. In fact, demonstrated beneficial effects of HT for human health include its antidiabetic,[3,4] antifungal,[5] antiinflammatory,[6] anti-parasitic,[7] cardio-[8] and neuroprotective[9] activities. HT has also beneficial effects on metabolic syndrome[10] and antitumoral effects of HT have been reported.[11,12]

On the other hand, HT has been shown to be an anti-angiogenic compound able to inhibit several key steps in the angiogenic process.[13] In fact, HT can behave as a protective compound against angiogenesis-dependent diseases, as in the case of rheumatoid arthritis.[14] The anti-angiogenic potential of HT is due to its shown inhibitory effects on multiple molecular targets, including inhibition of matrix metalloproteinase 2 (MMP-2),[15] MMP-9, cyclooxygenase 2 and vascular endothelial growth factor receptor-2 (VEGFR-2) phosphorylation.[6,16] Additional protective effects of HT against endothelial cell dysfunction have been reported.[8]

However, the hydrophilic nature of HT prevents its direct use in the protection of lipidic matrices. Currently available natural lipophilic AO are barely tocopherols and β-carotene, whereas synthetic ones, such as propyl gallate (PG), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) or *tert*-butylhydroquinone (TBHQ), have not shown to be completely safe.[17] For this reason, there is an increasing interest in the food industry to develop new lipophilic AO for use as functional ingredients in lipid food matrices. In this sense, several lipophilic HT derivatives have been prepared, among them: esters,[18] ethers,[19] nitro,[20,21] and isochroman-derivatives,[22] which have shown antioxidant activities, at least, as efficient as that of HT itself,[18,22-24] with improved physico-chemical properties regarding lipid solubility and miscibility. Recent reviews on the subject have been published.[25,26]

Some of these HT derivatives are particularly interesting. Thus, HT acetate (**2**, Figure 1) is an antioxidant naturally present in olive oils[27] that is transported across the small intestinal epithelial cell barrier more efficiently than HT,[28] showing higher hepatic bioavailability than HT.[29] Moreover, **2** has shown protective effects against oxidative DNA damage in blood cells,[30] iron-induced oxidative stress in human cervical cells,[31] oxidative stress in HepG2 cells[32] and a significant improvement in the oxidative state of human red blood cells.[33] In addition, HT ethyl ether (**3**, Figure 1),[19] with an alkyl chain of the same length as the acyl chain in **2**, was included in this study to assess the influence of different functional groups (ether *vs* ester) on the biological activity of the two phenolic compounds. **3** is also absorbed to a higher extent in Caco-2

a. *Research Support Central Services (SCAI) of the University of Málaga, Spain.*
b. *Department of Organic and Pharmaceutical Chemistry, Faculty of Pharmacy, University of Seville, Seville, Spain.*
c. *Universidad de Málaga, Andalucía Tech, Departamento de Biología Molecular y Bioquímica, Facultad de Ciencias, and IBIMA (Biomedical Research Institute of Málaga).*
d. *CIBER de Enfermedades Raras (CIBERER), E-29071 Málaga, Spain.*
** To whom correspondence should be sent.*

This journal is © The Royal Society of Chemistry 20xx

Please do not adjust margins

View Article Online
DOI: 10.1039/C8FO01140K

cells than its precursor HT,[34] being also broadly taken up by HepG2 cells.[35] In addition, **3** protects hepatic human HepG2 cells against oxidative stress[36] and inhibits platelet activation after oral administration in rats.[37] Recently, beneficial metabolic effects of these HT hydrophobic derivatives, **2** and **3**, in hypercholesterolemic rats have been shown.[38]

Otherwise, introduction of a nitro group (NO_2) in the cathecolic ring of HT induces a significant increase in their antioxidant activities,[20,21] maintaining a high *in vitro* bioavailability and hepatic metabolism.[39] Finally, a recent study has shown that some nitroderivatives associated with the Mediterranean diet have an important cardioprotective effect.[40] For these reasons, nitro homologues (compounds **4**-**6**, Figure 1) of above were selected for inclusion in this research.

Taking into account all this background, the aim of the present work was to study the potential anti-angiogenic effects of five HT derivatives, namely compounds **2**-**6**, using free HT (**1**) as standard reference. All selected compounds in this work maintain the *ortho*-diphenolic group intact and show higher antioxidant capacity than HT itself, preserving their potential application as antioxidants to stabilize foodstuffs or as functional food ingredients.

Materials and methods

Chemicals

Supplements and other chemicals not listed in this section were obtained from Sigma Chemicals Co. (St. Louis MO, USA). Cell culture media, penicillin, streptomycin and amphotericin were purchased from Biowhittaker (Walkersivlle, MD, USA). Fetal bovine serum (FBS) and human serum (HS) were products of Harlan-Seralb (Belton, United Kingdom). Plastics for cell culture were supplied by NUNC (Rockilde, Denmark) and VWR (West Chester, Pennsylvania, USA). Hydroxytyrosol derivatives used in the present work were hydroxytyrosyl acetate (**2**), ethyl hydroxytyrosyl ether (**3**), nitrohydroxytyrosol (**4**), nitrohydroxytyrosyl acetate (**5**), and ethyl nitrohydroxytyrosyl ether (**6**). HT derivatives were synthesized as previously described elsewhere.[18-21] As a control, free HT (**1**) was also tested. HT (>99%) was supplied by Seprox Biotech (Murcia, Spain). Figure 1 shows the chemical structure of the six tested compounds.

Cell culture

Bovine aorta endothelial cells (BAEC) were isolated from bovine aortic arches, as previously described,[41] and maintained in Dulbecco´s modified Eagle´s medium (DMEM) containing glucose (1 g/L), glutamine (2 mM), penicillin (50 IU/mL), streptomycin (50 mg/ L), amphotericin (1.25 mg/L), 10% fetal bovine serum.

Control assays of in vitro toxicity

With the exception of the in vitro MTT cell growth and the cell survival assays, the rest of the in vitro assays used in this study were carried out under conditions (hydroxytyrosol derivative concentrations and duration of treatments) that produced no cytotoxic effect on cells. This lack of cytotoxicity was determined by modified MTT survival assays, that is, MMT assays carried out at the same concentrations and duration of treatments used in the rest of the in vitro assays described in this study (routinary control results not shown).

MTT cell growth assay

The 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT) dye reduction assay in 96-well microplates was used. The assay is dependent on the reduction of MTT by mitochondrial dehydrogenases of viable cells to a blue formazan product, which can be measured spectrophotometrically. BAEC ($2.5x10^3$ cells in a total volumen of 100 µL of complete medium) were incubated in each well with serial dilutions of HT derivatives. After 3 days of incubation in the dark (37°C, 5% CO_2 in a humid atmosphere), 10 µL of MTT (5mg/mL in PBS) was added to each well, and the plate was incubated for a further 4 h (37°C). The formazan was dissolved in 150 µM of 0.04 N HCl-2 propanol, and samples were spectrophotometrically measured at 550 nm. All determinations were carried out in quadruplicate, and at least three independent experiments were carried out. IC_{50} values were calculated as those concentrations of compound yielding 50% cell survival, taking the values obtained for control as 100%.

Endothelial cell differentiation assay: tube formation on Matrigel

Wells of 96-well microplate were coated with 50 µL of Matrigel (10.5 mg/mL) at 4 °C and allowed to polymerize at 37 °C for a minimum of 30 min. Some 5×10^4 BAE cells were added in 200 µL of complete medium. Finally, different amounts of HT derivatives were added and incubated at 37 °C in a humidified chamber with 5% CO_2. After incubation for 7 h, cultures were observed and photographed with a Nikon DIAPHOT-TMD inverted microscope (Tokyo, Japan). Each concentration was tested in triplicate, and two different observers evaluated the results of chord formation inhibition. Those assays where no tubular structure could be observed were considered as positive in this assay. The minimal concentration required for this complete inhibitory effect will be referred as MIC (minimal inhibitory concentration).

Cell cycle analysis by flow cytometry

Cells at > 80% of confluence in 6-well plates were treated with different concentrations of tested compounds for 24 h. After incubation, attached and unattached HT derivative-treated and control cells were harvested, washed (PBS), and fixed (70% ethanol, 1 h on ice). Pelleted cells were incubated with RNase A (o.1 mg/mL) and propidium iodide (40 µg/mL) for 1 h with shaking and protected from light. Percentages of sub-G1, G1 and G2/M populations were determined using a Dako MoFlow cytometer and its software, Summit 4.3.

Hoechst staining of nuclei to detect apoptotic cells

Published on 04 July 2018. Downloaded by Universidad de Malaga on 7/4/2018 1:07:04 PM.
Food & Function Accepted Manuscript

This journal is © The Royal Society of Chemistry 20xx
Please do not adjust margins

View Article Online
DOI: 10.1039/C8FO01140K

Published on 04 July 2018. Downloaded by Universidad de Malaga on 7/4/2018 1:07:04 PM.
Food & Function Accepted Manuscript

Cells were seeded on 8-well chamber slides and grown to sub-confluence. After treatments with the indicated concentrations of HT derivatives for 14 h, cells were washed (PBS) and fixed (formalin solution, Sigma). Chamber slides were stained with Hoechst, mounted (DAKO Cytomation Fluorescent Mounting Medium, DAKO), and observed under a fluorescence microscope (Leica, TCS-NT).

Gelatinolytic activity

To prepare conditioned media and cell lysates, BAEC were grown at 75% confluency in 6-well plates. After 2 washes with PBS, each well received the indicated concentration of HT derivatives in 1.5 mL of DMEM/0.1% bovine serum albumin (BSA) containing 200 kallikrein inhibitor units (KIU) of aprotinin/mL. After 24 h of incubation, the conditioned media were collected, and the cells were washed twice with PBS and harvested by scrapping into 0.5 mL of 0.2% Triton X-100 in 0.1 M Tris/HCl containing 200 KIU of aprotinin. Duplicates were used to determine the cell number by using a Coulter counter. The media and cell lysates were centrifuged at 1000× *g* at 4 °C for 20 min, and the supernatants were collected and used for assayed gelatinolytic activity.

The gelatinolytic activities of MMP-2 delivered to the conditioned media or present in cell lysates were detected in gelatinograms. Aliquots of conditioned media and cell lysates normalized for equal cell numbers were subjected to non-reducing SDS-PAGE with gelatin (1 mg/mL) added to the 10% resolving gel. After electrophoresis, gels were washed twice with 50 mM Tris/HCl, pH 7.4, supplemented with 2% Triton X-100, and twice with 50 mM Tris/HCl, pH 7.4. Each wash was for 10 min and with continuous shaking. After the washes, the gels were incubated overnight at 37 °C immersed in a substrate buffer (50 mM Tris/HCl, pH 7.4, supplemented with 1% Triton X-100, 5 mM $CaCl_2$ and 0.02% Na_3N). Then, the gels were stained with Commassie Blue R-250, and the bands of gelatinase activity could be detected as non-stained bands in a dark, stained background. Quantitative analysis of gelatinograms was performed with the NIH Image 1.6 program.

In vivo chorioallantoic membrane (CAM) assay

Fertilized chick eggs were incubated horizontally at 38°C in a humidified incubator, windowed by day 3 of incubation and processed by day 8. The indicated amount of HT derivatives was added to a 1% solution of methylcellulose in water, and 10 µL drops of this solution were allowed to dry on a Teflon-coated surface in a laminar flow hood. Then, the methylcellulose disks were implanted on the CAMs, and the eggs were sealed with adhesive tape and returned to the incubator for 48 h. Negative controls were always made with ethanol (vehicle) mixed with methylcellulose. After the reincubation, CAMs were examined under a stereomicroscope. The assay was scored as positive when two independent observers reported a significant reduction of vessels in the treated area.

Statistical analysis

For all of the assays, at least three independent experiments were carried out. Results are expressed as means ± SD. Statistical significance was determined by using Student´s paired simple test. Values of $p<0.05$ were considered to be significant.

Results

HT derivatives have inhibitory effects on the growth of BAEC

Since many of the described anti-angiogenic compounds were firstly selected by their inhibitory effect on endothelial cell proliferation and HT has been reported to inhibit endothelial cell growth with IC_{50} values within the micromolar range,[13] we firstly tested the ability of HT derivatives to inhibit the proliferation of actively growing BAEC. Table 1 shows that IC_{50} values for four out of the five tested HT derivatives are lower than that of free HT.

HT derivative compounds 2 and 3 have a very potent inhibitory effect on the capillary tube formation by BAEC

The formation of a three-dimensional network of tubes by endothelial cells and its stabilization by recruiting pericytes are key events at the end of angiogenesis. *In vitro*, endothelial cells plated on Matrigel align themselves forming cords enclosing void spaces. This is the case for control, untreated BAEC shown in Figure 2. Compounds that inhibit this process totally without affection of cell viability give rise to small rounded patches of viable cells isolated from each other. The MIC for HT (compound **1**) in this assay was 250 µM. Figure 2 shows that 250 µM of compounds **4**, **5** and **6** was not enough to completely inhibit tube formation by BAEC. In contrast, compounds **2** and **3** exhibited much stronger inhibitory effects than HT, with their MICs as low as 30 and 15 µM, respectively. Based on the results obtained with this key assay, we decided to test the rest of the assays only with compounds **1**, **2** and **3**.

HT derivatives 2 and 3 exhibit mild pro-apoptotic effects on BAEC

The distribution of cell subpopulations in the different phases of cell cycle can be easily assessed in propidium iodide stained cells by flow cytometry. Figure 3A shows that HT (compound **1**), HT acetate (compound **2**) and ethyl HT ether (compound **3**) slightly increased the percentage of BAEC at the subG1 phase, suggesting their potential pro-apoptotic effects on endothelial cells. Hoechst dye staining of nuclei shows that compound **3** exhibited mild pro-apoptotic effects on a mean percentage of BAEC higher than HT (Figure 3B and Table 2).

HT derivatives 2 and 3 exhibit inhibitory effects on BAEC MMP-2 similar to those induced by free HT

We have previously shown that HT decreases the MMP-2 levels secreted to BAEC conditioned medium in a dose dependent manner.[13] Table 3 shows that compounds **1**, **2** and **3** produced similar decreases in the MMP- 2 levels in BAEC conditioned media and cell extracts.

View Article Online
DOI: 10.1039/C8FO01140K

Published on 04 July 2018. Downloaded by Universidad de Malaga on 7/4/2018 1:07:04 PM.
Food & Function Accepted Manuscript

HT derivatives 1 and 3 inhibit in vivo angiogenesis

Table 4 summarizes the results obtained with the CAM assay. Both HT derivatives, HT acetate (**2**) and ethyl HT ether (**3**) were stronger inhibitors of angiogenesis than compound **1** (free HT). Positive, inhibitory effects were observed in 100% of eggs treated with 200 nmol of **2**, 86% of eggs treated with 200 nmol of **3** and 53% of eggs treated with 200 nmol of free HT (**1**).

Discussion

As mentioned in the Introduction, HT exhibits pleiotropic effects on multiple targets related with many physiopathologic conditions. Available data suggest potential pharmacological uses of this compound and underscore the chemopreventive roles it might play. Based on these grounds, diverse HT derivatives have been synthesized.[18-22] The bioactivities of these HT derivatives have been studied, including their antiplatelet aggregation capacity,[4,42,43] neuroprotective activity,[44] DNA oxidative damage protection,[30] cytoprotection,[45] antiinflammatory effects,[38,46] and anticancer activity.[12,47] Most of these derivatives have shown to maintain or even improve the diverse activities of free HT, mainly due to the better lipophilic/hydrophilic balance exhibited by these derivatives.[25]

Our group has previously shown that HT behaves as an anti-angiogenic compound both in vitro and in vivo.[13,15] It should be underscored that these effects were observed with HT doses within the range of those absorbed from a sustained, moderate intake of VOO, like in a typical Mediterranean diet.[48] Important molecular targets for the antiangiogenic effect of HT are VEGFR-2,[16] and extracellular remodeling enzymes.[13,15] The anti-angiogenic effects of HT suggest that it might be protective against angiogenesis-dependent diseases, as it seems to be the case for age-related macular degeneration and rheumatoid arthritis.[14,49]

In spite of the fact that numerous HT derivatives have been so far described, up to now their anti-angiogenic potential has not been yet studied. In the present study, we have tested five HT compounds and we have compared their anti-angiogenic potential with that exhibited by free HT. Endothelial cells use to be quiescent in the adult. In contrast, they quickly become proliferative when the angiogenic phenotype is switched on. Any compound able either to inhibit endothelial cell proliferation or to induce endothelial cell death could be considered as a potential antiangiogenic compound. This is the case of HT and its five derivatives tested in the present study. It should be mentioned that the IC_{50} value for HT shown in Table 1 is almost half of that IC_{50} value reported for HT on BAEC in our previous article.[13] Perhaps this difference is due to the different suppliers (Extrasynthèse versus Seprox Biotech) and declared purity (98% versus 99%) of the HT samples used in the previous and the present work, respectively.

The formation of tubule-like structures by endothelial cells on Matrigel simulates the key final step of the angiogenic process. The assay is as sensitive and selective as to be useful for a primary screening of antiangiogenic compounds.[50] Our data clearly show that both compounds **2** and **3** behave as much more potent inhibitors than free HT (**1**) in this assay, decreasing the MIC values from 250 μM for HT to 30 and 15 μM for compounds **2** and **3**, respectively. In contrast, tested compounds containing a nitro group (compounds **4**-**6**) exhibited much weaker effects than HT, as shown by the fact that none of these three nitro-containing HT derivatives tested at 250 μM could abrogate tube formation on Matrigel. For this reason, compounds **4**-**6** were no longer analyzed in the present work. The presence in the cathecolic ring of an electron-withdrawing substituent as the NO_2 group, undoubtedly decrease the biological activity of these compounds, similarly to previously reported results for other phenolics compounds,[51,52] although these compounds have shown a good antioxidant activity in chemical media.[20,21]

We had previously shown that 0.5-1 mM HT treatment increased the sub-G1 population of immortalized human umbilical vein endothelial ECRF-24 cells.[13] In the present work, we confirm this effect showing that 0.5 mM HT also increased the sub-G1 population in BAEC treated cells. Similar effects were obtained in BAEC treated with 0.5 mM of either compound **2** or compound **3**. These data suggest that these three compounds could have mild proapoptotic effects on BAEC, as confirmed by Hoechst staining of nuclei.

To invade its surroundings during angiogenesis, endothelial cells have to produce increased levels of the extracellular matrix remodeling enzymes, mainly MMP-2 and uPA. We have recently published qPCR data regarding the effects of 1 mM HT treatment (24 h) on BAEC messenger RNA expression levels of extracellular matrix remodeling enzymes and their inhibitors.[15] Those published data clearly showed that MMP-2 and uPA levels, as well as MMP-2/TIMPs ratios were decreased by HT, which suggested that this compound could decrease the invasive potential of endothelial cells. In the present work, we confirm and extend that previous observation. In fact, herein we show that the three selected compounds **1**-**3** decreased the levels of MMP-2 secreted to BAEC conditioned media and present in BAEC cell extracts. The inhibitory effects of compound **3** on MMP-2 in BAEC conditioned media and cell extracts seem to be complementary (minimum effect in conditioned media samples and maximum effect in cell extract samples).

The CAM assay is the most frequently used in vivo assay of angiogenesis.[53] The results obtained in this *in vivo* angiogenesis assay confirm that hydroxytyrosol (compound **1**), hydroxytyrosyl acetate (compound **2**) and ethyl hydroxytyrosyl ether (compound **3**) are inhibitors of angiogenesis, with compound **2** exhibiting the most potent effect.

Conclusions

Taken altogether, the results presented in this work confirm that HT is an anti-angiogenic compound and they show for the first time that two HT derivatives, namely, HT acetate (compound 2) and ethyl HT ether (compound 3) can be considered new anti-angiogenic compounds with more potent inhibitory effects in both the in vitro tubule formation assay on Matrigel and the in vivo CAM assay. These new results add interest to the presence of compound

This journal is © The Royal Society of Chemistry 20xx

Please do not adjust margins
View Article Online
DOI: 10.1039/C8FO01140K

2 in VOO and to the potential use of compounds 2 and 3 as functional food ingredients.

Conflicts of interest

The authors declare that they have no financial or other conflict of interest.

Acknowledgements

This work was supported by grants BIO2014-56092-R (MINECO and FEDER), and P12-CTS-1507 and P09-AGR-5098 (Andalusian Government and FEDER). E.G. thanks Junta de Andalucía for a predoctoral fellowship. The "CIBER de Enfermedades Raras" is an initiative from the ISCIII (Spain). The funders had no role in the study design, data collection and analysis, decision to publish or preparation of the manuscript.

References

1 J. B. German, Antioxidants, in "Food Additives", A. L. Branen, P. M. Davidson, S. Salminen and J. H. Thorngate III (Eds.), 2nd Ed., Marcel Dehker, Inc., New York, 2002, pp. 523-541.
2 R. Mateos, M. Dominguez, J. L. Espartero and A. Cert, *J. Agric. Food Chem.* 2003, **51**, 7170-7175.
3 J. A. Lopez-Villodres, M. Abdel-Karim, J. P. De La Cruz, M. D. Rodriguez-Perez, J. J. Reyes, R. Guzman-Moscoso, G. Rodriguez-Gutierrez, J. Fernandez-Bolanos and J. A. Gonzalez- Correa, *J. Nutr. Biochem.*, 2016, **37**, 94-100.
4 J. J. Reyes, B. Villanueva, J. A. Lopez-Villodres, J. P. De La Cruz, L. Romero, M. D. Rodriguez-Perez, G. Rodriguez-Gutierrez, J. Fernandez-Bolanos and J. A. Gonzalez-Correa, *J. Agr. Food Chem.*, 2017, **65**, 4378-4383.
5 N. Zoric, I. Horvat, N. Kopjar, A. Vucemilovic, D. Kremer, S. Tomic and I. Kosalec, H *Current Drug Targets*, 2013, **14**, 992-998.
6 E. Scoditti, A. Nestola, M. Massaro, N. Calabriso, C. Storelli, R. De Caterina and M. A. Carluccio, *Atherosclerosis*, 2014, **232**, 17-24.
7 E. Belmonte-Reche, M. Martinez-Garcia, P. Penalver, V. Gomez-Perez, R. Lucas, F. Gamarro, J. M. Perez-Victoria and J. C. Morales, *Eur.J. Med. Chem.*, 2016, **119**, 132-140.
8 U. Catalan, L. Rubio, M. C. Lopez de Las Hazas, P. Herrero, P. Nadal, N. Canela, A. Pedret, M. J. Motilva and R. Sola, *Mol. Nutr. Food Res.*, 2016, **60**, 2114-2129.
9 M. C. Crespo, J. Tome-Carneiro, C. Pintado, A. Davalos, F. Visioli and E. Burgos-Ramos, *BioFactors*, 2017, **43**, 540-548.
10 H. Poudyal, N. Lemonakis, P. Efentakis, E. Gikas, M. Halabalaki, I. Andreadou, L. Skaltsounis and L. Brown, *Pharmacol. Res.*, 2017, **117**, 32-45.
11 M. C. Lopez de Las Hazas, C. Pinol, A. Macia and M. J. Motilva, *J. Agr. Food Chem.*, 2017, **65**, 6467-6476.
12 H. Zubair, A. Bhardwaj, A. Ahmad, S. K. Srivastava, M. A. Khan, G. K. Patel, S. Singh and A. P. Singh, *Nutr. Cancer*, 2017, **69**, 932-942.
13 C. Fortes, J. A. García-Vilas, A. R. Quesada and M. A. Medina, *Food Chem.*, 2012, **134**, 134-140.
14 S. Silva, B. Sepodes, J. Rocha, R. Direito, A. Fernandes, D. Brites, M. Freitas, E. Fernandes, M. R. Bronze and M. E. Figueira, *J. Nutr. Biochem.*, 2015, **26**, 360-368.
15 J. A. Garcia-Vilas, A. R. Quesada and M. A. Medina, *Food Chem.*, 2017, **221**, 1741-1746.
16 S. Lamy, A. Ouanouki, R. Beliveau and R. R. Desrosiers, *Exp. Cell Res.*, 2014, **322**, 89-98.
17 L. Fan and N. A. M. Eskin, *The use of antioxidants in the preservation of edible oils*, in "Handbook of Antioxidants for Food Preservation", F. Shaidi Ed., Woodhead Pub., Cambridge, U.K. 2015, pp 373-388.
18 M. Trujillo, R. Mateos, L. Collantes de Teran, J. L. Espartero, R. Cert, M. Jover, F. Alcudia, J. Bautista, A. Cert and J. Parrado, *J. Agr. Food Chem.*, 2006, **54**, 3779-3785.
19 A. Madrona, G. Pereira-Caro, R. Mateos, G. Rodriguez, M. Trujillo, J. Fernandez-Bolanos and J. L. Espartero, *Molecules*, 2009, **14**, 1762-1772.
20 M. Trujillo, E. Gallardo, A. Madrona, L. Bravo, B. Sarria, J. A. Gonzalez-Correa, R. Mateos and J. L. Espartero, *J. Agr. Food Chem.*, 2014, **62**, 10297-10303.
21 E. Gallardo, R. Palma-Valdes, B. Sarria, I. Gallardo, J. P. de la Cruz, L. Bravo, R. Mateos and J. L. Espartero, *Molecules*, 2016, **21**.
22 R. Mateos, A. Madrona, G. Pereira-Caro, V. Dominguez, R. M. Cert, J. Parrado, B. Sarria, L. Bravo and J. L. Espartero, *Food Chem.*, 2015, **173**, 313-320.
23 G. Pereira-Caro, A. Madrona, L. Bravo, J. L. Espartero, F. Alcudia, A. Cert and R. Mateos, *Food Chem.* 2009, **115**, 86-91.
24 R. Cert, A. Madrona, J. L. Espartero and M. C. Pérez-Camino, *Food Funct.* 2015, **6**, 1999-2007.
25 R. Bernini, M. S. Gilardini Montani, N. Merendino, A. Romani and F. Velotti, *J. Med. Chem.* 2015, **58** (23), 9089–9107.
26 J. G. Fernandez-Bolanos, O. Lopez, M. A. Lopez-Garcia and A. Marset, *Biological properties of hydroxytyrosol and its derivatives*, in "Olive Oil: Constituents, Quality, Health Properties and Bioconversions", D. Boskou (Ed.), 2012, pps.375-396.
27 M. Brenes, A. García, P. García, J. J. Rios and A. Garrido, *J. Agric. Food Chem.*, 1999, **47**, 3535–3540.
28 R. Mateos, G. Pereira-Caro, S. Saha, R. Cert, M. Redondo-Horcajo, L. Bravo and P. A. Kroon, *Food Chem.*, 2011, **125**, 865–872.
29 R. Mateos, L. Goya and L. Bravo, *J. Agric. Food Chem.*, 2005, **53**, 9897–9905.
30 S. Grasso, L. Siracusa, C. Spatafora, M. Renis and C. Tringali, *Bioorg. Chem*., 2007, **35**, 137–152.
31 Z. Bouallagui, M. Bouaziz, S. Lassoued, J. M. Engasser, M. Ghoul and S. Sayadi, *Appl. Biochem. Biotechnol*., 2011, **163**, 592–599.
32 G. Pereira-Caro, R. Mateos, B. Sarria, R. Cert, L. Goya and L. Bravo, *Food Chem*., 2012, **131**, 869–878.
33 M. Candiracci, A. Madrona, J. L. Espartero, G. Zappia and E. Piatti, *J. Funct. Foods*, 2016, **23**, 339–347.
34 G. Pereira-Caro, R. Mateos, S. Saha, A. Madrona, J. L. Espartero, L. Bravo and P. A. Kroon, *J. Agric. Food Chem.*, 2010, **58**, 11501–11509.
35 G. Pereira-Caro, L. Bravo, A. Madrona, J. L. Espartero and R. Mateos, *J. Agric. Food Chem*., 2010, **58**, 798–806.
36 G. Pereira-Caro, B. Sarria, A. Madrona, J. L. Espartero, L. Goya, L. Bravo and R. Mateos, *J. Agric. Food Chem.*, 2011, **59**, 5964–5976.
37 J. Munoz-Marin, J. P. De La Cruz, J. J. Reyes, J. A. Lopez-Villodres, A. Guerrero, I. Lopez-Leiva, J. L. Espartero, M. T. Labajos and J. A. Gonzalez-Correa, *Food Chem. Toxicol*., 2013, **58**, 295–300.
38 M. Tabernero, B. Sarria, C. Largo, S. Martinez-Lopez, A. Madrona, J. L. Espartero, L. Bravo and R. Mateos, *Food Funct*., 2014, **5**, 1556-1563.
39 E. Gallardo, B. Sarria, J. L. Espartero, J. A. Gonzalez Correa, L. Bravo-Clemente and R. Mateos, *J. Agric. Food Chem.*, 2016, **64**, 2289–2297.

Published on 04 July 2018. Downloaded by Universidad de Malaga on 7/4/2018 1:07:04 PM.

Food & Function Accepted Manuscript

This journal is © The Royal Society of Chemistry 20xx

Please do not adjust margins

View Article Online
DOI: 10.1039/C8FO01140K

40 R. L. Charles, O. Rudyk, O. Prysyazhna, A. Kamynina, J. Yangb, C. Morisseau, B. D. Hammock, B. A. Freeman and P. Eaton, *PNAS*, 2014, **111**, 8167–8172.
41 D. Gospodarowicz and J. S. Moran, *J. Cell Biol.*, 1975, **66**, 451-457.
42 J. A. Correa, J. A. Lopez-Villodres, R. Asensi, J. L. Espartero, G. Rodriguez-Gutierez and J. P. De La Cruz, *Br. J. Nutr.*, 2009, **101**, 1157-1164.
43 G. I. Togna, A. R. Togna, M. Franconi, C. Marra and M. Guiso, *J. Nutr.*, 2003, **133**, 2532-2536.
44 J. A. Gonzalez-Correa, M. D. Navas, J. A. Lopez-Villodres, M. Trujillo, J. L. Espartero and J. P. De La Cruz, *Neurosci. Lett.*, 2008, **446**, 143-146.
45 F. Echeverria, M. Ortiz, R. Valenzuela and L. A. Videla, *Int. J. Mol. Sci.*, 2017, **18**.
46 S. F. Ng, L. S. Tan and F. Buang, *Drug Develop. Industrial Pharm.*, 2017, **43**, 108-119.
47 G. Toteda, S. Lupinacci, D. Vizza, R. Bonofiglio, E. Perri, M. Bonofiglio, D. Lofaro, A. La Russa, F. Leone, P. Gigliotti, R. A. Cifarelli and A. Perri, *J. Endocrinol. Invest.*, 2017, **40**, 153-162.
48 E. Miro-Casas, M. I. Covas, M. Fito, M. Farre-Albadalejo, J. Marrugat and R. de la Torre, *Eur. J. clin. Nutr.*, 2003, **57**, 186-190.
49 T. Granner, S. Maloney, E. Antecka, J. A. Correa and M. N. Burnier, Jr., *Br. J. Ophthalmol.*, 2013, **97**, 371-374.
50 C. A. Staton, M. W. Reed and N. J. Brown, *Int. J. Exp. Pathol.*, 2009, **90**, 195-221.
51 K. Inami, M. Suzuki, A. Shimizu, M. Furukawa, M. Moritab and M. Mochizuk, *RSC Adv.*, 2014, **4**, 43882-43889.
52 J. S. Wright, E. R. Johnson and G. A. DiLabio, *J. Am. Chem. Soc.*, 2001, **123**, 1173-1183.
53 D. Ribatti, *Int. Rev. Cell Mol. Biol.*, 2008, **270**, 181-224.

Published on 04 July 2018. Downloaded by Universidad de Malaga on 7/4/2018 1:07:04 PM.

Food & Function Accepted Manuscript

This journal is © The Royal Society of Chemistry 20xx

View Article Online
DOI: 10.1039/C8FO01140K

Published on 04 July 2018. Downloaded by Universidad de Malaga on 7/4/2018 1:07:04 PM.

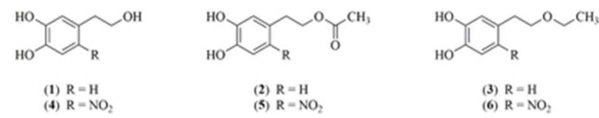

Figure 1

254x190mm (72 x 72 DPI)

Food & Function Accepted Manuscript

View Article Online
DOI: 10.1039/C8FO01140K

Published on 04 July 2018. Downloaded by Universidad de Malaga on 7/4/2018 1:07:04 PM.

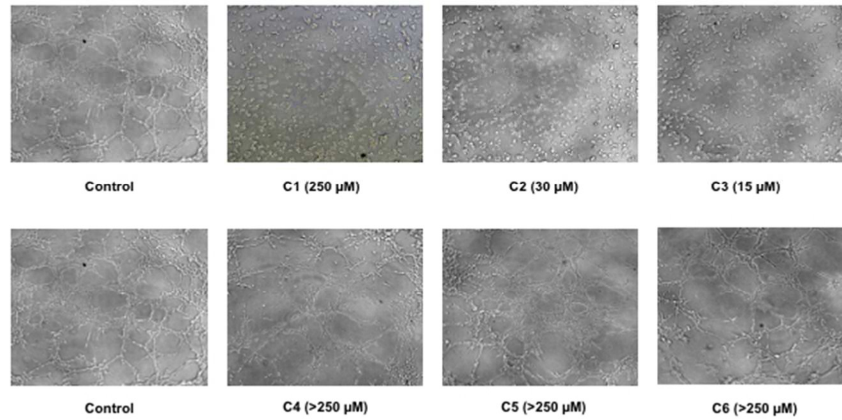

Figure 2

254x190mm (72 x 72 DPI)

Food & Function Accepted Manuscript

View Article Online
DOI: 10.1039/C8FO01140K

Published on 04 July 2018. Downloaded by Universidad de Malaga on 7/4/2018 1:07:04 PM.

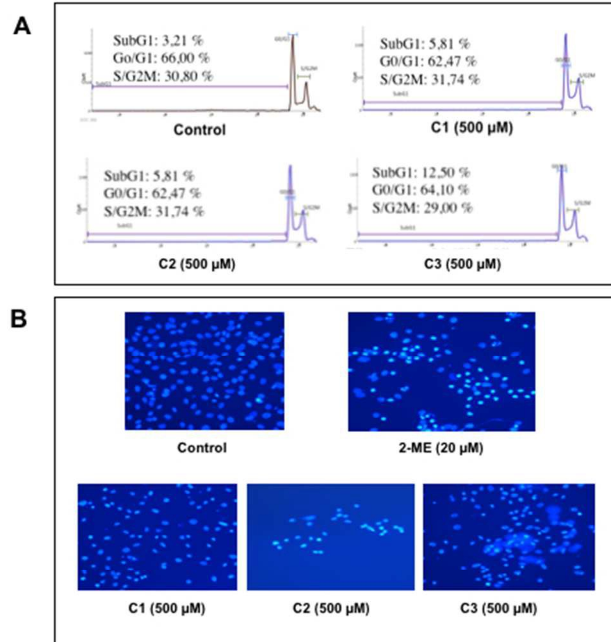

Figure 3

254x190mm (72 x 72 DPI)

Food & Function Accepted Manuscript