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# Inhibition of ex vivo VEGF-induced angiogenesis by tyrosol and hydroxytyrosol: a quantitative three-dimensional mouse aortic ring model

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Mediterranean diet foods such as olives, virgin olive oil and wine are sources of tyrosol (TOL) and hydroxytyrosol (HT), which are bioactive compounds. HT has already shown *in vitro* anti-angiogenic effects in HUVECs. Since TOL is structurally closely related to HT, the aim of the present study was firstly to evaluate the anti-angiogenic properties of TOL regarding inhibition of VEGF-induced VEGFR2 phosphorylation as well as its effects on the intracellular signaling cascade (PLC $\gamma$ 1, Akt and eNOS). Additionally, this paper aims to demonstrate the anti-angiogenic effects of HT and TOL using the *ex vivo* gold standard mouse aortic ring model. Our results demonstrated that TOL significantly inhibits VEGF-induced VEGFR2 activation in HUVECs, with an IC $_{50}$  value of 38.33  $\mu$ M. Additionally, TOL completely blocked PLC $\gamma$ 1 activation, a key component of the VEGFR-2-mediated signalling pathway, while simultaneously increasing the phosphorylation of Akt and eNOS, critical molecules in the regulation of angiogenesis and vasodilation. This study is the first to use the mouse aortic ring model to demonstrate the anti-angiogenic effects of TOL and HT. A significant reduction of capillary sprouting at 68% and 96% was observed for TOL and HT, respectively. These results not only support the potential of TOL and HT as natural anti-angiogenic agents but also offer a new perspective on how diet, especially the Mediterranean diet, may influence the prevention and treatment of angiogenesis-related diseases, such as cancer and cardiovascular diseases.

#### Introduction

Scientific evidence proves that diet plays a relevant role in preventing diseases, especially fruits and vegetables, since they are a rich source of bioactive compounds. However, the mechanisms by which the compounds present in foods, or their metabolites, exert their action are not always well understood. Certainly, there is a need to unravel how bioactives act in biological processes involved in disease prevention.

Angiogenesis is the process of forming new blood vessels from existing ones, which plays a crucial role in various pathologies, such as tumour growth and cardiovascular diseases. For example, in cancer, angiogenesis is involved in supplying nutrients to tumours and promoting their metastasis,<sup>3</sup> while, in atherosclerosis, impaired angiogenesis contributes to

plaque growth and instability.4 Vascular endothelial growth

factor (VEGF) and its receptor VEGFR-2 are essential in the

cules in the diet have been tested using different assays, as summarized by Marrero et al.7 Certain polyphenols, such as epigallocatechin gallate (EGCG) and procyanidins, among others, have demonstrated their molecular mechanisms in vitro by binding specifically to VEGF and consequently inhibiting its capacity for signalling in human umbilical vein endothelial cells (HUVECs).8 Compounds such as melatonin, other indolic compounds and hydroxytyrosol (HT) might interact with the cell surface components of the endothelial membrane in a way that prevents VEGF from activating its receptor. 9,10 In fact, HT has proved to be a potent inhibitor of VEGF-induced VEGFR-2 activation<sup>11</sup> through the inhibition of PLCγ1 phosphorylation. On the other hand, in vitro experiments showed that HT, as well as melatonin, serotonin and fisetin, inhibits HUVEC migration. 9,10,12,13 Like HT, stilbenes such as astringin, pallidol, ω-viniferin, and ε-viniferin have also shown potential anti-VEGF effects in endothelial cells

regulation of this process, mediating the activation of intracellular pathways that finally promote proliferation, migration, and tube formation.<sup>5,6</sup>

The anti-angiogenic properties of naturally occurring molecules in the diet have been tested using different assays, as

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(most of them with  $IC_{50}$  < 10  $\mu$ M), inhibiting the downstream VEGF-induced PLC $\gamma$ 1 phosphorylation, <sup>14</sup> which is responsible for cell proliferation. Additionally, HT, EGCG, dp4 procyanidin and  $\epsilon$ -viniferin have been demonstrated to simultaneously stimulate Akt and eNOS phosphorylation and consequently prevent the hypertensive side effects caused by anti-VEGF drug treatments on nitric oxide (NO) bioavailability. Additionally, HT and its 3-glucuronide conjugated form, as well as TOL-sulfate, have been proven to activate eNOS in human aortic endothelial cells (HAECs). <sup>15</sup> For this reason, the study of these compounds in more complex angiogenesis models could be highly beneficial for the prevention/treatment of angiogenic pathologies. Due to the complexity of angiogenesis, different

methods have been developed to test each step of the process,

which have been comprehensively discussed elsewhere.<sup>16</sup>

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The aortic ring assay has been proposed as a gold standard model to assess the efficacy of anti-angiogenic drugs. 16-18 The aortic ring assay replicates ex vivo cellular and molecular mechanisms essential for regulating the angiogenic process. This assay encompasses endothelial cell differentiation, migration, proliferation, tube formation, microvessel branching, perivascular recruitment and remodeling-all without the need for cellular dissociation-thus providing a more complete picture of angiogenic processes compared with traditional cell-based assays. 19 Consequently, it reproduces the complete process that generates tubular vascular structures. Therefore, this assay provides an invaluable platform to demonstrate the efficacy of the next generation of angiogenesis-targeting compounds. One of the advantages of the aortic ring model is that it maintains the original three-dimensional structure and vascular architecture of the tissue, similar to that observed in vivo.20 These interactions allow for more faithful reproduction of physiological processes, such as cell migration and proliferation, endothelial cell organization into tubes, and the formation of vascular structures.<sup>21</sup> However, this model has been scarcely used to test the anti-angiogenic effects of bioactive compounds due to its methodological complexity. Among the references found, Wen et al. 22 used the chicken aortic ring model, showing that grape seed extract decreased capillary sprouting in a dose-dependent manner. Similarly, treatment with cinnamon extract resulted in a dosedependent decrease in sprout length and density.23 However, there is no further literature in the field of bioactive compounds.

Tyrosol (TOL) and HT are bioactive compounds that have attracted attention due to their presence in Mediterranean diet foods. In particular, HT is abundant in olives ranging from 14.5 to 3833 mg kg<sup>-1</sup> and in extra virgin olive oil (0.09–200 mg kg<sup>-1</sup>). For TOL, the values in olives are between 0.435 and 353 mg kg<sup>-1</sup>, and in extra virgin olive oil from 0.2 to 180 mg kg<sup>-1</sup>. Conversely, wines are richer in TOL, ranging from 1.1 to 48.3 mg L<sup>-1</sup>, while the values for HT are 0.000071–9.6 mg L<sup>-1</sup>.<sup>24</sup> HT has shown *in vitro* anti-angiogenic effects in HUVECs as mentioned above. Since TOL is structurally closely related to HT, both bioactives are present in the same foods, and the anti-angiogenic effect of TOL has not been studied so

far, the aim of the present study was firstly to evaluate the anti-angiogenic properties of TOL to provide a more complete picture of the potential anti-angiogenic effect after the intake of the above-mentioned foods. For this purpose, its anti-VEGF properties were determined as well as its effects on the intracellular signalling cascade (PLCγ1, Akt and eNOS). Additionally, this paper aims to test the effects of HT and TOL in the *ex vivo* gold standard mouse aortic ring model, which has not been evaluated so far.

#### Materials and methods

#### Chemicals and reagents

HT (purity: ≥98%) was acquired from Extrasynthese (Genay, France). TOL (≥98%), dimethyl sulfoxide (DMSO), bicinchoninic acid (BCA), BS1 lectin-FITC and monoclonal anti-actin α-smooth muscle Cy3 were purchased from Sigma Aldrich (St Louis, MO, USA). DAPI Fluoromount-G® was purchased from Southern Biotech (Birmingham, AL, USA; Art. 0100-20).

Human umbilical vein endothelial cells (HUVECs), endothelial cell growth medium-2 (EGM-2) and endothelial basal medium (EBM) were obtained from Lonza (Slough, UK). Recombinant human VEGF $_{165}$  was bought from R&D Systems (Minneapolis, MN, USA). Opti-MEM culture medium was acquired from Gibco (Waltham, MA).

A PathScan® Phospho-VEGFR-2 (Tyr1175) ELISA sandwich kit and the p-PLCγ1 (Tyr783), PLCγ1, p-Akt (Ser 473), Akt, p-eNOS (Ser 1177), and eNOS antibodies were purchased from Cell Signalling Technology (Danvers, MA, USA). NuPAGE lithium dodecyl sulfate (LDS) sample buffer (4×), NuPAGE DTT (10×) and 4–12% Bis-Tris gels were obtained from Invitrogen (Loughborough, UK). Nitrocellulose 0.2 μm membranes were acquired from Bio-Rad (Hercules, CA, USA). The SuperSignal West Pico chemiluminescent substrate was obtained from Thermo Scientific<sup>TM</sup> (Hitchin, UK). Ketamine (Ketamidor, Richter Pharma AG, Wels, Austria; 100 mg mL<sup>-1</sup>) and diazepam (Diazedor, Richter Pharma AG, Wels, Austria; 5 mg mL<sup>-1</sup>) were purchased to anesthetize the mice described in this manuscript.

#### Cell culture conditions

HUVECs were used between passages 4 and 5. The cells were cultured in EGM-2. Endothelial cell cultures were maintained at 37 °C under a humid atmosphere enriched with 5% CO<sub>2</sub>.

#### **Treatment of HUVECs**

Confluent HUVECs were washed twice with warm PBS before the treatments were added. Either vehicle control ( $\leq$ 0.1% DMSO), or TOL at concentrations ranging from 30  $\mu$ M to 100  $\mu$ M (eight different concentrations) in endothelial basal medium (EBM) was incubated for 4 h with HUVECs, prior to stimulation with VEGF at 25 ng mL<sup>-1</sup> for 5 min to determine VEGFR-2 phosphorylation, for 10 min in the case of PLC $\gamma$ 1, and for 60 min for Akt and eNOS evaluation. After that, the cells were lysed with radioimmunoprecipitation assay (RIPA)

buffer containing protease and phosphatase inhibitors. The

protein content of the lysates was determined through a bicinchoninic acid assay. The TOL concentration range was selected for comparative purpose with the previosuly described anti-VEGF effect of HT<sup>10,11</sup> since they share similar structures.

#### Phosphorylated VEGFR-2 (ELISA)

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Phosphorylated VEGFR-2 in the lysates was quantified using a PathScan Phospho-VEGFR-2 (Tyr1175) sandwich ELISA kit, following the manufacturer's instructions.

#### Western blot analysis for PLC<sub>7</sub>1, AKT and eNOS

Electrophoresis was performed with denatured proteins in NuPAGE 4-12% Bis-Tris gels before being transferred to 0.2 µm nitrocellulose membranes. The membranes were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline with Tween 20 (TBST) buffer and incubated overnight at 4 °C with primary antibodies (p-PLCγ1, PLCγ1, p-Akt, Akt, p-eNOS, and eNOS). Subsequently, the membranes were incubated for 1 h at room temperature with a secondary antibody anti-rabbit IgG-HRP in 5% bovine serum albumin (BSA) in Tris-buffered saline with Tween® 20 (TBST). The bands were detected using the SuperSignal West Pico chemiluminescent substrate and visualized on a ChemiDoc Imaging System from Bio-Rad. The band intensities were quantified using Image J® software.

#### Animal ethical approval

Animal experiments adhered to the European Union (EU) Directive 2010/63/EU and the national guidelines (RD 53/2013) for laboratory animal care and use. The Institutional Animal Care and Use Committee reviewed and approved these experiments, with approval reference #02/08/2023/65, issued by the Junta de Andalucía, Dirección General de la Producción Agrícola y Ganadera. 10-12-week-old male C57B/6J mice were obtained from the Centre for Animal Production and Experimentation at the University of Seville (Spain). All animals were housed under standard conditions in a controlled environment (23 ± 1 °C, 12 h light/dark cycles).

#### Ex vivo mouse aortic ring assay

The anti-angiogenic effects of TOL and HT were measured ex vivo using the mouse aortic ring assay protocol described by Baker et al. 19 Mice were deeply anesthetized with a mix of ketamine (75 mg kg<sup>-1</sup> i.p.) and diazepam (10 mg kg<sup>-1</sup> i.p.) followed by thoracotomy and removal of the aorta. The thoracic aorta was dissected from untreated 10-12-week-old male C57B/6J mice (described above) and the surrounding fibro-adipose tissue was completely removed gently under a binocular stereoscopic microscope. Then, aortic rings of 0.5 mm in diameter were cut, and embedded on collagen-coated (Millipore, cat. no. 08-115, Burlington, Massachusetts, USA) and placed in 96-well plates individually. Each well was incubated in Opti-MEM culture medium supplemented with 2.5% (vol/vol) FBS, together with a final concentration of 6 ng mL<sup>-1</sup> of VEGF to induce angiogenesis except for the negative control, VEGF +

TOL and VEGF + HT at the final concentration of their IC<sub>50</sub> values (38 µM and 72 µM, respectively). Media with their corresponding treatments were changed first on day 3 and then approximately every other day until the end of the experiment (day 6). After 6 days of treatments, vessel growth was quantified by epifluorescence microscopy counting of all sprouts on each aortic ring stained by immunofluorescence with BS1 lectin-FITC and monoclonal anti-actin α-smooth muscle Cy3, an endothelial and smooth muscle cell marker, respectively. A drop of DAPI Fluoromount-G® was added per well to counterstain the nuclei. Each experiment was repeated at least three times using three mice each time and between 4 and 7 rings per treatment. In this study, a total of 144 aortic rings were used.

#### Statistical analysis

Statistical analyses were carried out using GraphPad Prism software 8.0.2 (GraphPad Software, Inc., San Diego, CA, USA). Student's t test was used to analyse significant differences between samples. The degree of significance of the analysis was as follows: \*\* p < 0.01, \*\*\* p < 0.001, and \*\*\*\* p < 0.0001. Data are presented as mean  $\pm$  standard deviation.

#### Results

#### Anti-angiogenic effect of TOL by inhibition of VEGFR-2 activation

Firstly, the effect of TOL against activation of VEGFR-2 was investigated using ELISA. Fig. 1 shows that VEGF stimulates VEGFR-2 phosphorylation. However, TOL at 30 µM, 50 µM and 100 μM was capable of inhibiting VEGF-mediated VEGFR-2 activation by 44%, 63% and 77%, respectively. Therefore, the IC<sub>50</sub> value was determined at concentrations ranging between 30  $\mu$ M and 100  $\mu$ M, showing an IC<sub>50</sub> value of 38.33  $\mu$ M (36.01-40.54 at 95% confident intervals).

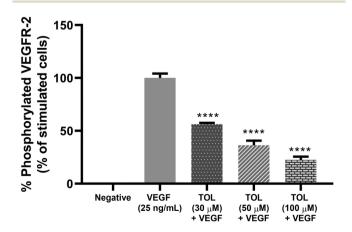


Fig. 1 TOL inhibits VEGF-induced VEGFR-2 activation. HUVECs were treated with TOL at different concentrations for 4 hours before stimulation with VEGF (25 ng mL<sup>-1</sup>) for 5 minutes. Phosphorylated VEGFR-2 was determined by ELISA. Data are expressed as mean + SD (n = 4). \*\*\*\*

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#### Effects of TOL on PLCγ1, Akt and eNOS

Once TOL inhibition of VEGF-induced VEFGR-2 phosphorylation was demonstrated, we evaluated whether TOL at its IC<sub>50</sub> value (38 µM) could regulate downstream signalling events of p-VEGFR-2. First, we evaluated whether the anti-angiogenic properties of TOL were mediated by the inhibition of PLCy1, the main protein involved in cell proliferation. The results show that after VEGF stimulation, PLC<sub>γ</sub>1 became phosphorylated, but pre-incubating the cells with TOL prior to VEGF stimulation caused a significant decrease in the pPLCy1/ PLCy1 ratio compared to the positive control with only VEGF (Fig. 2A and D). TOL inhibited PLC<sub>7</sub>1 phosphorylation by 81%, without affecting total protein levels. These data demonstrate that TOL not only inhibits VEGFR-2 activation but also prevents downstream signalling through PLCy1, therefore counteracting the angiogenesis process.

Secondly, we evaluated the effect of TOL on Akt and eNOS activation, proteins responsible for vasodilation (by means of nitric oxide formation), which are later activated in the VEGF signalling cascade. The results obtained in this work demonstrate that VEGF alone activates Akt (Fig. 2B and E) and increases the ratio between eNOS phosphorylation/total eNOS (Fig. 2C and F). However, TOL does not inhibit VEGF-induced phosphorylation of Akt and eNOS but maintains them significantly activated (Fig. 2B, C, E and F). In fact, TOL alone caused significant increases in the p-Akt/Akt (Fig. 2B) and p-eNOS/ eNOS (Fig. 2C) ratios.

#### Inhibition of microvessel sprouting in mouse aortic rings by HT and TOL

Compared with in vitro ELISA and western blot assay, organ culture methods, such as the mouse aortic ring assay, are thought to more closely mimic multiple stages of in vivo angiogenesis. To further demonstrate the anti-angiogenic activities of HT and TOL, we next performed an aortic ring assay. In addition to TOL, HT has been included in this trial, since its anti-angiogenic properties have been demonstrated in previous experiments by our group. 10 Additionally, they are structurally closely related and both are present in the same foods.

Sprout formation was examined under an epifluorescence microscope after 6 days of treatment. Microvessels were noticed in VEGF samples alone (Fig. 3A). However, no sprouts were observed in the negative control without VEGF. Treatment with HT  $(72 \mu M)^{10}$  or TOL  $(38 \mu M)$  at their IC<sub>50</sub> values resulted in a significant decrease in the number of VEGF-induced capillary sprouts by 96% and 68%, respectively (Fig. 3), indicating that HT and TOL inhibited ex vivo VEGF-induced angiogenesis. Furthermore, the length of the sprouts was not affected in the presence of TOL or HT (data not shown).

#### Discussion

There is no doubt that it is necessary to develop new strategies to modulate angiogenesis due to its importance in physiological and pathological processes. For example, human tumours

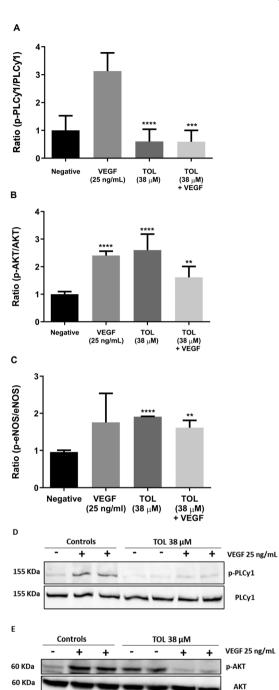


Fig. 2 TOL treatment significantly inhibits PLCγ1 phosphorylation while activating Akt and eNOS. HUVECs were treated with (A-F) TOL (38 µM) for 4 h and then incubated with VEGF (25 ng mL<sup>-1</sup>) for 10 min (A and D) and 60 min (B, C, E and F). Western blot membranes were incubated with PLC $\gamma$ 1 and p-PLC $\gamma$ 1 (A and D), Akt and Akt (B and E) and eNOS and p-eNOS (C and F) antibodies. Data representation of the phosphorylated antibody/total antibody ratio is indicated as mean + SD (n = 5). \*\* p <0.01, \*\*\* p < 0.001, \*\*\*\* p < 0.0001 vs. VEGF alone (A) and versus negative control (B and C).

140 KD

TOL 38 uM

VEGF 25 ng/ml

p-eNOS

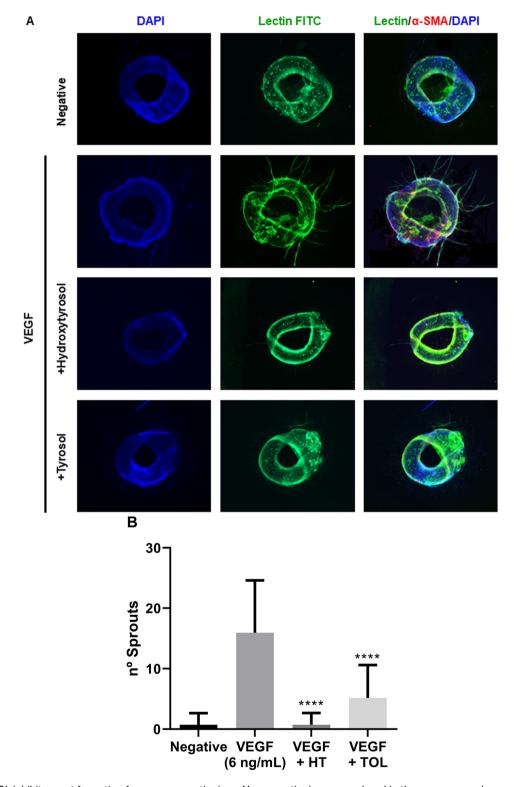


Fig. 3 HT and TOL inhibit sprout formation from mouse aortic rings. Mouse aortic rings were placed in the presence or absence of HT (72 µM) and TOL (38 µM). The effects of HT and TOL on cell sprout formation from 144 aortic rings have been assessed by quantifying the epifluorescence microscopy images (A). Nuclei staining with DAPI (blue) was utilized alongside endothelial and smooth muscle cell markers, lectin FITC (green) and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA, red), respectively. (B) Graphical representation of the number of sprouts formed in mouse aortic rings with and without HT (72  $\mu$ M) and TOL (38  $\mu$ M). Data representation of the number of sprouts is indicated as mean  $\pm$  SD (n = 9). \*\*\*\* p < 0.0001 against VEGF alone and between VEGF + HT and VEGF + TOL.

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can remain dormant for years due to the balance between cell proliferation and apoptosis. Blockade of angiogenesis is therefore an important approach for cancer treatment and prevention. 25 Furthermore, in atherosclerotic areas, the local specific conditions (relative anoxia, inflammation, oxidative stress) induce classical and non-classical angiogenic factors that promote sprouting angiogenesis from preexisting vasa vasorum.4 In the same way, angiogenesis plays a crucial role in cardiovascular disease.26 While VEGF, a crucial endogenous factor in angiogenesis induction, has emerged as an attractive molecular target for anti-angiogenic treatment, the chronic therapeutic use of anti-VEGF agents is limited due to side effects. Hypertension is a common adverse effect of anti-VEGF therapies, such as sorafenib, 27 sunitinib, 28 pazopanib 29 and axitinib,30 among others.

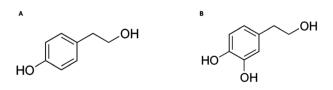
The present study evaluates the anti-VEGF effects of HT and TOL, both present in a limited number of foods such as table olives, olive oil and wine, all three characteristic components of the Mediterranean diet.<sup>24</sup> Furthermore, it has been shown in the literature that TOL is a precursor of HT during alcoholic fermentation.31 TOL can also be converted to HT in vivo, so it may be an additional source of HT in circulation after TOL consumption.<sup>32,33</sup> In a previous study, we estimated that a daily dietary intake of four spoons of virgin extra olive oil (7.2 mg of TOL, 8 mg of HT), 2 glasses of wine (9.66 mg of TOL, 1.92 mg of HT) and 7 olives (7.06 mg of TOL, 76.66 mg of HT) provides a total of 23.92 mg of TOL and 86.58 mg of HT, largely above the tested dose of 5 mg of HT in previous studies on HT bioavailability.<sup>24,34</sup> Indeed, these authors determined 0.6 μM of HT in human plasma after the intake of 20 g of extra virgin olive oil, providing 5 mg of HT.34 Additionally, the bioavailability of TOL and HT in humans has been reported as dose-dependent.35 Therefore, concentrations of 2.9 µM and 10.4 µM could be assumed for TOL and HT, respectively, in plasma after the intake of Mediterranean diet foods as mentioned above. It has to be considered that a large number of metabolites derived from HT have been described (glucuronides, sulfates, O-methylated forms, homovanillic acid, acetylated and sulfated derivatives and N-acetylcysteine derivatives), with the glucuronidation pathway being the most relevant when a dose of 1 mg kg<sup>-1</sup> was tested.<sup>35</sup> However, few studies have been devoted to TOL metabolism, describing glucuronide and sulfate metabolites. 36,37 Additionally, these metabolites can be easily deconjugated within HUVECs, potentially leading to higher TOL and HT concentrations in the target location.<sup>38</sup> Since our results showed an anti-VEGF IC $_{50}$  value of 38.33  $\mu M$ for TOL and 72.4 µM for HT, 10 food supplements enriched with TOL and HT would be needed to achieve an anti-VEGF effect. Therefore, different strategies to increase bioactive compounds in food are currently in the spotlight. For instance, in fermented products, the selection of strains of yeast, and adequate concentrations of substrates can increase the concentrations of TOL and HT. 39,40 Moreover, HT is an authorized novel food ingredient in the EU that can be added to different foods, as the intake is far below toxicological concern. 41 Therefore, it is reasonable to expect that the intake of HT would increase in the future, as its use as a food ingredient is becoming more and more frequent.

Lamy et al. 11 have reported that HT has an inhibitory effect on VEGF-induced VEGFR-2 activation, cell proliferation, cell migration, and tubular formation in HUVECs. Additionally, in a previous study by our group, HT exhibited an anti-VEGF IC<sub>50</sub> value of 72.40 μM. 10 Besides, TOL has a similar chemical structure to that of HT, the only difference being that TOL has a hydroxyl group (-OH group) attached to the benzene ring, while HT has a catechol group (two -OH groups at the orthoposition) (Fig. 4).

In the present study, we have determined the TOL IC<sub>50</sub> value against VEGFR-2 phosphorylation at 38.33 µM for the first time. When an electron-donating group, such as the -OH group, is attached to the ring, the electron density of benzene is higher, and its reactivity will increase. In fact, the catechol group, with two -OH groups, was one of the chemical characteristics strongly related to a potent VEGF inhibition by flavonoid polyphenols such as quercetin, quercetagetin, luteolin, and orobol. 42 Therefore, it would be expected that HT with a catechol group would exhibit greater anti-VEGFR-2 activity than TOL, as it has been referenced for flavonoids such as catechin gallate, luteolin or quercetin. 42 However, our results show that TOL has nearly half the IC<sub>50</sub> value of HT (72.40 μM), despite HT having a catechol group. This structure-function differences might be supported by the fact that HT and certain flavonoids differ in their anti-angiogenic molecular mechanisms, since flavonoids bind directly to VEGF,8 while HT interacts with components of the cell surface (VEGFR-2, neuropilins, etc.).<sup>10</sup>

We have evaluated the role of TOL in regulating cell proliferation by studying the activation of PLC<sub>γ</sub>1, the first constituent of the main VEGFR-2 pathway. The results showed that, after VEGF stimulation, PLC<sub>γ</sub>1 became phosphorylated. However, pre-incubating the cells with TOL (38 µM) plus VEGF stimulation blocked PLCy1 activation without affecting total protein, compared to the positive control with only VEGF (Fig. 2A and D). The ability to inhibit the PLCγ1 phosphorylation of TOL agrees with that described for HT, whose anti-angiogenic effect is also mediated by PLCγ1 inhibition. 10 However, TOL (38 μM) completely inhibits PLCγ1 phosphorylation, while HT (50 μM) inhibits it by 41%. 10 In addition, these results agree with other bioactives such as procyanidin dp4 and EGCG (at 1 μM), which have been proven to prevent VEGF-induced VEGFR-2 activation downstream signalling through blocking PLCy1 phosphorylation.8

Vasodilation is also stimulated through VEGF-induced VEGFR-2 activation. This binding activates eNOS by means of



Chemical structures of TOL (A) and HT (B).

Akt, triggering the production of NO.43 Therefore, the inhibition of VEGF-induced VEGFR-2 phosphorylation would be expected to decrease AkT and eNOS activation. In fact, anti-VEGF drugs such as bevacizumab, sorafenib and sunitinib have been shown to increase the risk of developing hypertension by decreasing the production of NO.<sup>27,44</sup> However, TOL (38 µM) inhibits VEGF-induced VEGFR-2 activation simultaneously activating eNOS (Fig. 2C) via AkT activation (Fig. 2B) in the presence and absence of VEFG at a similar level to that of VEGF alone. It may be expected, therefore, that TOL would induce NO bioavailability, avoiding the adverse hypertensive effects associated with current anti-VEGF drugs. In this regard, previous studies conducted by our research group demonstrated that a diet enriched in extra virgin olive oil, which contains a high amount of TOL, prevented the increase in blood pressure and intraocular pressure in a mouse model of arterial hypertension. 45,46 We postulated that these effects were partly due to the activation of eNOS, which exhibited increased phosphorylation at its active site (Ser1177) compared to its inhibitory site (Thr495), and the higher concentration of NO in the animals fed with olive oil. This observation aligns with the results presented in this manuscript, supporting the potential of TOL in these effects. Additionally, some authors have already demonstrated that NO was not affected in breast cancer cells MCF-7 treated with HT (5-200 µM) under hypoxic conditions. 47 Moreover, these results agree with Cerezo et al., 10 since they showed that HT (50 µM) significantly increased both Akt and eNOS phosphorylation while simultaneously inhibiting VEGF-induced VEGR-2 phosphorylation. Similarly, the polyphenols EGCG from green tea and procyanidin dp4 from apples have been shown to potently inhibit VEGFinduced VEGFR-2 signalling but still may induce NO bioavailability by increasing phosphorylation of both AKT and eNOS at concentrations achievable through diet.8 Therefore, Moyle et al.8 stated that it is possible that polyphenols can effectively inhibit VEGF signalling at physiologically achievable concentrations but retain or even activate Akt and eNOS. In fact, certain polyphenols such as EGCG, epicatechin, ellagic acid, and procyanidins activate eNOS in endothelial cells by the PI3K/AKT/eNOS pathway, which can be initiated not only by the stimulation of receptor tyrosine kinases (RTKs) such as VEGFR-2 but also by G-protein-coupled receptors (GPCRs) or unidentified specific cell surface receptors. 48,49 Additionally, epicatechin, resveratrol and rosmarin have been proven to activate eNOS via the CaMKII/AMPK pathway in endothelial cells. 49-51 More recently, polyphenol-rich Aronia melanocarpa juice has been demonstrated to persistently stimulate sustained eNOS phosphorylation through intricate redox-sensitive pathways, which activates key kinases such as PI3K/Akt, JNK, and p38 MAPK.<sup>52</sup> In fact, TOL and HT have been shown to be active in enhancing Akt1/eNOS activation, leading to an increase in the cellular NO balance by superoxide suppression in different ways through both direct scavenging properties and NADPH oxidase inhibition.<sup>53</sup> All these studies have been developed in the absence of VEGF. The novelty of our findings lies in the ability of TOL to inhibit VEGF-induced VEGFR-2

activation while simultaneously promoting the phosphorylation of Akt and eNOS.

When comparing the activity of TOL and HT against eNOS activation, we observed that TOL significantly increases the peNOS/eNOS ratio to a greater degree than HT in both the presence and absence of VEGF, agreeing with its IC<sub>50</sub> values. These results suggest that TOL is more effective than HT in activating eNOS and, consequently, enhancing NO production, with the associated beneficial effects discussed throughout this manuscript (*e.g.*, preventing the hypertensive adverse effects of anti-VEGF therapies).

To confirm the *in vitro* anti-angiogenic effects of TOL and HT, we have evaluated their capacity to inhibit new blood vessel formation using an  $ex\ vivo$  mouse aortic ring model. Our results demonstrate for the first time the significant inhibition of VEGF-induced microvessel sprouting by TOL and HT  $ex\ vivo$  (68% and 96%, respectively) at their IC<sub>50</sub> values (Fig. 3). These results complement the anti-angiogenic *in vitro* data of TOL and HT. Although TOL showed a higher anti-VEGF effect by inhibiting VEGFR-2 and PLC $\gamma$ 1 phosphorylation than HT *in vitro*, the  $ex\ vivo$  model, which incorporates all angiogenic functions, showed that HT has higher potential. This could be because these compounds would not only influence the first steps of angiogenesis but also the further stages, in which HT should have a more relevant impact.

Only a few studies have shown the effect of polyphenols on the formation of sprouts in aortic rings. The study by Lavaud  $et~al.^{54}$  is one of the few works confirming the ex~vivo anti-VEGF effect of certain polyphenols. They found that 2-deprenylrheediaxanthone (DRX) at 8  $\mu$ M, isolated from *Garcinia vieillardii*, significantly reduced the vessel area of mouse aortic rings, although they did not disclose the part of the tree from which the compound was extracted, nor whether it was edible.

Another study found in the literature about polyphenols and mouse aortic rings was conducted by Lu  $et~al.^{23}$  They evaluated the effect of cinnamon extract (30  $\mu g~mL^{-1}$ ) on the formation of sprouts in chicken aortic rings and observed an inhibitory effect on the formation of new blood vessels. However, the bioactive profile of the extract and their concentrations were not disclosed; therefore, the effect cannot be attributed to known compounds.

Taken together, our data revealed for the first time a novel biological function of TOL and HT by an *ex vivo* aortic ring assay, which confirms previous studies and provides new insights into the inhibitory effects of TOL and HT against VEGFR-2. As a natural inhibitor of VEGFR-2, TOL and HT have the potential to be routine diet-based strategies for cancer and cardiovascular prevention or treatment.

#### Conclusions

This study is the first to use the aortic ring model to demonstrate the effects of TOL and HT on *ex vivo* angiogenesis inhibition. The aortic ring model is, therefore, a fundamental tool for evaluating angiogenesis *ex vivo*, as it not only allows for the

observation of new vessel formation but also provides a controlled environment where the impact of different treatments on the underlying molecular mechanisms, such as VEGFR-2 activation and associated pathways, can be assessed. In addition, in this study, we observed that TOL completely blocks PLC $\gamma$ 1 activation, a key component of the VEGFR-2-mediated signalling pathway, and significantly increased the phosphorylation of Akt and eNOS, critical molecules in the regulation of angiogenesis and vasodilation, in a higher extent than that previously observed with HT.

These results not only support the potential of these compounds as natural anti-angiogenic agents but also offer a new perspective on how diet, especially the Mediterranean diet, may influence the prevention and treatment of angiogenesis-related diseases, such as cancer and cardiovascular diseases.

#### **Author contributions**

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Conceptualization: ABC, MCGP, AMT, and CMV. Data curation: ABC, MGF, ASG, and RHO. Formal analysis: ABC, MGF, ASG, and RHO. Funding acquisition: MCGP, AMT, ABCL, and CMV. Investigation: ABC, MGF, ASG, and RHO. Methodology: ABC, ASG, RHO, AMT, MCGP, and CMV. Project administration: ABCL, MCGP, AMT, and CMV. Supervision: ABCL, MCGP, AMT, CMV, and ASG. Writing—original draft: ABC, MGF, and RHO. Writing—review and editing: ABC, MCGP, AMT, ASG, and CMV.

#### Conflicts of interest

There are no conflicts to declare.

## Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Supplementary information (SI) is available. Raw images of the full blots for phospho (left) and total (right) PCLγ1 (SI 1), Akt (SI 2) and eNOS (SI 3) antibodies. See DOI: https://doi.org/10.1039/d5f002820e.

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