This is the peer reviewed version of the article accepted for publication in J. Agric. Food Chem. 2019, 67, 3909−3918, which has been published in final form at https://10.1021/acs.jafc.9b00282.

Inhibition of VEGFR-2 Phosphorylation and Effects on Downstream Signaling Pathways in Cultivated Human Endothelial Cells by Stilbenes from Vitis Spp

Fernandez-Cruz, Edwin.[1], Cerezo, Ana B.[1], Cantos-Villar, Emma.[2], Richard, Tristan[3], Troncoso, Ana M[1], Garcia-Parrilla, M. Carmen[1]*.

[1]Departamento de Nutrición y Bromatología, Toxicología y Medicina Legal. Facultad de Farmacia. Universidad de Sevilla. C/ Profesor García González 2, 41012, Sevilla, Spain

[2]Instituto de Investigación y Formación Agraria y Pesquera (IFAPA), Centro Rancho de la Merced, Consejería de Agricultura, Pesca y Desarrollo Rural (CAPDA), Junta de Andalucía. Ctra. Trebujena, Km 2.1, 11471 Jerez de la Frontera, Spain.

[3]Université de Bordeaux, ISVV Bordeaux-Aquitaine, 71 Avenue Edouard Bourleaux, 33883 Villenave d'Ornon Cedex, France.

*Corresponding author. Tel.: +34 95 4556759.

•E-mail address: mcparrilla@us.es (M.C. Garcia-Parrilla)

ABSTRACT

Stilbenes are phenolic compounds present in different higher plant families, which have shown different biological activities such as antioxidant properties, anti-tumoral and anti-atherosclerotic effects, among others. Angiogenesis is a key process involved in both cancer and cardiovascular diseases, the vascular endothelial growth factor (VEGF) and its receptor VEGFR-2 being the main triggers. Certain polyphenol compounds such as flavonoids have shown a potent capacity to inhibit VEGF and consequently, angiogenesis. The present work, therefore, aims to evaluate the potential effect of stilbenes on inhibiting VEGF and their subsequent effect on the downstream signaling pathway (PLCγ1, Akt and eNOS). VEGFR-2 activation was study through ELISA assay in HUVEC line while the phosphorylation of intracellular downstream proteins PLCγ1, Akt and eNOS were tested by western-blot. Student's t-test was used to determine significant differences between samples. On the one hand, astringin, pallidol and ω-viniferin showed the lowest IC_{50} values (2.90 ± 0.27, 4.42 ± 0.67 and 6.10 ± 1.29 µM, respectively) against VEGFR-2 activation. Additionally, VEGF-induced PLCγ1 phosphorylation was significantly inhibited by ε-viniferin, astringin and ω-viniferin. However, ε-viniferin and pallidol simultaneously enhanced eNOS activation, proving to be via Akt activation in the case of ε-viniferin. . For the first time, these data suggest that stilbenes such as astringin, pallidol, ω-viniferin and ε-viniferin have a potential anti-angiogenic effect and they could be further considered as anti-VEGF ingredients in food and beverages. In addition, ε-viniferin and pallidol significantly allowed eNOS activation and could likely prevent the side effects caused by anti-VEGF hypertension drugs.

Keywords: Antiangiogenic, Astringin, HUVEC, Stilbenes, VEGF

1. INTRODUCTION

Stilbenes are secondary metabolites classified as non-flavonoid polyphenols with a monomeric structure comprising two aromatic rings joined by an ethylene ring[1]. These compounds derive from the amino acid phenylalanine via the phenylpropanoid pathway, through the enzyme stilbene synthase[2] and they are synthesized by different higher plant families such as *Leguminoseae*, *Pinaceae* and *Vitaceae* among others[3]. The most representative compound of the stilbenes family is resveratrol, found in foods such as cranberries, peanuts, pistachios or chocolate. However, table grapes and red wine are the main source of dietary intake[4]. As well as resveratrol, other stilbenes, such as astringin, piceatannol, pallidol, ε-viniferin and hopeaphenol, have also been described in grapes and wines (Table 1)[5-18].

Moreover, not only is ε-viniferin present in diet sources, it is also present in grape cane, grapevine and root extracts of *Vitis vinifera*, as well as other stilbenoids compounds such as ω-viniferins, *r*-viniferin, *r2*-viniferin and ampelopsin A. These are currently being studied for their promising biological effects[19] and also as an alternative to SO_2 in winemaking[20].

Resveratrol has shown antioxidant, anti-inflammatory, antidiabetic, neuroprotective, antiaging, anti-cancer and cardioprotective effects[5, 21]. With regard to its cardioprotective effect, resveratrol has been proven to reduce atherosclerotic plaques formation and it restores flow-mediated dilation in rabbits fed on a high-cholesterol diet[22]. Resveratrol supplementation in mice also delayed spontaneous mammary tumor development and reduced metastasis[23]. Additionally, pterostilbene, a resveratrol methoxylated monomer, has shown antioxidant properties[24]; it reduces blood pressure[25], improves cardiac function[26] and exerts inhibiting an effect on aortic vascular smooth muscle cells growth[27].

Furthermore, piceatannol has demonstrated an inhibitory effect on the proliferation and migration of human aortic smooth muscle cells[28].

Angiogenesis, which involves the formation of new capillary vessels from pre-existing ones[29], plays a crucial role in both cancer and cardiovascular diseases. It drives tumor cell growth as well as the development and destabilization of atherosclerotic plaques[30, 31]. The most pro-angiogenic factor involved in this process is the vascular endothelial growth factor (VEGF)[32] by binding to its receptor VEGFR-2, which is the main mediator of the proliferation, migration, survival and permeability process on endothelial cells[33]. In fact, VEGF inhibition is an objective for current pharmacological cancer therapies. Anti-VEGF antibodies such as Avastin®, among other drugs, have recently been developed and approved for the treatment of colon, lung, breast, kidney and liver cancer[34] However, their lengthy treatment causes serious side effects, increasing the risk of hypertension, since, as a consequence of VEGF signaling inhibition[35], anti-VEGF drugs also inhibit nitric oxide (NO) production, this latter being a potent vasodilator..

Recently, it has been reported that VEGF is the key molecular target for certain polyphenol compounds, such as epigallocatechin gallate (EGCG), procyanidin oligomers and quercetin, among others, which potently inhibit VEGF signaling and angiogenesis at physiological concentrations (IC_{50}: 0.08-1 µM)[36, 37]. Additionally, EGCG and tetrameric procyanidin inhibited phospholipase gamma 1 (PLCγ1), the principal regulator of the cell proliferation, while they increased activation (phosphorylation) of endothelial nitric oxide synthase (eNOS) via protein kinase B (Akt), which still might induce NO bioavailability[36]. Such data show that these bioactive compounds might be a promising alternative for cancer and cardiovascular diseases prevention, reducing VEGF-induced angiogenesis while avoiding eNOS inhibition, and the subsequent hypertension risk caused by treatment with drugs[38].

Resveratrol has been the only stilbene compound evaluated for inhibiting VEGF-induced VEGFR-2 activation, showing a weak inhibitory effect (24 % of inhibition at 50 μM)[37]. However, the inhibitory effect of the different compounds of this polyphenol family needs to be explored, since different substituents on the phenolic rings (total OH, catechol, methyl and glycosyl groups) affect their potential inhibitory effect[37]. The aim of this work is, therefore, to evaluate the potential anti-VEGF effect of twelve different stilbene compounds present in food, including monomers, dimers and tetramers, on VEGF inhibition and their subsequent effect on the downstream signaling pathway (PLCγ1, Akt and eNOS) in endothelial cells.

2. MATERIAL AND METHODS

2.1. Chemicals

Human Umbilical Vein Endothelial Cells (HUVEC) and the Endothelial Cell Growth Medium-2 (EGM-2) were provided by Lonza (Slough, UK). Rhapontin and pterostilbene standards, as well as dimethyl sulfoxide, were purchased from Sigma-Aldrich (Steinheim, Germany). Methanol and acetonitrile were provided by Merck (Darmstadt, Germany) and formic acid by Prolabo® (Obregón, México). $VEGF_{165}$ was supplied by R&D Systems (Minneapolis, USA). The PathScan Phospho-VEGFR-2 (Tyr1175) sandwich ELISA kit was provided by Cell Signaling Technology (Danvers, MA, USA). For western blot assay, sample buffer LDS (4x), sample reducing agent DTT (10x), antioxidant and 4-12 % Bis-Tris gels were purchased from ThermoFisher Life Technologies (NuPAGE, Waltham, Massachusetts, USA). Nitrocellulose membrane for transfer step was provided by Bio Rad Laboratories (Hercules, California, USA). Bovine serum albumin (BSA) was supplied by Sigma-Aldrich (Steinheim, Germany). Phospho-PLCγ1 (Tyr 783), PLCγ1, phospho-Akt (Ser 473), Akt, phospho-eNOS (Ser 1177), eNOS and anti-rabbit IgG-HRP antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

SuperSignal West Pico chemiluminescent substrate was supplied by Thermo Scientific (Hitchin, UK).

2.2. Cell culture

HUVEC were cultured at 37 ºC in a 5 % CO_2 atmosphere between passages 4 and 5 in 6-well plates until completely confluence.

2.3. Stilbenes extraction and purification

Figure 1 shows the chemical structure of the stilbenes included in the present work: ampelopsin A, astringin, ε-viniferin, hopeaphenol, isohopeaphenol, pallidol, piceatannol, pterostilbene, rhapontin, *r*-viniferin (vitisin B), *r2*-viniferin (vitisin A) and ω-viniferin. Additionally, resveratrol and piceid are included in Figure 1 for comparative purposes. Except for piceatannol and rhapontin, the rest of stilbenes were extracted and purified (> 92% purity) from a raw grapevine shoot following the method described by Biais et al.[39], except for astringin, whose extraction was based on Gabaston et al. method[40]. Purity was determined using UPLC with UV detection at 280 nm in an Agilent Zorbax C18 column (100 × 2.1 mm) with a particle size of 1.8 μm. Two mobile phases consisting of water with 0.1% of formic acid (A) and acetonitrile (B) were used, with a flow rate of 0.4 mL/min. The chromatograms for the stilbenes tested are shown in Supplementary material (Figure S1). Before cell treatment, each compound was diluted in dimethyl sulfoxide (DMSO) at different stock concentrations (between 1 and 50 mM) and stored at -20 ºC until HUVEC treatment.

2.4. Determination of VEGFR-2 phosphorylation by ELISA

Vehicle control (≤ 0.1 % DMSO) and VEGF at 25 ng/mL concentration (≤ 0.1 % DMSO) were used as negative and positive controls, respectively. We followed the previously described plausible molecular mechanism for polyphenols VEGF inhibition[36] on

endothelial cells in which VEGF is proven to be the molecular target for different polyphenols. Confluent HUVEC cells were incubated separately for 5 minutes with different concentrations of stilbenes (1-50 µM for monomers and dimers; 1 µM for tetramers) which had been pre-incubated (for 5 minutes) with VEGF (25 ng/ml) using Endothelial Basal Medium (EBM) (DMSO final concentration < 0.1 %). . Every sample was performed in duplicate. HUVECs were then lysed with radioinmunoprecipitation assay (RIPA) buffer and subsequently centrifuged at 4 ºC, 13000 rpm for 10 minutes. Finally, a bicinchoninic acid assay (BCA) was performed in order to determine protein content in the supernatant. Every experiment was carried out in duplicate.

To measure the phosphorylated VEGFR-2 levels in the lysates, the Phospho-VEGFR-2 (Tyr1175) sandwich ELISA kit was used following the manufacturer's instruction. Half maximal inhibitory concentration (IC_{50}) with confidence intervals was then determined for each stilbene with the GraphPad Prism software v. 6.00 (GraphPad Software, La Jolla California USA). Each sample was also analyzed in duplicate.

2.5. Western Blot analysis for PLCγ1, Akt and eNOS

Compounds with the highest anti-VEGF effect were evaluated for their potential effect on modulating the activity of the dowmstream signaling proteins PLCγ1, Akt and eNOS. The experimental conditions were the same as those explained above in section 2.4, except for the HUVEC cells which were incubated with the stilbene+VEGF mixture or stilbene alone for 10 minutes in the case of PLCγ1, and 60 minutes for Akt and eNOS evaluation. The stilbene doses used were, according to their respective IC_{50} values for VEGFR-2 inhibition, as follows: 10 µM for pallidol, ω-viniferin and astringin; and 50 µM for ε-viniferin and piceatannol. All compounds were tested in duplicate. The protein lysates (26.8 µg) were mixed with the sample buffer and the reducing agent before heating them for 10 minutes at 70 ºC to denature proteins. Subsequently, electrophoresis was

167 performed in the Bis-Tris gel and then, proteins were transferred into nitrocellulose
168 membranes (0.2 µM). Tris-buffered saline with Tween® 20 (TBST) was mixed with BSA
169 to a final concentration of 5 % for blotting the membranes before adding primary
170 antibodies (p-PLCγ1, PLCγ1, p-Akt, Akt, p-eNOS, eNOS). The primary antibodies were
171 then incubated overnight with the membranes at 4 ºC. Subsequently, a secondary antibody
172 (anti-rabbit IgG-HRP) in TBST+BSA (5 %) was added to incubate membranes for 1h at
173 room temperature. After treating membranes with the chemiluminescent substrate, image
174 analysis of bands was performed using an Amershan Imager 600 station (GE Healthcare
175 life sciences, Marlborough, MA, USA). Samples were analyzed in triplicate.

176 2.6. Statistical analysis

177 GraphPad Prism software V.6.00 (GraphPad Software, La Jolla California USA) was
178 used to determine significant differences between samples through Student's t-test. The
179 degree of significance of the analysis was as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p<0.001$
180 (***) and $p<0.0001$ (****). Data were displayed as mean ± standard deviation in all cases.

181 3. RESULTS & DISCUSSION

182 3.1. Inhibition of VEGFR-2 activation by stilbenes

183 It has already been demonstrated that certain polyphenols inhibit VEGFR-2 activation by
184 binding tightly to VEGF. As a consequence, it is prevented from binding to the receptor[36].
185 More than fifty polyphenols compounds from different families have been tested for their
186 anti-VEGF effect. The only stilbene reported, however, was resveratrol which showed a
187 weak inhibition of VEGFR-2 phosphorylation (24 % at 50 µM). Since different structural
188 features affect potential VEGF inhibition, the present work assesses for the first time the
189 potential anti-angiogenic effect of this family of polyphenols on twelve different
190 stilbenes, including monomers (astringin, piceatannol, pterostilbene and rhapontin),

dimers (ampelopsin A, ε-viniferin, pallidol and ω-viniferin) and tetramers (hopeaphenol, isohopeaphenol, *r*-viniferin and *r2*-viniferin) (Table 2).

3.1.1. Monomers

Piceatannol has quite a similar structure to resveratrol with an additional OH group forming a catechol group which enhances the inhibition of the VEGFR-2 phosphorylation, showing a 3.5-fold higher inhibitory effect than resveratrol (IC_{50} = 39.7 µM). Similarly, the presence of a catechol group on flavonoids such as luteolin, orobol or quercetin showed an enhanced inhibitory effect on VEGFR-2 activation[37]. Piceatannol has been previously reported to reduce VEGF expression in cancer cells, indirectly preventing angiogenesis[41]. However, this is the first time that piceatannol has been proven to inhibit directly VEGF-induced VEGFR-2 phosphorylation on endothelial cells. Considering the highest piceatannol concentration presents in grapes (702 µg/kg, Table 1) and its bioavailability (50 %)[42], 3 portions of grapes (average grapes portion = 125 g) would be enough to reach its IC_{50} concentration in plasma. These data are in line with a balanced diet.

Astringin, which is the piceatannol glucoside (Figure 1), was the tested stilbene that exhibited the highest VEGFR-2 phosphorylation inhibitory activity (IC_{50} = 2.90 µM), showing a 14-fold greater effectiveness against VEGFR-2 phosphorylation than its aglycone piceatannol (IC_{50} = 39.7 µM). In contrast, piceid, which is the main natural resveratrol glucoside form, did not show significant differences on the inhibition of VEGFR-2 phosphorylation compared with resveratrol (28 % and 24 % respectively, at 50 µM)[37]. Additionally, Cerezo et al. (2015) have previously shown that different glycosylated forms of quercetin were completely ineffective in inhibiting VEGFR-2 phosphorylation[37] compared with quercetin (IC_{50} = 0.75 µM). In the case of stilbenes, the presence of the catechol group in one of the rings of the astringin and a 3-*O*-glucoside

groupappears to be the structural combination that best enhances anti-VEGF activity. Therefore, our present results prove for the first time that a glycoside polyphenol can be also a good candidate for anti-VEGF activity.

Considering the highest astringin concentration reported in red wine (38.1 mg/L, Table 1) and the bioavailability data for related stilbenoids (piceatannol, pterostilbene and resveratrol 50 %, 80 % and 30 % of bioavailability, respectively)[42, 43], and, since there is no available dataon astringin bioavailability, between 2 and 4 glasses of red wine (average glass of wine = 150 mL) would be sufficient to reach an active astringin concentration in plasma (3.4-4.5 μM). Since, for a healthy diet, alcohol content limits wine consumption, food supplements would be a good option for achieving an active astringin concentration in plasma without the limitations imposed by alcohol.

Pterostilbene and rhapontin, the other two monomers tested, present methyl groups in their structures. Pterostilbene (3',5'-dimethoxy-resveratrol) results from a double methylation of resveratrol in positions 3' and 5'. Although pterostilbene provoked a low inhibitory activity against VEGFR-2 phosphorylation at 50 μM (40 %), it was more effective than resveratrol (24 %). In flavonoids, methyl groups on the B-ring strongly diminished bioactivity against VEGF phosphorylation[37]. In stilbenes, it appears that the presence of methyl groups allows certain bioactivity against VEGFR-2 phosphorylation. On the other hand, rhapontin, which presents a methyl group on 4’ position and a 3-O-glucosyde group, was completely ineffective at 50 μM.. In this case, it appears that the presence of both glycoside and methyl groups strongly diminished the effect against VEGFR-2 phosphorylation.

3.1.2. Dimers

The order of anti-VEGF potency for stilbenes dimers were as follows: pallidol (IC_{50} = 4.42 μM) > ω-viniferin (IC_{50} = 6.10 μM) > ε-viniferin (IC_{50} = 18.8 μM). Ampelopsin A

241 was completely ineffective at 50 µM. At 10 µM ε-viniferin has previously shown an anti-
242 proliferative effect () on vascular smooth muscle cells[44]. This, however, is the first time
243 that ε-viniferin and ω-viniferin have shown anti-VEGF activity by inhibiting VEGF-
244 induced VEGFR-2 phosphorylation. Comparing the anti-VEGF activity of stilbenoid
245 dimers with previously reported procyanidins dimers[37], the former present geather
246 effectiveness (IC_{50} = 4.42 – 18.8 µM, IC_{50} = 52.6 µM, respectively). The reactivity
247 differences between stilbene dimers could be related to their three-dimensional structures.
248 For example, ω- and ε-viniferin have the same plane structure and differ only by the
249 orientation of their phenol rings. Cerezo et al. (2015) have demonstrated that flavonoids
250 with near-planarity on their structure such as quercetin or myricetin displayed potent anti-
251 VEGF activity[37], since near-planar structures have been shown to enter hydrophobic
252 pockets in proteins more easily[45]. In addition, the complexity of the ampelopsin A
253 structure might not enter the pocket of the VEGF molecule, rendering it completely
254 ineffective[36]. Structure-activity relationship studies are needed to better understand the
255 properties of these compounds.

256 Bioavailability data for dimers have been reported for ε-viniferin only (0.77 %)[46].
257 Considering also its highest concentration described in wine (4.35 mg/L, Table 1), 8
258 glasses of wine would be necessary to reach its IC_{50} value in plasma. If, given the
259 similarity in structure, we assume a similar bioavailability for pallidol which is the most
260 active dimer, and its highest concentration found in wine (2.22 mg/L, Table 1), 4 glasses
261 of wine would be needed to achieve the IC_{50} value in plasma. In both cases, the doses
262 exceed the acceptable quantity of wine in a balanced diet (1-2 glasses). Food supplements
263 including ε-viniferin and pallidol dimmers would, therefore, be a good strategy to achieve
264 an active concentration in plasma.

265 3.1.3. Tetramers

High molecular weight stilbenes (tetramers) such as hopeaphenol, isohopeaphenol, *r*-viniferin and *r2*-viniferin caused a low inhibitory effect (<25 %) on VEGFR-2 phosphorylation at 1 μM (Table 2). These results contrast with those obtained in procyanidins, where the more polymerized structures confer the greatest activity against VEGFR-2 phosphorylation, reaching IC_{50} values of 0.28 μM for the procyanidin tetramer[37].

Considering that resveratrol (monomer) and ε-viniferin (resveratrol dimer) bioavailability (30 % and 0.77 %, respectively)[42,46] decrease with the degree of polymerization, bioavailability can be expected to be much lower for hopeaphenol, isohopeaphenol, *r*-viniferin and *r2*-viniferin (resveratrol tetramers). Additionally, the low anti-VEGF activity of the tetramers at 1 μM (Table 2) render them irrelevant as anti-VEGF agents, since an insufficient concentration for them to be active would be expected in plasma.

3.2. Modulation of downstream signaling proteins (PLCγ1, Akt and eNOS) in HUVEC by stilbenes

To check whether the inhibition of VEGF-induced VEGFR-2-activating activity by stilbenes also regulates signaling events downstream of pVEGFR-2, the inhibition of PLCγ1 phosphorylation, (PLCγ1 being the first protein activated in response to VEGFR-2 activation and responsible for cell proliferation), by the most potent stilbenes (piceatannol, astringin, pallidol, ε -viniferin and ω-viniferin) was evaluated. In addition, Akt and eNOS phosphorylation, activated later in the VEGF signaling cascade and accountable for NO production were also tested. Two sets of concentrations were assessed, depending on the IC_{50} values of stilbenes, to ensure that VEGFR-2 phosphorylation was inhibited completely (10 μM for astringin, pallidol and ω-viniferin; and 50 μM for piceatannol and ε-viniferin).

3.2.1. Phosphorylation of PLCγ1

Pallidol and piceatannol presented IC_{50} values for VEGFR-2 inhibition below 10 μM and 50 μM respectively (Table 2), but they had either no effect or a very low effect (6 %), respectively, against the inhibition of the PLCγ1 phosphorylation in the presence of VEGF (Figure 2A and 2E). The data, therefore, suggest that these compounds might be acting in other proteins also involved in endothelial cell migration and as a consequence in angiogenesis mediation, such as Shb-Sck-PI9K-Rac-IQGAPI (also mediated by VEGFR-2 tyrosin 1175)[47]. Further research should, therefore, be carried out on the effects of pallidol and piceatannol on the inhibition of the Shb protein phosphorylation.

Astringin, ε- and ω-viniferins showed a significant inhibition of VEGF-induced PLCγ1 phosphorylation (86, 55 and 68 %, respectively), astringin being the most effective (Figure 2 C-F). Astringin treatment in the presence of VEGF presented a pPLCγ1/PLCγ1 7-fold lower ratio than VEGF alone (Figure 2C), while ε-viniferin reached a 3 fold lower ratio (Figure 2E). These data are in agreement with Moyle et al. (2015)[36] who showed that certain polyphenols such as a procyanidin tetramer isolated from apple (dp4) and EGCG from green tea inhibited VEGFR-2 phosphorylation and as a consequence they totally inactivated PLCγ1 phosphorylation at 1 μM[36]. As far as we are concerned, our data show for the first time the potential of certain stilbenes for the inhibition of PLCγ1 (at 10 and 50 μM) and, as a likely consequence, the cell proliferation of endothelial cells.

3.2.2. Phosphorylation of Akt and eNOS

The importance of activating eNOS lies on the fact that anti-VEGF drugs currently used in the treatment of colon, lung, breast, kidney and liver cancer[34] have been demonstrated to cause serious side effects, increasing the risk of hypertension by inhibiting the production of NO (a potent vasodilator) as a consequence of VEGF signaling inhibition[35]. Akt is a protein prior activated in the signaling cascade by VEGF that has been proven to activate eNOS phosphorylation, among different alternative pathways.

316 Although ε- and ω-viniferins inhibited VEGF-induced VEGFR-2 and PLCγ1
317 phosphorylation (Table 2 and Figure 2), VEGF-induced phosphorylation of Akt was not
318 inhibited but significantly enhanced by these compounds, both in the presence and
319 absence of VEGF (more than twenty-fold higher for ε-viniferin and more than two-fold
320 higher for ω-viniferin) regarding negative control (Figure 3A-D). On the other hand,
321 pallidol and piceatannol only increased the Akt activation in presence of VEGF (Figure
322 3 E-F), likely due to the fact that neither pallidol at 10 μM or piceatannol at 50 μM
323 completely inhibited VEGFR-2 (84.4 % and 85 % of inhibition, respectively). In spite of
324 the above, the relevant point is that while pallidol and piceatannol are almost completely
325 inhibiting VEGFR-2 phosphorylation, they are able to maintain Akt activation similar to
326 VEGF alone (positive control). However, astringin, which inhibited VEGFR-2 and
327 PLCγ1 phosphorylation, did not enhance the Akt activation in presence or absence of
328 VEGF (Figure 3A).

329 Since pAkt is known to activate the eNOS enzyme (peNOS), we evaluated the effects of
330 the stilbenes on eNOS activation. The present data show that VEGF alone increased the
331 peNOS/eNOS ratio (Figure 4). ε-Viniferin increased the peNOS/eNOS by a 3.2 and 3-
332 fold higher ratio in both the presence and absence of VEGF, respectively (Figure 4A).
333 The ε-viniferin data are in agreement with those previously reported for EGCG and dp4
334 also at 10 μM, which, although inhibiting VEGFR-2 and PLCγ1, were able to increase or
335 retain pAkt and peNOS activation in both the presence and absence of VEGF[36]. The
336 hypothesis postulated was that polyphenol compounds were able to activate Akt and
337 eNOS by means of other surface receptors or by directly generating ROS in a receptor-
338 independent fashion, since Kim et al. (2007) demonstrated that EGCG activated Akt,
339 eNOS and NO production in BAECs via these possible ways[48]. Similarly, resveratrol has
340 been reported to activate eNOS via AMP-activated protein kinase, estrogen receptors and

sirtuin 1 on endothelial cells[38]. A similar unexplored mechanism might be involved in the activation of Akt and eNOS by ε- viniferin.

Additionally, pallidol also significantly activated the peNOS/eNOS ratio in both the presence and absence of VEGF (Figure 4C), although our data demonstrate that it is not Akt-mediated (Figure 3E). Considering that not only can eNOS be activated by Akt but also by PKA, AMPK and CAMKII proteins[49], further studies should be performed to determine the protein involved.

On the other hand, only in the presence of VEGF did ω-viniferin slightly increase the peNOS/eNOS ratio regarding negative control (Figure 4B). This could be because ω-viniferin at 10 µM did not inhibited VEGFR-2 phosphorilation completely; there is still a 27.5 % of VEGFR-2 activity (data not shown). The residual VEGFR-2 activity could therefore be responsible for the eNOS activation. In addition, VEGF can also activate eNOS via VEGFR1[50]. Thus, the eNOS activation observed in the presence of VEGF and ω-viniferin could also be VEGFR-1 activation mediated. The present results reinforce the notion that certain polyphenols are potent VEGF inhibitors but may still induce NO production by increasing eNOS phosphorylation, revealing for the first time that ε-viniferin and pallidol show the same trend.

Although, ε-viniferin is mainly present in grapevine shoot, root and grape cane extracts[19], they are gaining importance due to their promising use in winemaking as an alternative to SO_2 in order to avoid its adverse effects[20]. Further research should, therefore, be conducted in order to focus on determining this mechanism and to evaluate the final concentration of these compounds in the final product, and the different winemaking conditions necessary to improve their content in wines.

The other compounds investigated, such as astringin or piceatannol did not show any significant eNOS stimulation (Figures 4), although they have demonstrated their potential

366 to be VEGF inhibitors (Table 2). These data demonstrate that not all of the VEGF-
367 inhibiting polyphenols are able to show the beneficial effect of activating eNOS and thus
368 likely induce NO production.

369 This is the first time that stilbenes such as astringin, pallidol, ω-viniferin and ε-viniferin
370 have shown potential anti-VEGF effects in endothelial cells (most IC_{50} < 10 μM).
371 Additionally, ε- viniferin have been proven to inhibit the downstream VEGF-induced
372 PLCγ1 phosphorylation which is responsible for cell proliferation, while stimulating Akt
373 and eNOS phosphorylation. Pallidol also showed itself able to inhibit VEGFR-2
374 activation while inducing eNOS phosphorylation. The present data suggest for the first
375 time that stilbenes, such as ω-viniferin and pallidol, possess potential anti-angiogenic
376 effect, likely preventing the side effects caused by anti-VEGF drugs on NO
377 bioavailability. Therefore, these compounds present great potential for future exploitation
378 as anti-VEGF ingredients in foods and beverages.

379 **Abbreviations**:

380 Akt, protein kinase B; BCA, bicinchoninic acid; DMSO, dimethyl sulfoxide; EGM-2,
381 endothelial cell growth medium-2; eNOS, endothelial nitric oxide synthase; EGCG,
382 epigallocatechin gallate; HUVEC, human umbilical vein endothelial cells; NO, nitric
383 oxide; PLCγ1, phospholipase gamma 1; RIPA, radioinmunoprecipitation assay buffer;
384 TBST, Tris-buffered saline with Tween® 20; VEGF, vascular endothelial growth factor;
385 VEGFR-2, vascular endothelial growth factor receptor 2.

386 **Acknowledgements**

387 The authors are grateful for the financial assistance provided by the European Regional
388 Development Funds (Project: Research and Technological Innovation in Vitiviniculture,
389 PP.AVA.AVA201601.03) and the Spanish Ministry of Education, Culture and Sport

(MECD scholarship FPU13/04820). The authors also thank the assistance of the University of Seville Biology's central service (CITIUS) and its technical staff (M.J. Carballo Álvarez and L. Navarro Sampedro). The work was supported by the ANR LabCom program (ANR-14-LAB5-0005-01), the Bordeaux Metabolome Facility, and MetaboHUB (ANR-11-INBS-0010 project).

Supporting information:

Funding: This work was funded by the European Regional Development Funds (Project: PP.AVA.AVA201601.03). The authors declare no competing financial interest

Authors' contributions: A.B.C., E.F.C., M.C.G.P., A.M.T., T. R. and E.C.V. designed the research; A.B.C., E.F.C. and T.R. conducted the research; A.B.C., E.F.C., M.C.G.P., A.M.T., T. R. and E.C.V. analyzed the data and wrote the paper. All of the authors read and approved the final manuscript.

Conflicts of interest: E. Fernandez-Cruz, A. B. Cerezo, E. Cantos-Villar, T. Richard, A.M. Troncoso and M.C. García-Parrilla declare that they have no conflicts of interest.

Ethical approval: This study does not contain any studies performed on human participants by any of the authors.

Informed consent: Not applicable.

REFERENCES

1 Rivière, C., Pawlus, A.D., Mérillon, J.M., Natural stilbenoids: distribution in the plant kingdom and chemotaxonomic interest in Vitaceae. *Nat. Prod. Rep.* **2012**, *29*, 1317.

2 Chong, J., Poutaraud, A., Hugueney, P., Metabolism and roles of stilbenes in plants. *Plant Sci.* **2009**, *177*, 143–155.

3 Harborne, J.B., The comparative biochemistry of phytoalexin induction in plants. *Biochem. Syst. Ecol.* **1999**, *27*, 335–367.

4 Guerrero, R.F., García-Parrilla, M.C., Puertas, B., Cantos-Villar, E., Wine, resveratrol and health: a review. *Nat. Prod. Commun.* **2009**, *4*, 635–658.

5 Flamini, R., De Rosso, M., De Marchi, F., Dalla Vedova, A., Panighel, A., Gardiman, M., Maoz, I., Bavaresco, L., An innovative approach to grape metabolomics: Stilbene profiling by suspect screening analysis. *Metabolomics.* **2013**, *9*, 1243–1253.

6 Vitrac, X., Monti, J.P., Vercauteren, J., Deffieux, G., Mérillon, J.M., Direct liquid chromatographic analysis of resveratrol derivatives and flavanonols in wines with absorbance and fluorescence detection. *Anal. Chim. Acta.* **2002**, *458*, 103–110.

7 Vitrac, X., Bornet, A., Vanderlinde, R., Valls, J., Richard, T., Delaunay, J.C., Mérillon, J.M., Teissédre, P.L., Determination of stilbenes (δ-viniferin, trans-astringin, trans-piceid, cis- and trans-resveratrol, ε-viniferin) in Brazilian wines. *J. Agric. Food Chem.* **2005**, *53*, 5664–5669.

8 Naugler, C., McCallum, J.L., Klassen, G., Strommer, J., Concentrations of trans-resveratrol and related stilbenes in Nova Scotia wines. *Am. J. Enol. Vitic.* **2007**, *58*,

117–119.

9 Buiarelli, F., Coccioli, F., Jasionowska, R., Merolle, M., Terracciano, A., Analysis of some stilbenes in Italian wines by liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 2955–2964.

10 Ribeiro De Lima, M.T., Waffo-Téguo, P., Teissedre, P.L., Pujolas, A., Vercauteren, J., Cabanis, J.C., Mérillon, J.M., Determination of stilbenes (trans-astringin, cis- and trans-piceid, and cis- and trans-resveratrol) in Portuguese wines. *J. Agric. Food Chem.* **1999**, *47*, 2666–2670.

11 Rimando, A.M., Kalt, W., Magee, J.B., Dewey, J., Ballington, J.R., Resveratrol, pterostilbene, and piceatannol in Vaccinium berries. *J. Agric. Food Chem.* **2004**, *52*, 4713–4719.

12 Bavaresco, L., Fregoni, M., Trevisan, M., Mattivi, F., Vrhovsek, U., Falchetti, R., The occurrence of piceatannol in grape. *Vitis.* **2002**, *41*, 133–136.

13 Cantos, E., Espín, J.C., Fernández, M.J., Oliva, J., Tomas-Barberan, F.A., Postharvest UV-C-irradiated grapes as a potential source for producing stilbene-enriched red wines. *J. Agric. Food Chem.* **2003**, *51*, 1208–1214.

14 Guebailia, H.A., Chira, K., Richard, T., Mabrouk, T., Furiga, A., Vitrac, X., Monti, J.P., Delaunay, J.C., Mérillon, J.M., Hopeaphenol: The first resveratrol tetramer in wines from North Africa. *J. Agric. Food Chem.* **2006**, *54*, 9559–9564.

15 Landrault, N., Larronde, F., Delaunay, J.C., Castagnino, C., Vercauteren, J., Mérillon, J.M., Gasc, F., Cros, G., Teissédre, P.L., Levels of stilbene oligomers and astilbin in French varietal wines and in grapes during noble rot development. *J. Agric. Food Chem.* **2002**, *50*, 2046–2052.

16 Amira-Guebailia, H., Valls, J., Richard, T., Vitrac, X., Monti, J.P., Delaunay, J.C., Mérillon, J.M., Centrifugal partition chromatography followed by HPLC for the isolation of cis-ε-viniferin, a resveratrol dimer newly extracted from a red Algerian wine. *Food Chem.* **2009**, *113*, 320–324.

17 Hurtado-Gaitán, E., Sellés-Marchart, S., Martínez-Márquez, A., Samper-Herrero, A., Bru-Martinez, R., A focused multiple reaction monitoring (MRM) quantitative method for bioactive grapevine stilbenes by ultra-high-performance liquid chromatography coupled to triple-quadrupole mass spectrometry (UHPLC-QqQ). *Molecules.* **2017**, *22*, 1–15.

18 Arraki, K., Renouf, E., Waffo-Téguo, P., Mérillon, J.M., Richard, T., Decendit, A., Identification and quantification of stilbenes in some Tunisian red wines using UPLC-MS and HPLC-DAD, *Oeno One*, **2017**, 51 (3), 231-236.

19 Guerrero, R.F., Biais, B., Richard, T., Puertas, B., Waffo-Téguo, P., Mérillon, J.M., Cantos-Villar, E., Grapevine cane's waste is a source of bioactive stilbenes. *Ind. Crops Prod.* **2016**, *94*, 884–892.

20 Guerrero, R.F., Cantos-Villar, E., Demonstrating the efficiency of sulphur dioxide replacements in wine: A parameter review. *Trends Food Sci. Technol.* **2015**, *42*, 27–43.

21 Smoliga, J.M., Baur, J.A., Hausenblas, H.A., Resveratrol and health - A comprehensive review of human clinical trials. *Mol. Nutr. Food Res.* **2011**, *55*,

1129–1141.

22 Wang, Z., Zou, J., Cao, K., Hsieh, T.C., Huang, Y., Wu, J.M., Dealcoholized red wine containing known amounts of resveratrol suppresses atherosclerosis in hypercholesterolemic rabbits without affecting plasma lipid levels. *Int. J. Mol. Med.* **2005**, *16*, 533–540.

23 Provinciali, M., Re, F., Donnini, A., Orlando, F., Bartozzi, B., Di Stasio, G., Smorlesi, A., Effect of resveratrol on the development of spontaneous mammary tumors in HER-2/neu transgenic mice. *Int. J. Cancer.* **2005,** *115*, 36–45.

24 McCormack, D., McFadden, D., A review of pterostilbene antioxidant activity and disease modification. *Oxid. Med. Cell. Longev.* **2013**, *2013*.

25 Riche, D.M., Riche, K.D., Blackshear, C.T., McEwen, C.L., Sherman, J.J., Wofford, M.R., Griswold, M.E., Pterostilbene on Metabolic Parameters : A Randomized , Double-Blind , and Placebo-Controlled Trial. **2014**, *2014*.

26 Yu, Z., Wang, S., Zhang, X., Li, Y., Zhao, Q., Liu, T., Pterostilbene protects against myocardial ischemia/reperfusion injury via suppressing oxidative/nitrative stress and inflammatory response. *Int. Immunopharmacol.* **2017**, *43*, 7–15.

27 Park, E.S., Lim, Y., Hong, J.T., Yoo, H.S., Lee, C.K., Pyo, M.Y., Yun, Y.P., Pterostilbene, a natural dimethylated analog of resveratrol, inhibits rat aortic vascular smooth muscle cell proliferation by blocking Akt-dependent pathway. *Vascul. Pharmacol.* **2010**, *53*, 61–67.

28 Choi, K.H., Kim, J.-E., Song, N.R., Son, J.E., Hwan, M.K., Byun, S., Kim, J.H., Lee, K.W., Lee, H.J., Phosphoinositide 3-kinase is a novel target of piceatannol for inhibiting PDGF-BB-induced proliferation and migration in human aortic smooth muscle cells. *Cardiovasc. Res.* **2010**, *85*, 836–844.

29 Celletti, F.L., Waugh, J.M., Amabile, P.G., Brendolan, A., Hilfiker, P.R., Dake, M.D., Vascular endothelial growth factor enhances atherosclerotic plaque progression. *Nat. Med.* **2001**, *7*, 425–429.

30 Camaré, C., Pucelle, M., Nègre-Salvayre, A., Salvayre, R., Angiogenesis in the atherosclerotic plaque. *Redox Biol.* **2017**, *12*, 18–34.

31 Hicklin, D.J., Ellis, L.M., Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. *J. Clin. Oncol.* **2005**, *23*, 1011–1027.

32Ferrara, N., Vascular endothelial growth factor: Basic science and clinical progress. *Endocr. Rev.* **2004**, *25*, 581–611.

33 Cébe-Suarez, S., Zehnder-Fjällman, A., Ballmer-Hofer, K., The role of VEGF receptors in angiogenesis; complex partnerships. *Cell. Mol. Life Sci.* **2006**, *63*, 601–615.

34 Roviello, G., Bachelot, T., Hudis, C.A., Curigliano, G., Reynolds, A.R., Petrioli, R., Generali, D., The role of bevacizumab in solid tumours: A literature based meta-analysis of randomised trials. *Eur. J. Cancer* **2017**, *75*, 245–258.

35 Li, M., Kroetz, D.L., Bevacizumab-induced hypertension: Clinical presentation and molecular understanding. *Pharmacol. Ther.* **2018**, *182*, 152–160.

36 Moyle, C.W.A., Cerezo, A.B., Winterbone, M.S., Hollands, W.J., Alexseev, Y., Needs, P.W., Kroon, P.A., Potent inhibition of VEGFR-2 activation by tight binding of green tea epigallocatechin gallate and apple procyanidins to VEGF: Relevance to angiogenesis. *Mol. Nutr. Food Res.* **2015**, *59*, 401–412.

37 Cerezo, A.B., Winterbone, M.S., Moyle, C.W.A., Needs, P.W., Kroon, P.A., Molecular structure-function relationship of dietary polyphenols for inhibiting

525 VEGF-induced VEGFR-2 activity. *Mol. Nutr. Food Res.* **2015**, *59*, 2119–2131.

526 38 Escalante, C.P., Zalpour, A., Vascular endothelial growth factor inhibitor-induced
527 hypertension: Basics for primary care providers. *Cardiol. Res. Pract.* **2011**, *1*.

528 39 Biais, B., Krisa, S., Cluzet, S., Da Costa, G., Waffo-Teguo, P., Mérillon, J.M.,
529 Richard, T., Antioxidant and cytoprotective activities of grapevine stilbenes. *J.*
530 *Agric. Food Chem.* **2017**, *65*, 4952–4960.

531 40 Gabaston, J., Richard, T., Biais, B., Waffo-Teguo, P., Pedrot, E., Jourdes, M.,
532 Corio-Costet, M.F., Mérillon, J.M., Stilbenes from common spruce (*Picea abies)*
533 bark as natural antifungal agent against downy mildew (*Plasmopara viticola*). *Ind.*
534 *Crops Prod.* **2017**, *103*, 267–273.

535 41 Tang, Y.-L., Chan, S.-W., A review of the pharmacological effects of piceatannol
536 on cardiovascular diseases. *Phytother. Res.* **2014**, *28*, 1581–1588.

537 42 Akinwumi, B.C., Bordun, K.A.M., Anderson, H.D., Biological activities of
538 stilbenoids. *Int. J. Mol. Sci.* **2018**, *19*, 1–25.

539 43 Lin, H.S., Tringali, C., Spatafora, C., Wu, C., Ho, P.C., A simple and sensitive
540 HPLC-UV method for the quantification of piceatannol analog trans-3,5,3′,4′-
541 tetramethoxystilbene in rat plasma and its application for a pre-clinical
542 pharmacokinetic study. *J. Pharm. Biomed. Anal.* **2010**, *51*, 679–684.

543 44 Zghonda, N., Yoshida, S., Araki, M., Kusunoki, M., Mliki, A., Ghorbel, A.,
544 Miyazaki, H., Greater effectiveness of ε-Viniferin in red wine than its monomer
545 resveratrol for inhibiting vascular smooth muscle cell proliferation and migration.
546 *Biosci. Biotechnol. Biochem.* **2011**, *75*, 1259–1267.

547 45 Atrahimovich, D., Vaya, J., Khatib, S., The effects and mechanism of flavonoid-

rePON1 interactions. Structure-activity relationship study. *Bioorganic Med. Chem.* **2013**, *21*, 3348–3355.

46 El Khawan, T., Courtois, A., Valls, J., Richard, T., Krisa, S., A review of dietary stilbenes: sources and bioavailability, *Phytochem. Rev*, **2018**, 17 (5), 1007-1029.

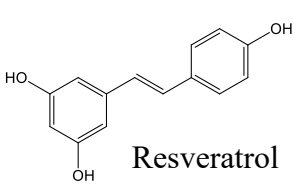
47 Olsson, A.K., Dimberg, A., Kreuger, J., Claesson-Welsh, L., VEGF receptor signalling- in control of vascular function. *Nat Rev Mol Cel Biol*. **2006**, 7, 359-371.

48 Kim, J.A., Formoso, G., Li, Y., Potenza, M.A., Marasciulo, F.L., Montagnani, M., Quon, M.J., Epigallocatechin gallate, a green tea polyphenol, mediates NO-dependent vasodilation using signaling pathways in vascular endothelium requiring reactive oxygen species and fyn. *J. Biol. Chem.* **2007**, *282*, 13736–13745.

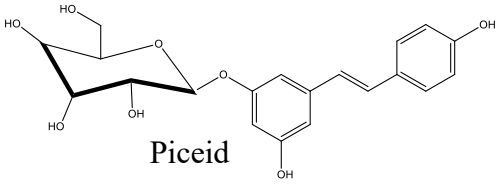
49 Fleming, I., Molecular mechanisms underlying the activation of eNOS, *Pflügers Archiv - European Journal of Physiology*, **2010**, 459 (6), 793-806.

50 Kimura, Y., Sumiyoshi, M., Baba, K., Antitumor activities of synthetic and natural stilbenes thorugh antiangiogenic action, *Cancer Sci*, **2008**, 99 (10), 2083-2096.

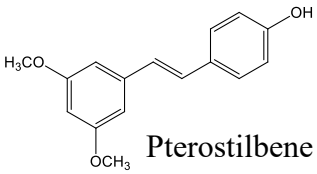
Figure 1



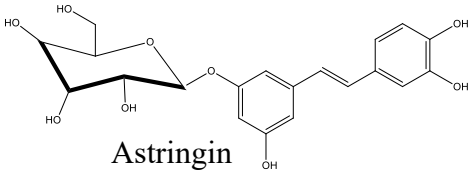
Resveratrol



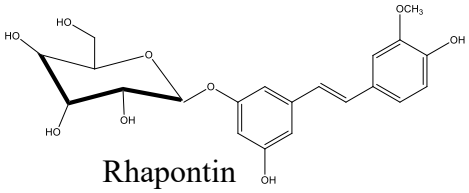
Piceid



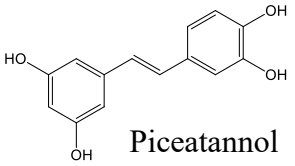
Pterostilbene



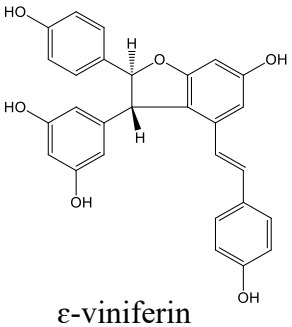
Astringin



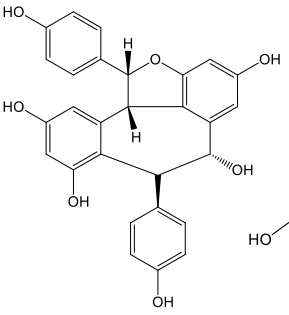
Rhapontin



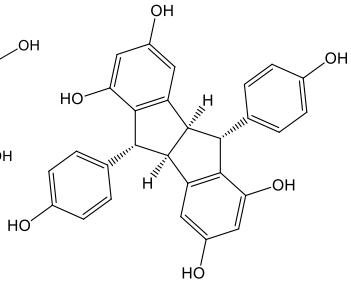
Piceatannol



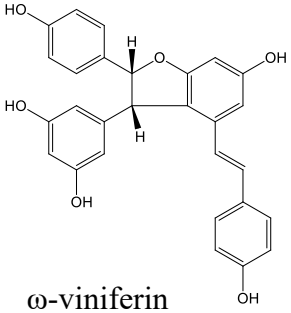
ε-viniferin



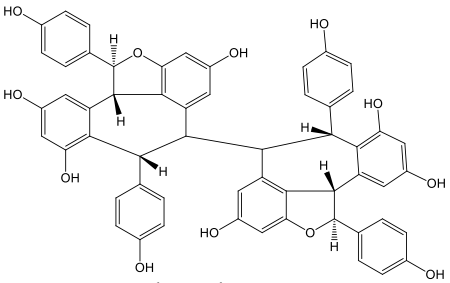
Ampelopsin A



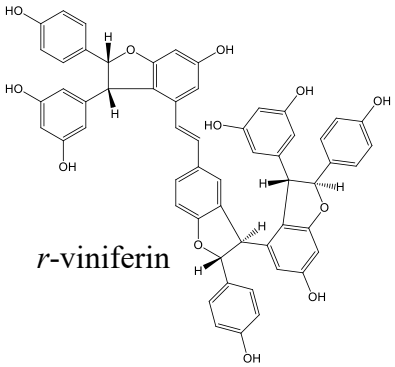
Pallidol



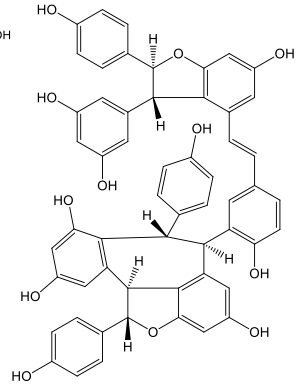
ω-viniferin



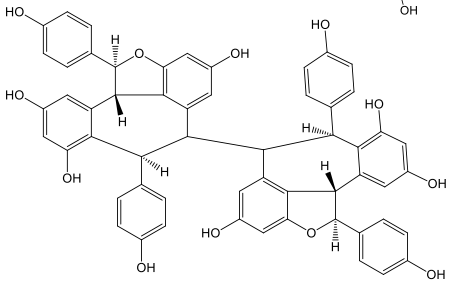
Hopeaphenol



r-viniferin

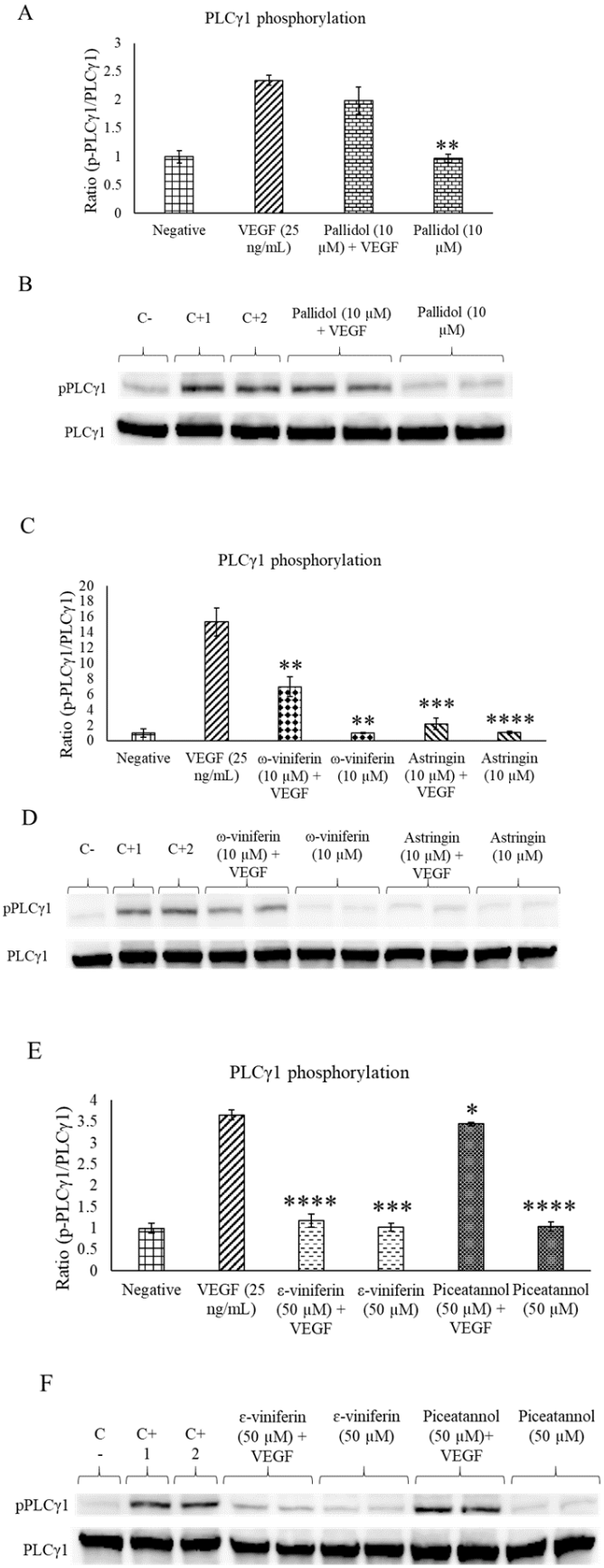


r2-viniferin



Isohopeaphenol

564 **Figure 2**



565

566

Figure 3

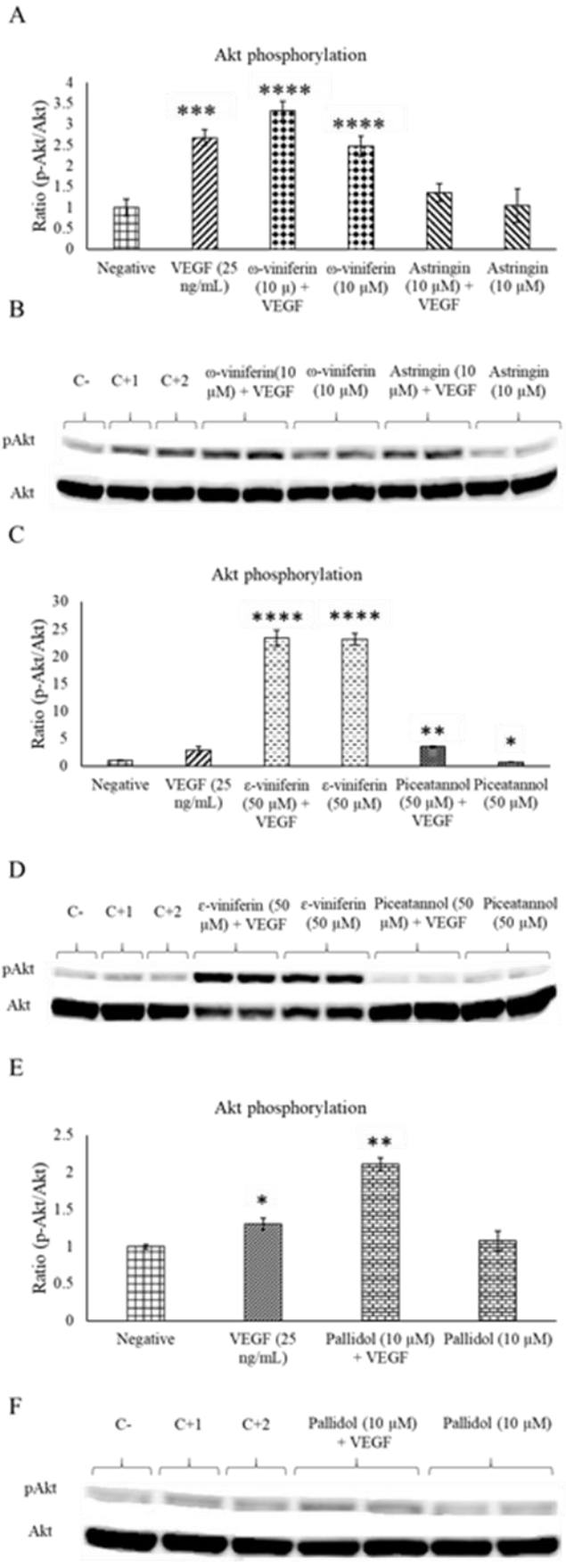
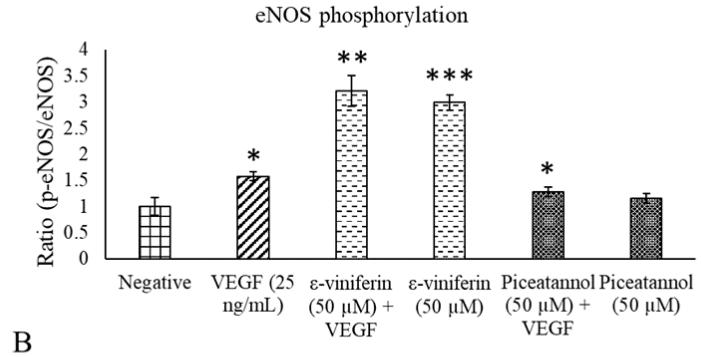
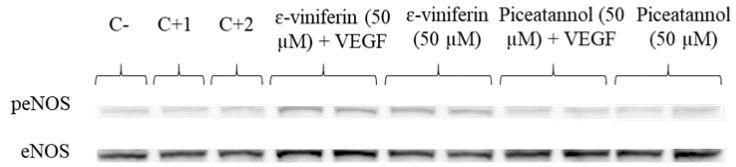

A
Akt phosphorylation
Ratio (p-Akt/Akt)
***
****
****
Negative
VEGF (25 ng/mL)
ω-viniferin (10 μ) + VEGF
ω-viniferin (10 μM)
Astringin (10 μM) + VEGF
Astringin (10 μM)
B
C-
C+1
C+2
ω-viniferin(10 μM) + VEGF
ω-viniferin (10 μM)
Astringin (10 μM) + VEGF
Astringin (10 μM)
pAkt
Akt
C
Akt phosphorylation
Ratio (p-Akt/Akt)
****
****
**
*
Negative
VEGF (25 ng/mL)
ε-viniferin (50 μM) + VEGF
ε-viniferin (50 μM)
Piceatannol (50 μM) + VEGF
Piceatannol (50 μM)
D
C-
C+1
C+2
ε-viniferin (50 μM) + VEGF
ε-viniferin (50 μM)
Piceatannol (50 μM) + VEGF
Piceatannol (50 μM)
pAkt
Akt
E
Akt phosphorylation
Ratio (p-Akt/Akt)
*
**
Negative
VEGF (25 ng/mL)
Pallidol (10 μM) + VEGF
Pallidol (10 μM)
F
C-
C+1
C+2
Pallidol (10 μM) + VEGF
Pallidol (10 μM)
pAkt
Akt

Figure 4

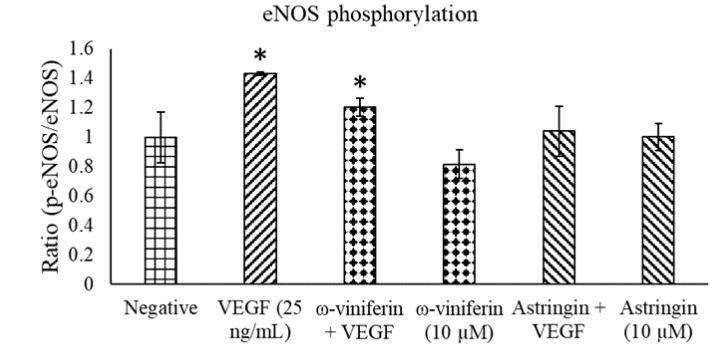
A


eNOS phosphorylation
Ratio (p-eNOS/eNOS)
4
3.5
3
2.5
2
1.5
1
0.5
0
**
***
*
*
Negative
VEGF (25 ng/mL)
ε-viniferin (50 µM) + VEGF
ε-viniferin (50 µM)
Piceatannol (50 µM) + VEGF
Piceatannol (50 µM)

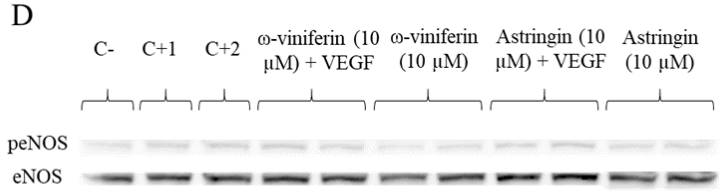
B


C-
C+1
C+2
ε-viniferin (50 µM) + VEGF
ε-viniferin (50 µM)
Piceatannol (50 µM) + VEGF
Piceatannol (50 µM)
peNOS
eNOS

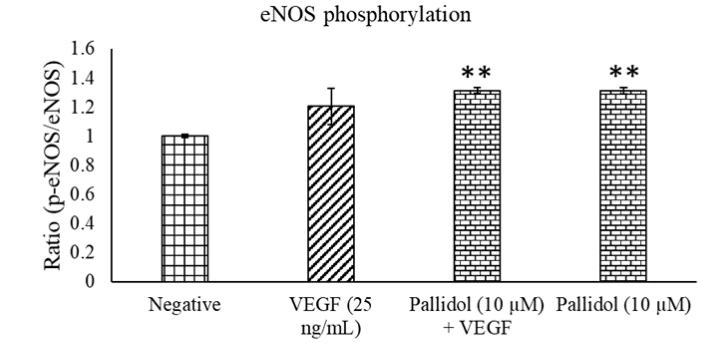
C


eNOS phosphorylation
Ratio (p-eNOS/eNOS)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Negative
VEGF (25 ng/mL)
ω-viniferin + VEGF
ω-viniferin (10 µM)
Astringin + VEGF
Astringin (10 µM)

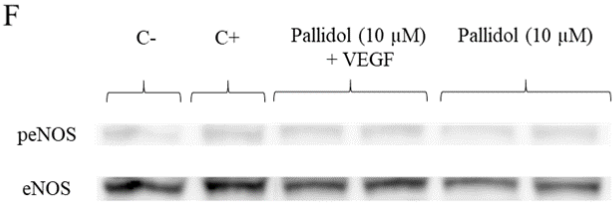
D


C-
C+1
C+2
ω-viniferin (10 µM) + VEGF
ω-viniferin (10 µM)
Astringin (10 µM) + VEGF
Astringin (10 µM)
peNOS
eNOS

E


eNOS phosphorylation
Ratio (p-eNOS/eNOS)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
**
Negative
VEGF (25 ng/mL)
Pallidol (10 µM) + VEGF
Pallidol (10 µM)

F


C-
C+
Pallidol (10 µM) + VEGF
Pallidol (10 µM)
peNOS
eNOS

571 **Figure captions**

572 **Figure 1.** Chemical structure of different stilbenes.

573

574 **Figure 2.** Pallidol caused no inhibition of VEGF-induced PLCγ1 phosphorylation (A).
575 Astringin, ω-viniferin (C) and ε-viniferin (E) showed a significant inhibition of VEGF-
576 induced PLCγ1 phosphorylation (86, 68 and 55% respectively). Basal medium containing
577 VEGF (25 ng/mL) was pre-incubated with astringin, ω-viniferin, pallidol (10 μM), ε-
578 viniferin and piceatannol (50 μM) separately. HUVECs were subsequently incubated
579 for 10 minutes with the pre-incubated solution. Cell were lysed and proteins were
580 separated on an SDS-PAGE gel and probed with the corresponding PLCγ1 antibodies.
581 Generated bands (B, D, F) and ratios for pPLCγ1/PLCγ1 (A, C, E) are displayed, *$p<0.05$,
582 **$p<0.01$, *** $p<0.001$, ****$p<0.0001$ versus VEGF positive control. Western blot
583 analysis was performed in triplicate (n=3).

584

585 **Figure 3.** ω-Viniferin (A), ε-viniferin (C), piceatannol (C) and pallidol (E) did not inhibit,
586 but even enhanced the VEGF-induced Akt phosphorylation, while astringin (A) caused
587 inhibition of VEGF-induced Akt activation. HUVEC were incubated for 60 minutes in
588 basal medium containing preincubated VEGF (25 ng/mL) with astringin, pallidol, ω-
589 viniferin (10 μM), ε-viniferin and piceatannol (50 μM) separately. Then, HUVEC were
590 lysed and proteins were separated on and SDS-PAGE gel to be treated with specific Akt
591 antibodies. Western blot bands (B, D, F) and ratios for pAkt/Akt are presented, *$p<0.05$,
592 **$p<0.01$, *** $p<0.001$, ****$p<0.0001$ versus negative control. Analysis was performed
593 in triplicate (n=3).

594

Figure 4. ε-Viniferin (A) and pallidol (C) significantly enhances eNOS phosphorylation, while piceatannol (A), ω-viniferin (B) and astringin (B) did not induce eNOS phosphorylation. VEGF (25 ng/mL) was incubated in basal medium with astringin, pallidol, ω-viniferin (10 μM), ε-viniferin and piceatannol (50 μM) separately. HUVEC were treated for 60 min with the VEGF/stilbene mixture and the subsequent lysed proteins were separated on a gel (SDS-PAGE) and treated with eNOS antibodies. Ratios for peNOS/eNOS are displayed, *$p<0.05$, **$p<0.01$, *** $p<0.001$ versus VEGF positive control. Western blot analysis was performed in triplicate (n=3).

Tables

Table 1. Dietary sources of stilbenes derived from resveratrol (mean ± standard deviations and rank).

Compound	*Source*	*Concentration*	*Reference*
Astringin	Raboso Piave grape	106 ± 5.8 µg/Kg	5
	French red wines	N.D–38.1 mg/L	6
	French white wines	N.D–8.5 mg/L	
	Brazilian red wines	4.35-25.7 mg/L	7
	Canadian red wines	0.04-0.35 mg /L	8
	Italian red wines	N.D-1.83 mg /L	9
	Italian white wines	N.D-0.72 mg /L	
	Portuguese red wines	N.D-35.9 mg /L	10
	Portuguese white wines	N.D.-15.6 mg/L	
	French red wines	2.5-26.1 mg/L	
Pterostilbene	Berries	99-520 ng/g dw	11
Piceatannol	Raboso Piave grapes	41.8 ± 0.5 µg/Kg	5
	Primitivo grapes	282 ± 10.2 µg/Kg	
	Italian red wines	N.D-5.22 mg/L	9
	Italian white wines	N.D-0.59 mg/L	
	Cabernet Sauvignon berries (without seeds)	0.05 mg/kg fw	12
	Monastrell grapes	0.78 ± 0.1 mg/kg fw	13
	Monastrell wines	208 ± 3.6 µg/L	
Pallidol	Raboso Piave grapes	21.7 ± 0.2 µg/Kg	5

	French red wines	0.5–4.8 mg/L	6
	Canadian red wines	0.06-0.40 mg /L	8
	South African red wines	0.20-9.20 mg /L	14
	Primitivo grapes	356 ± 2.6 µg/Kg	5
	French red wines	1.33-2.22 mg /L	15
	French rosé wines	0.38 mg /L	
ε-viniferin	Raboso Piave grapes	593 ± 11.6 µg/Kg	17
	Primitivo grapes	702 ± 3.4 µg/Kg	
	North African red wines	0.2-1.2 mg/L	14
	Merlot wine	1.20 ± 0.05 mg/L	16
	Cabernet Sauvignon wine	0.69 ± 0.08 mg/L	
	Ksar wine	0.49 ± 0.08 mg/L	
	Amjad wine	0.20 ± 0.04 mg/L	
	Red wine	0.01 mg/L	17
	Brazilian red wines	0.19-4.35 mg/L	7
	French red wines	0.10-1.63 mg/L	15
	French botrytized sweet white wines	0.08-0.17 mg/L	
Hopeaphenol	South African red wines	0.30-3.80 mg/L	14
Isohopeaphenol	Tunisian red wines	0-2.9 mg/L	18

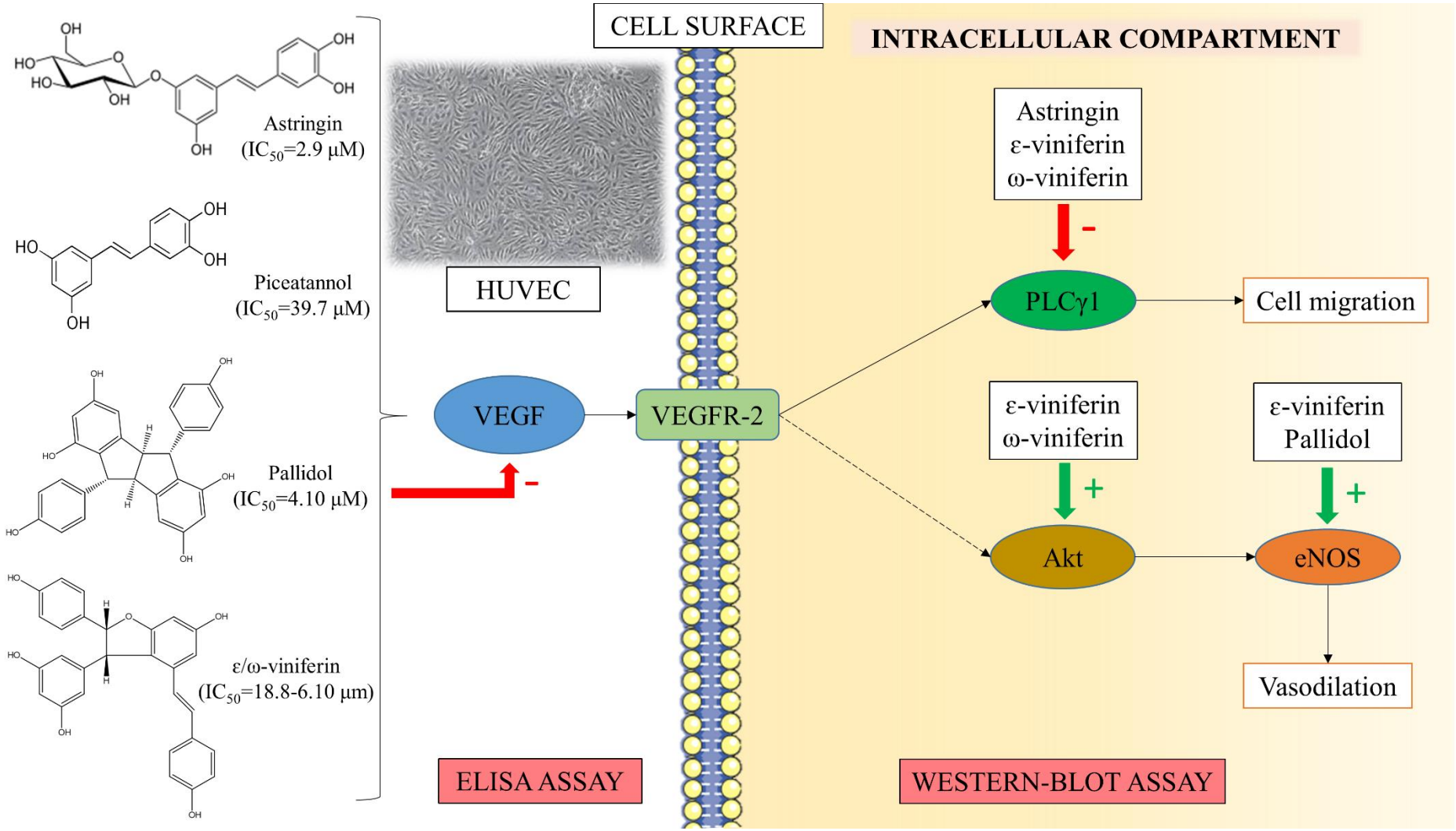
Footnote: fw, fresh weight; dw, dry weight; N.D, non determined

Table 2. Inhibition of 12 different stilbenes on VEGF phosphorylation induced via VEGFR-2.

Compound	*Total OH (Position)*	*Others (Position)*	*IC_{50} (μM)*	*Inhibition (%)*
Monomers				
Astringin	7	Glucoside (3)	2.90 (2.65-3.17)	
Piceatannol	4 (3,5,3',4')	---	39.7 (37.6-41.9)	
Pterostilbene	1 (4')	OCH_3 (3, 5)	N/D	40.7 (50 μM)
Rhapontin	6 (5, 5')	OCH_3 (4'), Glucoside (3)	N/D	Ineffective (50 μM)
Dimers				
Pallidol	6	---	4.42 (3.84-5.09)	
ω-viniferin	5	---	6.10 (5.04-7.39)	
ε-viniferin	5	---	18.8 (17.4-20.4)	
Ampelopsin A	6	---	N/D	Ineffective (50 μM)
Tetramers				
r2-viniferin	10	---	N/D	Ineffective (1 μM)
r-viniferin	9	---	N/D	23.4 (1 μM)
Isohopeaphenol	10	---	N/D	16.2 (1 μM)
Hopeaphenol	10	---	N/D	Ineffective (1 μM)

95% confidence intervals for the IC_{50} values are shown in parentheses; N/D: non determined

603


Astringin
(IC50=2.9 μM)
Piceatannol
(IC50=39.7 μM)
Pallidol
(IC50=4.10 μM)
ε/ω-viniferin
(IC50=18.8-6.10 μm)
CELL SURFACE
INTRACELLULAR COMPARTMENT
HUVEC
VEGF
VEGFR-2
Astringin
ε-viniferin
ω-viniferin
PLCγ1
Cell migration
ε-viniferin
ω-viniferin
Akt
ε-viniferin
Pallidol
eNOS
Vasodilation
ELISA ASSAY
WESTERN-BLOT ASSAY