Flavan-3-ols, flavonoids, anthocyanidins and triterpenoids induces TIE2 phosphorylation -a candidate target for the vascular protective effects

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Vascular system is essential for the body to maintain health. Dysregulated vascular system leads to cardiovascular diseases and are observed in ischemic stroke, Alzheimer's disease, amyotrophic lateral sclerosis, and diabetes. TIE2 is a tyrosine kinase receptor expressed on vascular endothelial cells and contributes to the maintenance of a vascular system. In this paper, we screened for natural products with an activity to induce phosphorylation of TIE2, which will be beneficial for protection of a vascular system. Employing HeLa cells expressing TIE2, flavan-3-ols, flavonoids, anthocyanidins and triterpenoids were identified as active compounds that induce TIE2 phosphorylation. Several of the identified compounds are previously reported to protect endothelial cells from inflammation. Thus, the result provided TIE2 as the candidate receptor protein of those compounds for the protective effect of endothelial cells and the identified compounds will be a good candidate for maintenance of a vascular system.

Keywords: vascular system; cardiovascular disease; TIE2; catechin; flavonoid; anthocyanidin; triterpenoid

1. Introduction

The vascular system carries blood and lymphatic fluid throughout the body and is essential for the body to maintain health. Dysregulation of the vasculature, including angiogenesis and barrier function, is associated with several diseases. Tumor development is accompanied by progressive angiogenesis (Saharinen et al. 2017). Increased permeability of the vascular system due to dysregulation of the endothelial barrier function leads to leakage of blood components that can cause cardiovascular disease (Saharinen et al. 2017). In ischemic stroke, Alzheimer's disease, and amyotrophic lateral sclerosis, the blood-brain barrier is disrupted (Uemura et al. 2020). The increased leakage is also associated with diabetic complications ; diabetic macular edema is caused by vascular leakage within the eye (Saharinen et al. 2017).

TIE2 is a tyrosine kinase receptor expressed in vascular endothelial cells that regulates vascular inflammation, permeability, and angiogenesis (Saharinen et al. 2017). Binding of the endogenous ligand angiopoietin-1 (ANG-1) to TIE2 induces phosphorylation of TIE2 and activates its downstream signalling factors. It then supports endothelial cell survival, upregulates intracellular adhesion molecules, stabilizes endothelial cell-cell junctions, and protects against inflammation, thereby contributing to the formation of a stable vasculature (Parikh 2017; Saharinen et al. 2017). Therefore, ANG-1 and its receptor TIE2 is a candidate of therapeutic target for preventing vascular problems.

ANG-1 is a potential ligand that activates TIE2, but its application is limited because it is a protein that cannot be applied via oral administration. Furthermore, Ang-1 oligomerizes to obtain its activity, but aggregation also results in poor solubility. The development of cartilage oligomeric matrix protein (COMP)-ANG-1, a soluble and stable ANG-1, solved the latter problem, but oral administration is still limited (Cho et al. 2004). Here, we screened small molecule ligands of TIE2 from nature as alternative candidates for ANG-1 for the prevention and treatment of vascular dysfunction.

2. Results and Discussion

Test compounds were selected from the component of Indian dates (*Tamarindus indica*) (Bhatia et al. 1966; Sudjaroen et al. 2005; Razali et al. 2015; Wandee et al. 2022) and Guava leaf (*Psidium guajava*) (Wang et al. 2010; Chang et al. 2013; Li et al. 2021), which extracts induced phosphorylation of TIE2 (Supplementary Figure S1-S6). The selected compounds were tested against HeLa cells expressing TIE2, with significantly higher p-TIE2/TIE values estimated from Western blotting analysis compared to controls were considered active. The active compounds found to induce phosphorylation of TIE2, and their minimum effective concentrations are summarized

in Figure 1 (Figure S7, S8 for p-TIE2/TIE2 value and Figure S9-S15 for western blotting photo).

Of the tested compounds classified as flavon-3-ols, epigallocatechin-3-gallate (EGCG) and gallocatechin-3-gallate (GCG) were active at the lowest concentration (25 μM). Compared to the structure of the inactive flavan-3-ols, the 3-O-gallate appears to be important for activity and the lower activity of CG and ECG suggests that the phenolic group in the B-ring is important for the activity. In the case of flavan-3-ol dimers, activity was observed for A-type procyanidin and not for B-type procyanidins. However, the B-type procyanidin trimer and tetramer showed activity, suggesting that other factors, such as the spatial arrangement of the flavan-3-ol, rather than the type of bond, may be important.

Among the flavonoids, tricetin and myricetin are active at 50 μM and 200 μM, indicating that the pyrogallol moiety of B-ring is important. However, morin is active at 50 μM, indicating that pyrogallol is one of the favourable structures. The lower activity of myricetin compared to tricetin suggest that 3-OH has a negative effect on activity, and the negative result of myricitrin indicates that steric hindrance may be a problem.

Anthocyanins had no effect on inducing TIE2 phosphorylation, but anthocyanidins were active. This is a similar trend with flavonoids, where steric hindrance of 3-OH has a negative effect on activity. The number of hydroxyl groups on the B-ring does not appear to strongly affect activity. However, as shown with peonidin, methylation of the hydroxyl group was shown to decrease activity and affect activity.

Triterpenoids showed activity to induce phosphorylation of TIE2, with the exception of erythrodiol. Maslinic acid (MA) acted at the lowest concentration (12.5 μM). A comparison of the triterpenoid structures shows that the oleanan-type (MA, OA) is the most suitable, followed by the ursane-type (AA, UA), and the lupan-type (BA) is

the least suitable, indicating that the E-ring is important. The higher activity of MA compared to OA, and AA compared to UA, suggest that the presence of 2-OH has a positive effect on activity. The lack of activity with erythrodiol indicates that a carboxyl group is required for activity.

Among the other tested compounds, casuarinin induced phosphorylation of TIE2 at 5 μM, the lowest concentration among the identified compounds. Casuarinin, a type of ellagitannin, have five gallic acids in their structure. Based on the activity of GCG and EGCG, it is presumed that the five gallic acids of casuarinin are important for the activity, since the gallate groups are thought to contribute to the activity.

Several of the compounds identified as TIE2 activators in this study have been reported to protect vascular endothelial cells. EGCG suppresses inflammation induced by tumor necrosis factor-α in human coronary artery endothelial cells (Reddy et al. 2020), apoptosis induced by high-glucose treatment in human umbilical vein endothelial cells (HUVECs) (Zhang and Zhang 2020), and endothelial barrier dysfunction induced by angiotensin II in glomerular endothelial cells (Bi et al. 2016). The cellular pathways involved in those activities are inhibition of NF-κB activation, activation of the PI3K/AKT pathway, and inhibition of p38 MAPK, respectively. Tricetin suppresses oxidized LDL-induced monocyte adhesion to HUVEC through reduced phosphorylation of ERK1/2, increased protein level of Egr-1, and downregulation of intercellular adhesion genes [intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1)] (Cai et al. 2020). Morin inhibits the NFκB signaling pathway in LPS-stimulated HUVECs and suppresses inflammatory responses (Meng et al. 2021). Maslinic acid inhibits monocyte adhesion in HUVECs by suppressing activation of NFκB by TNF-α and ameliorating increased expression of monocyte chemotactic protein-1 (MCP-1), VCAM-1 and scavenger receptor type A (SR-A) (Ooi et al. 2021).

ANG-1, a native agonist of TIE2, suppresses inflammatory responses induced by lipopolysaccharide (LPS) treatment of cultured endothelial cells through suppression of p38 MAPK activation and reduction of NF-κB activity (Echavarria et al. 2015). ANG-1 also activates PI3K/AKT pathway to counter VEGF-mediated inflammatory response in endothelial cells (Sako et al. 2009). ANG-1 reduces VEGF-stimulated leukocyte adhesion to HUVECs through reduction of ICAM-1 and VCAM-1 (Kim et al. 2001). A designed ANG-1 variant, COMP-Ang-1 (Cho et al. 2004), induces Egr-1 protein expression in HUVECs (Abdel-Malak et al. 2009).

The action of ANG-1 on endothelial cells and its intracellular signalling pathway is similar to the action and pathway of EGCG, tricetin, morin and MA. These similarities suggest that the protective effects of those compounds on endothelial cells may be induced via TIE2. Thus, the present results provide a candidate receptor for the vasoprotective effects of those compounds, and further studies may reveal the importance of TIE2 in the effects of those compounds.

3. Experimental

See Supplemental material.

4. Conclusions

After screening various natural products, several candidate substances were found to induce TIE2 activation. Since the TIE2 expression system was used to evaluate the activity, its efficacy in vascular endothelial cells needs to be investigated. However, the results do provide potential receptors for the vasoprotective effects of natural products on endothelial cells, and the identified compounds would be good candidates for protecting the vascular system.

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Declaration of Interest statement

Authors declare no potential conflicts of interests.

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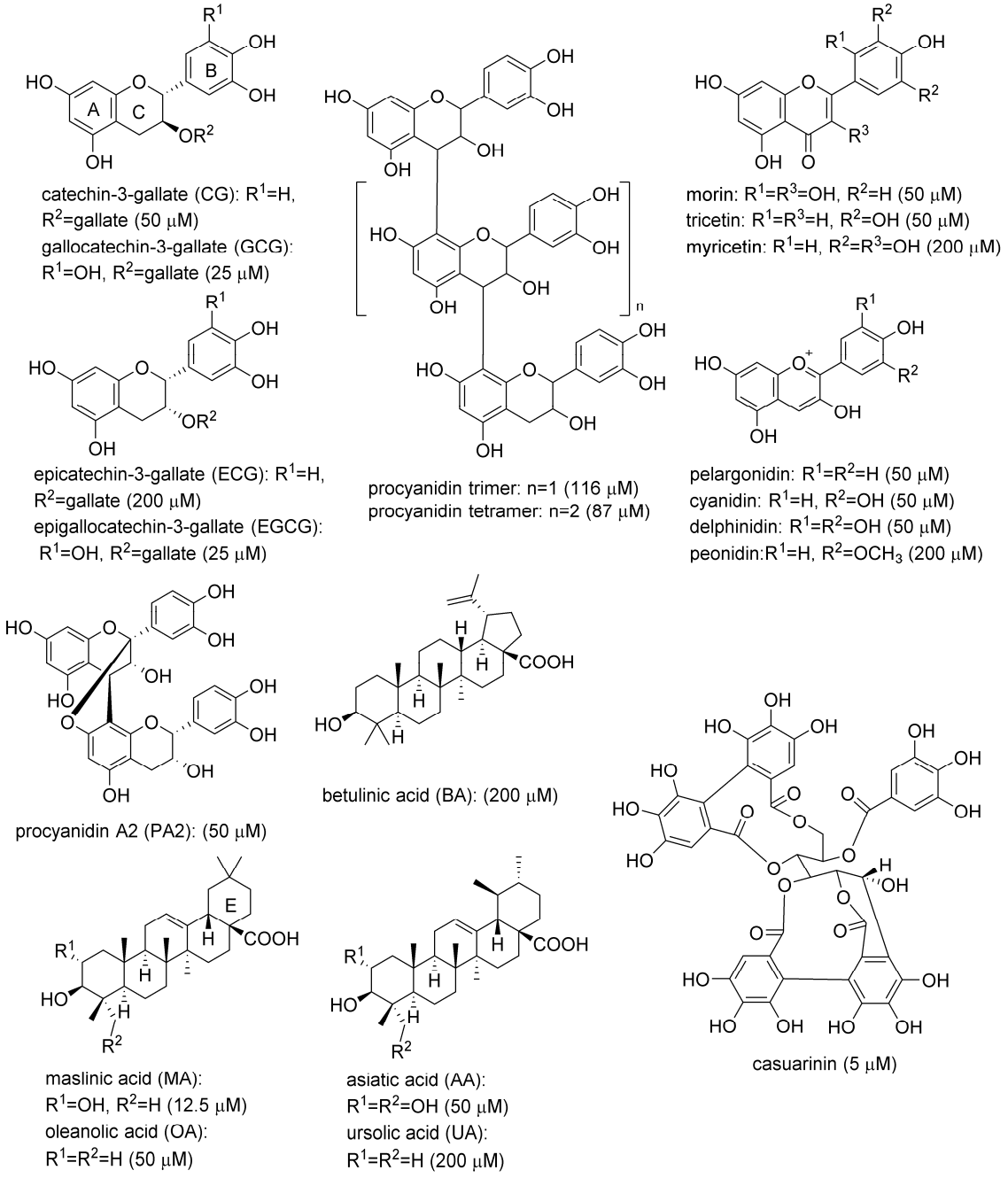

Figure 1. Structure of the compounds which induced TIE2 phosphorylation. Number in the bracket shows the minimal effective concentration to phosphorylate TIE2.

Supplementary material

Flavan-3-ols, flavonoids, anthocyanidins and triterpenoids induces TIE2 phosphorylation -a candidate target for the vascular protective effects

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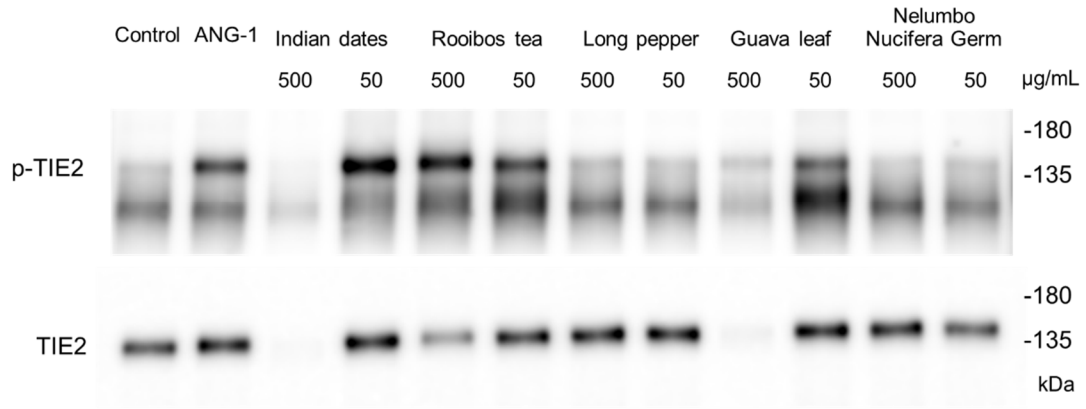


Figure S1. TIE2 phosphorylation activity of plant extracts. ANG-1 was tested at 500 ng/mL. The band above 135 kDa is the phosphorylated-TIE2 (p-TIE2).

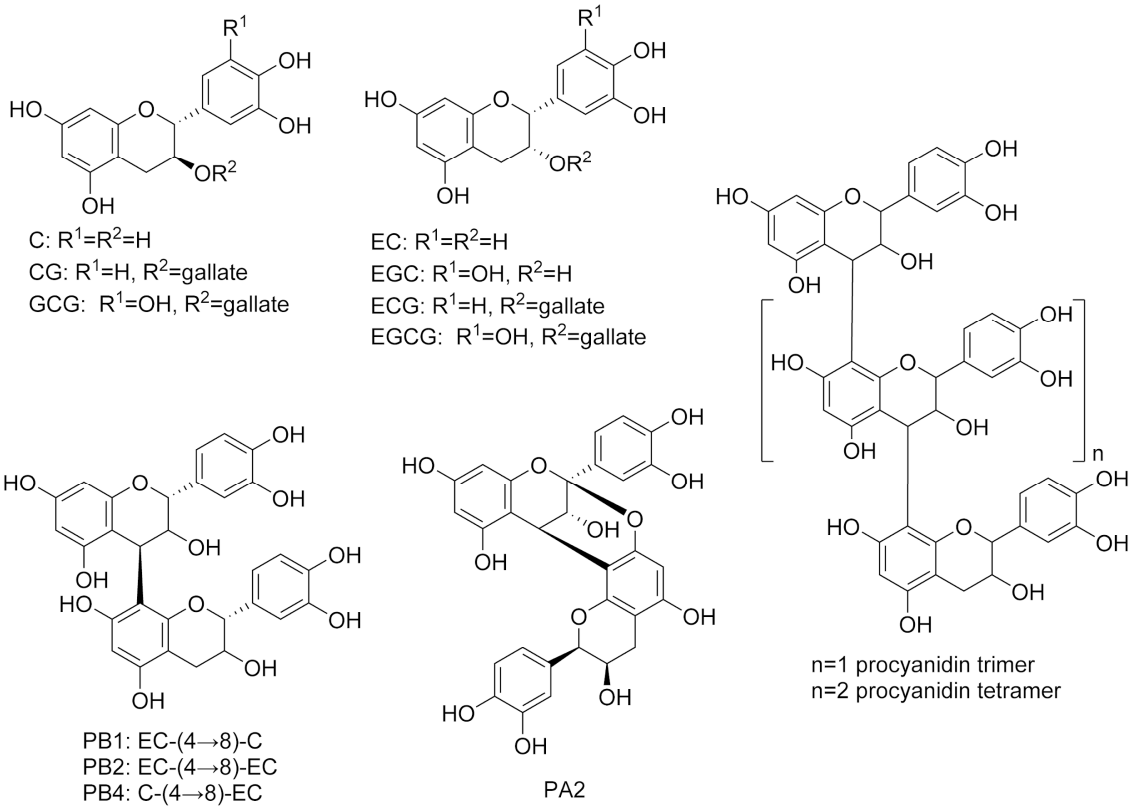


Figure S2. Flavan-3-ols selected for screening.

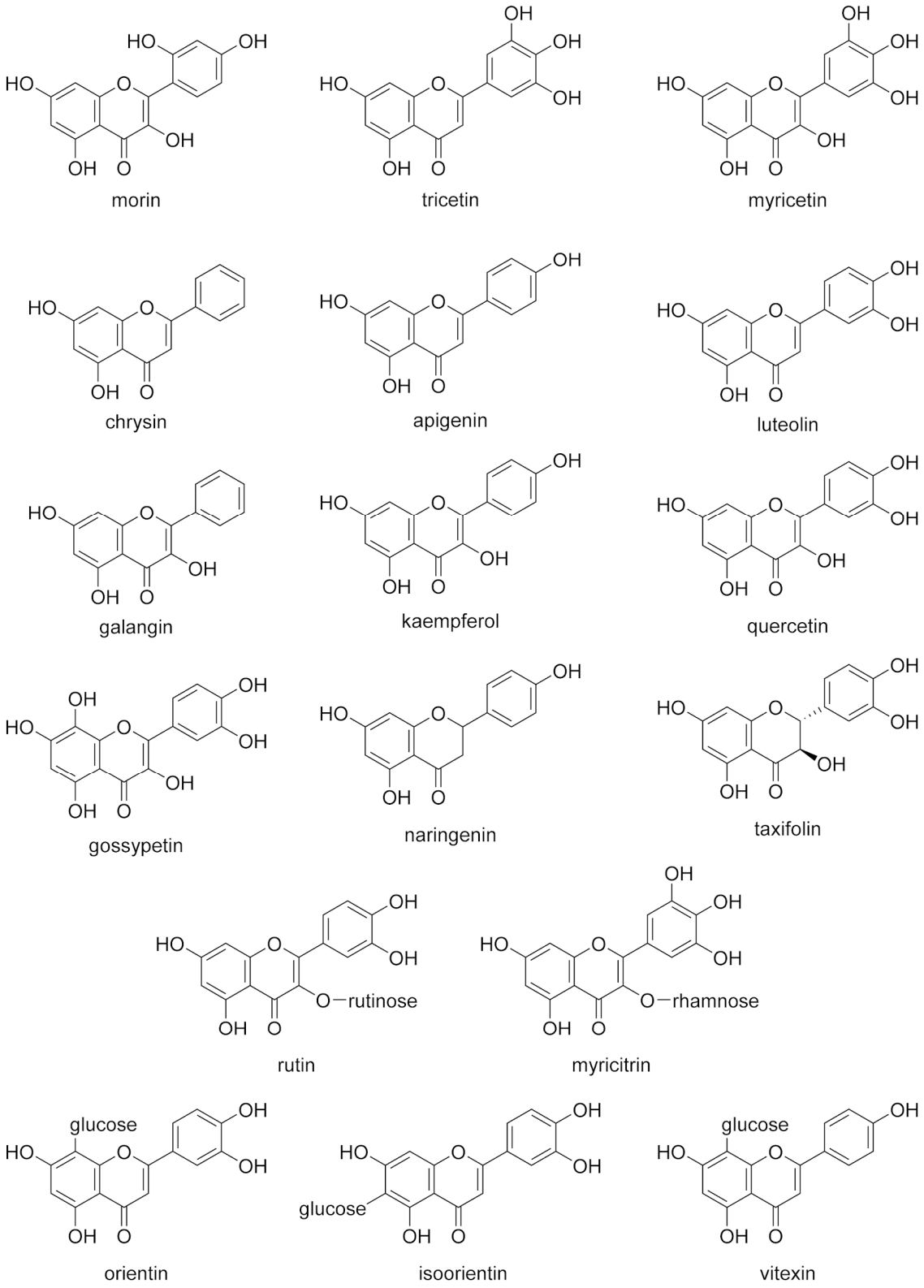


Figure S3. Flavonoids selected for screening.

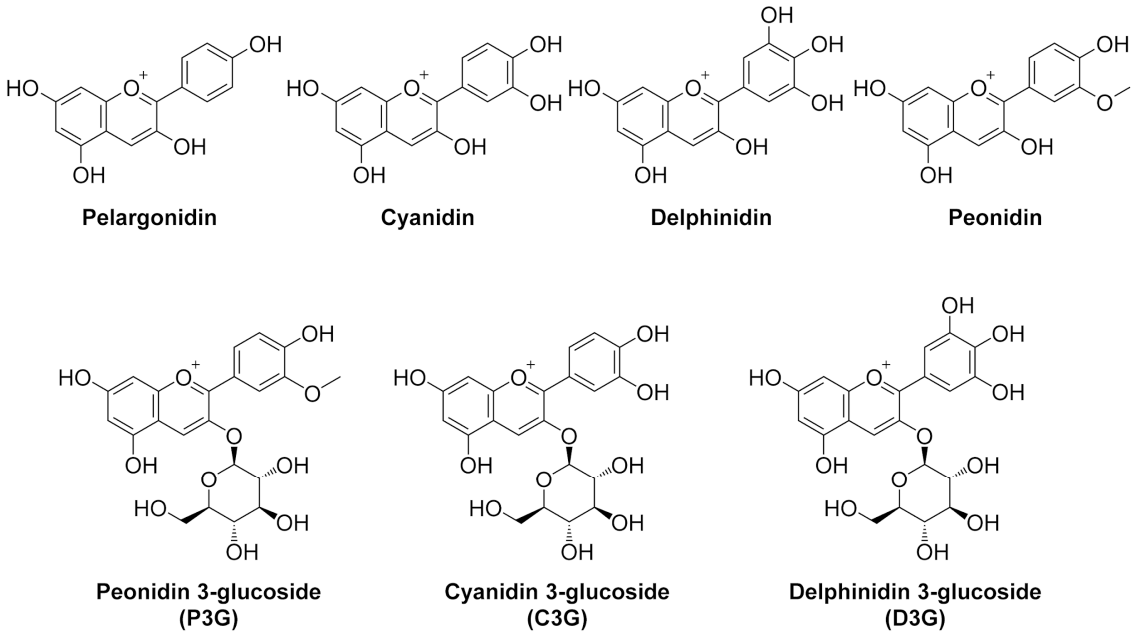


Figure S4. Anthocyanins and anthocyanidins selected for screening.

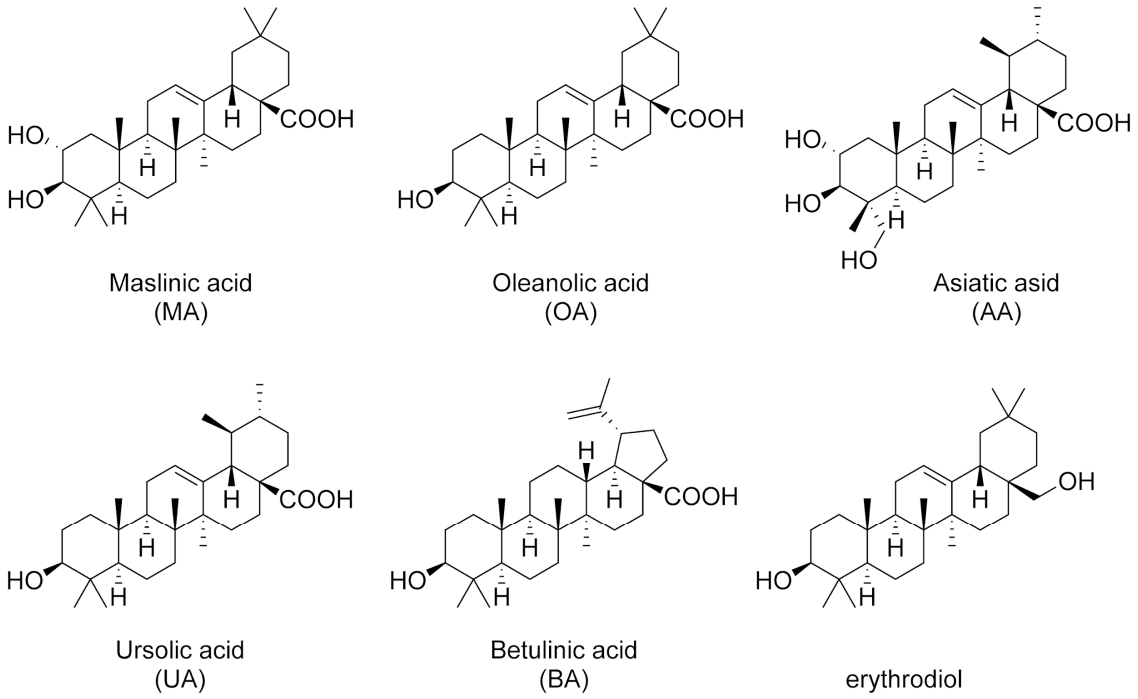


Figure S5. Triterpenoids selected for screening.

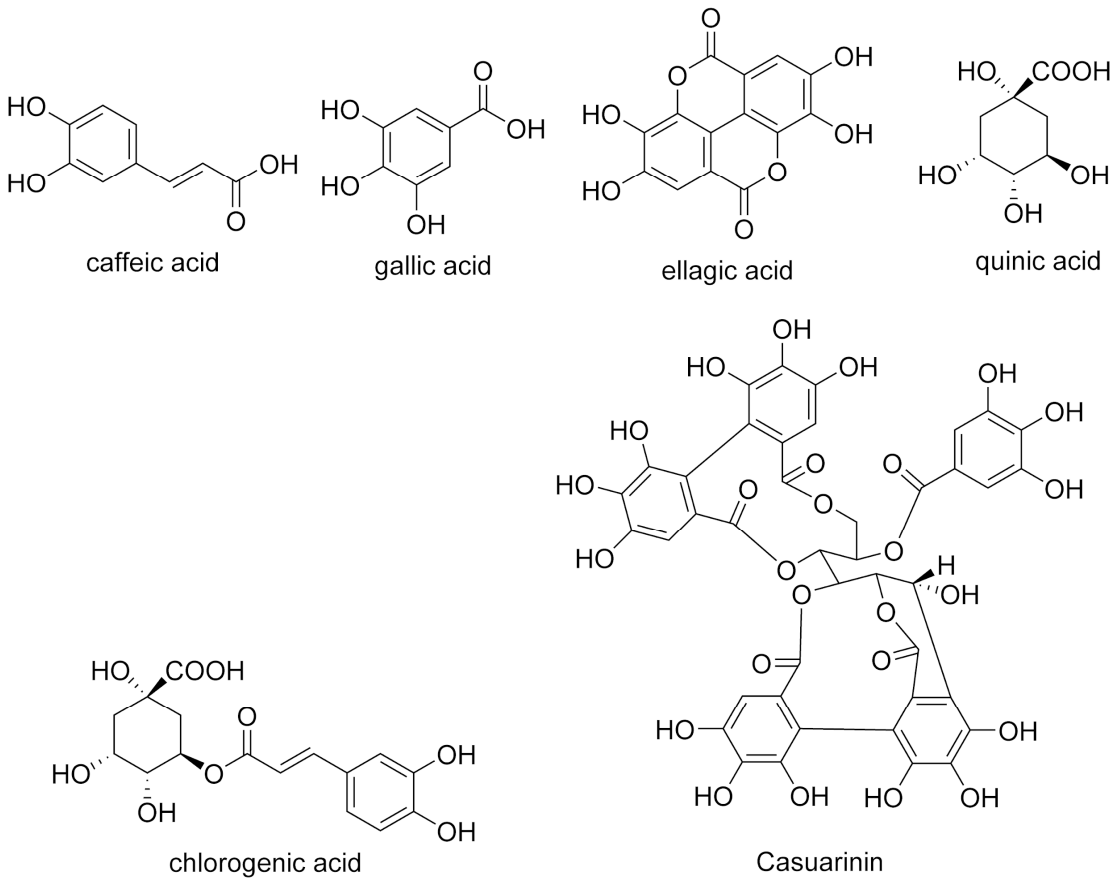


Figure S6. Other compounds selected for screening.

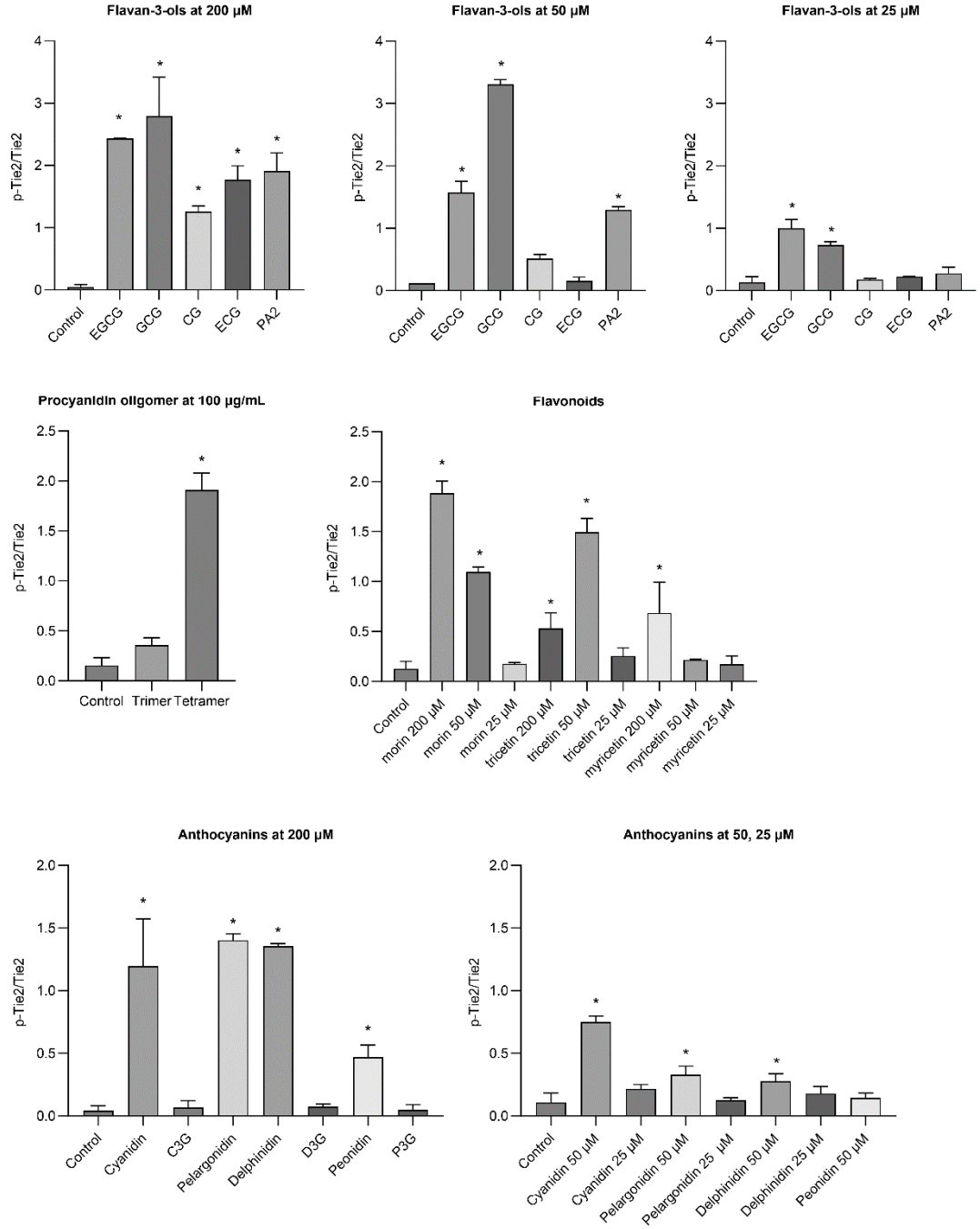


Figure S7. Quantitated p-TIE2/TIE2 after stimulation by compounds. Significantly higher p-TIE2/TIE2 values compared to controls were considered active (N=4).*$p<0.05$ (Tukey test)

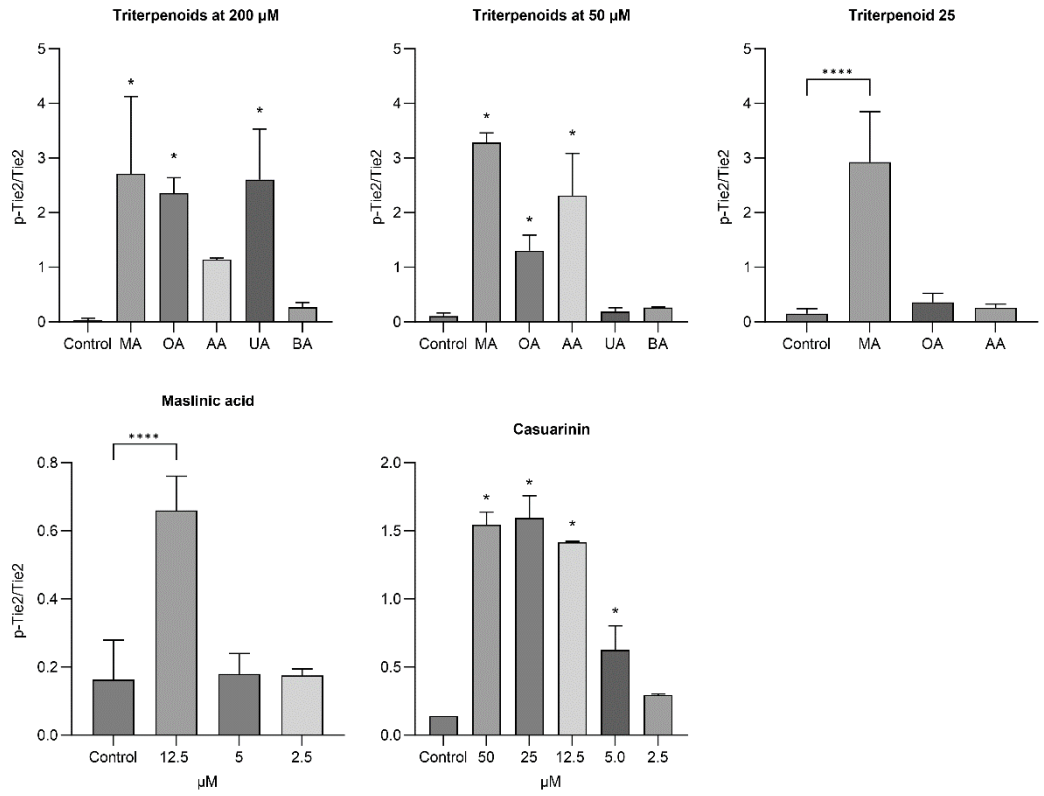


Figure S8. Quantitated p-TIE2/TIE2 after stimulation by compounds. Significantly higher p-TIE2/TIE2 values compared to controls were considered active (N=4).*$p<0.05$ (Tukey test)

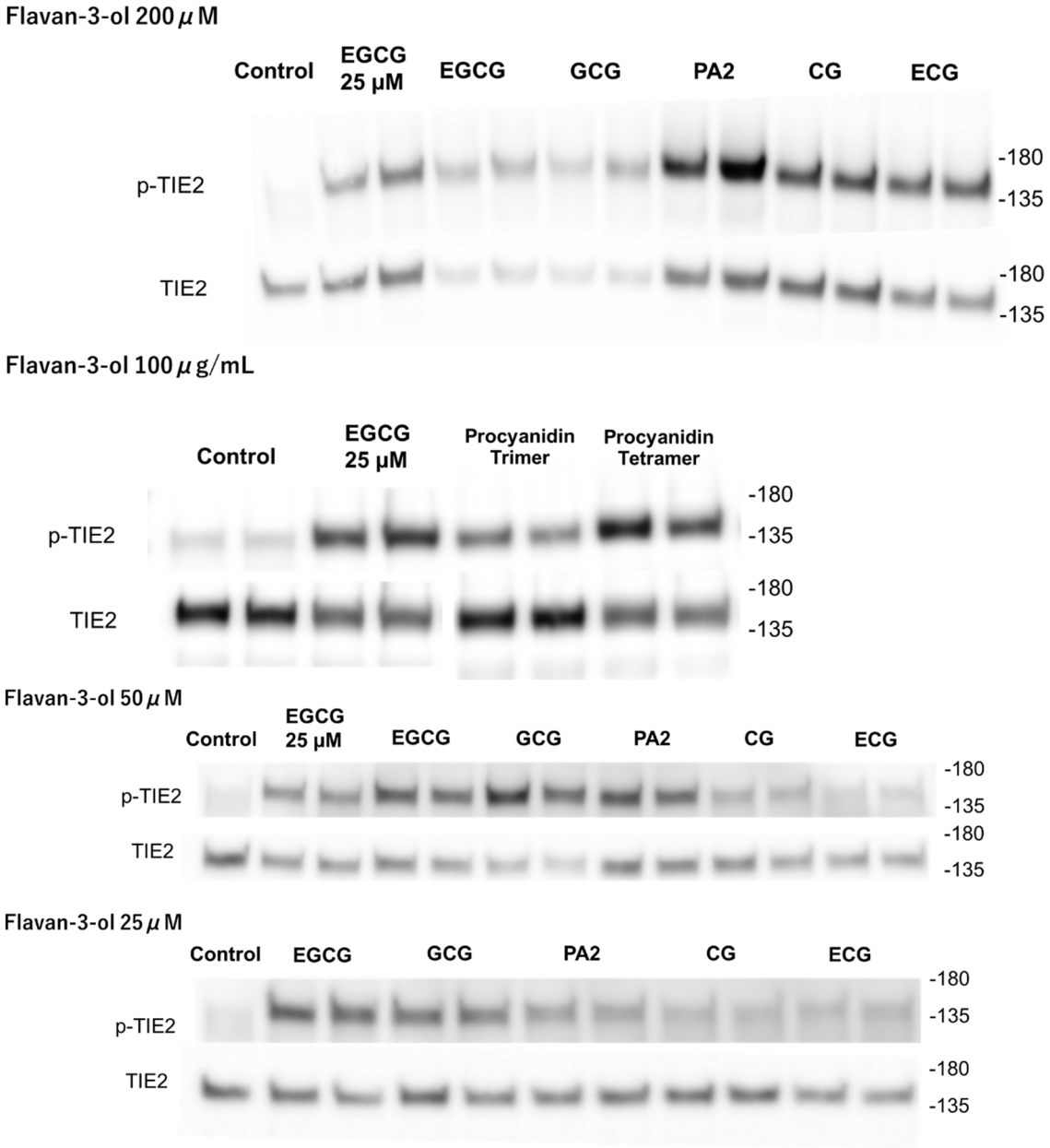


Figure S9. Western blotting analysis of p-TIE2 and TIE2 (flavan-3-ol)

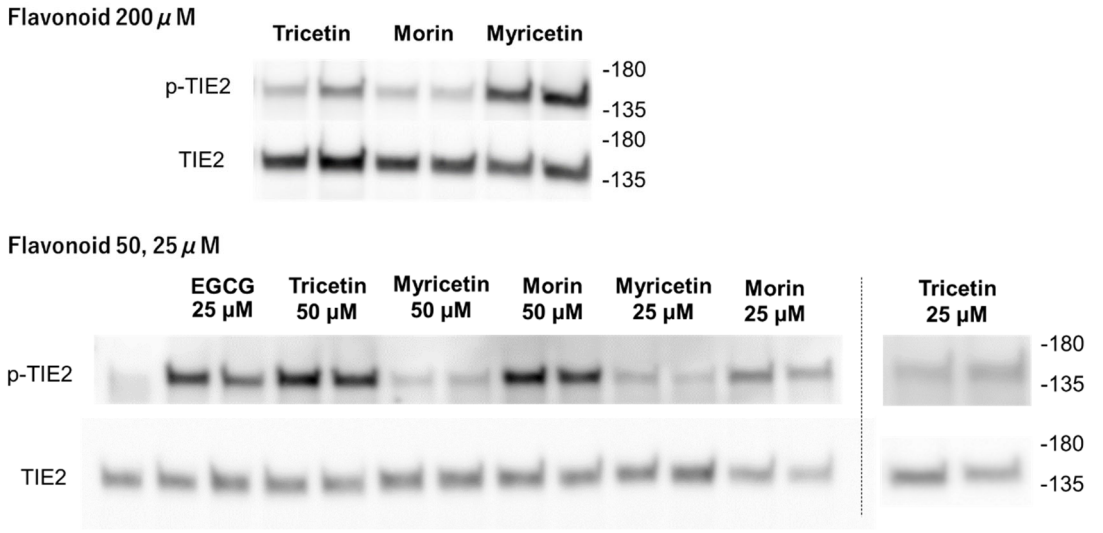


Figure S9. Western blotting analysis of p-TIE2 and TIE2 (flavonoids)

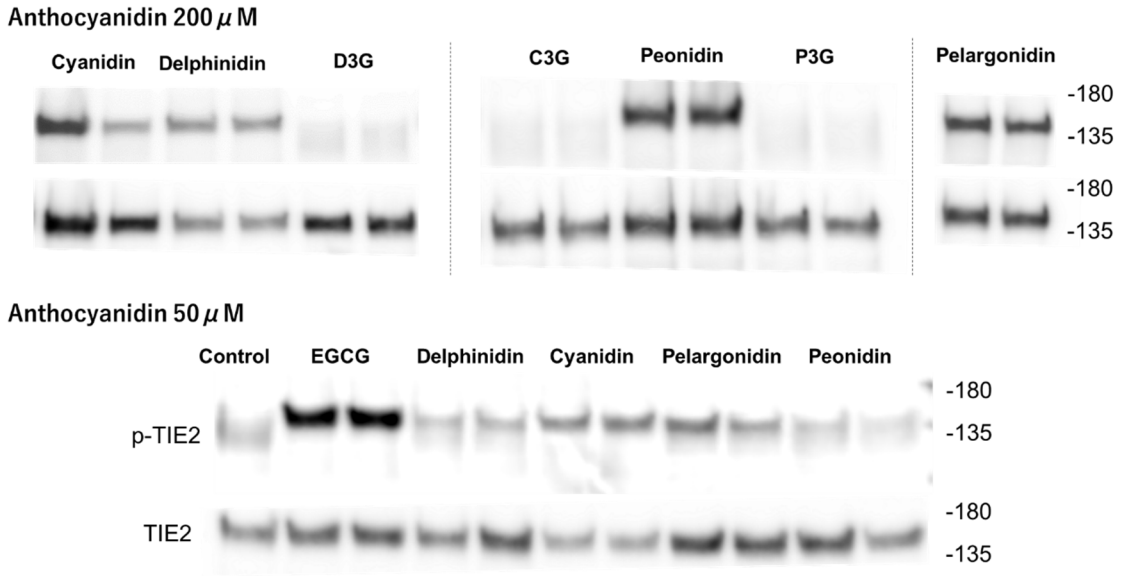


Figure S10. Western blotting analysis of p-TIE2 and TIE2 (anthocyanins)

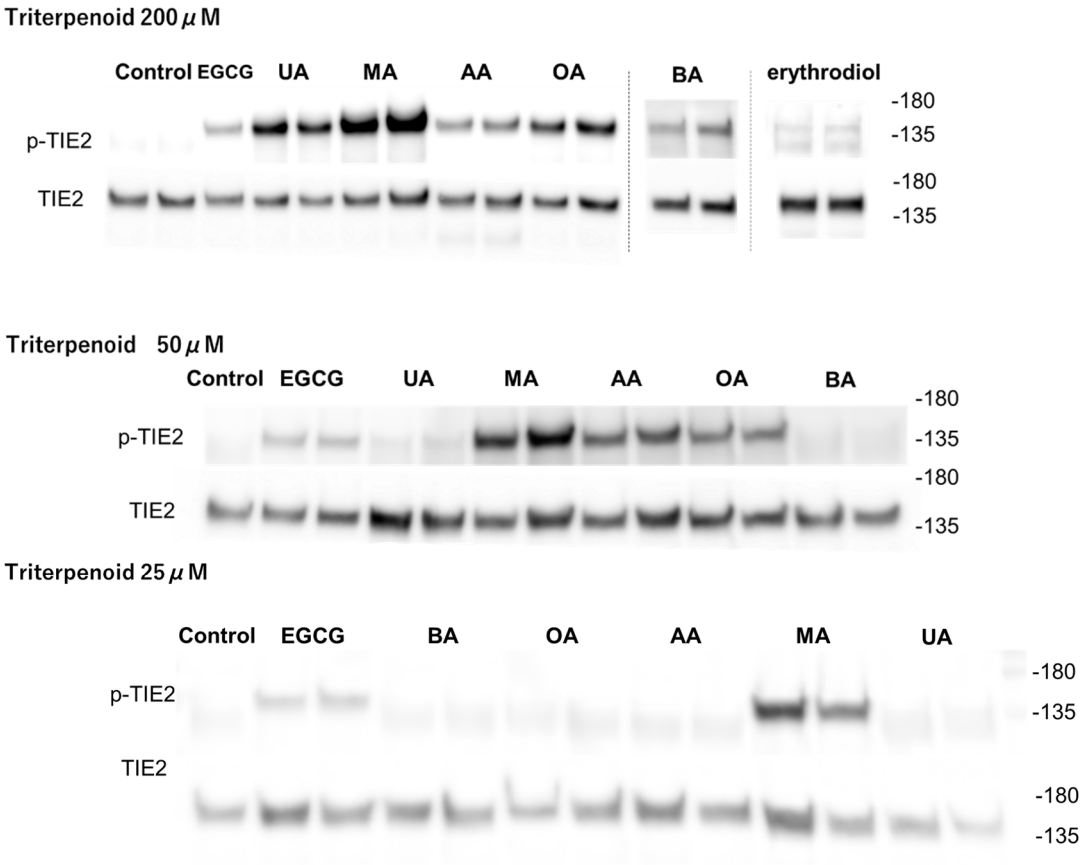


Figure S11. Western blotting analysis of p-TIE2 and TIE2 (triterpenoids)

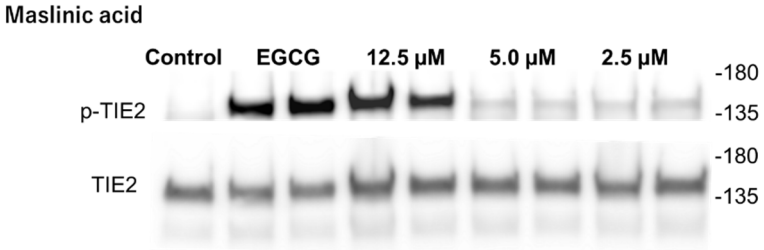


Figure S12. Western blotting analysis of p-TIE2 and TIE2 (maslinic acid)

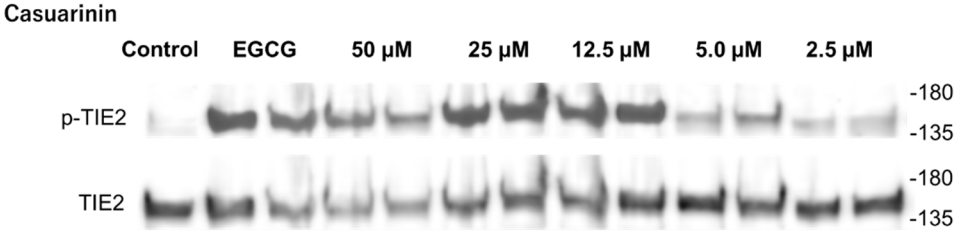


Figure S13. Western blotting analysis of p-TIE2 and TIE2 (casuarinin)

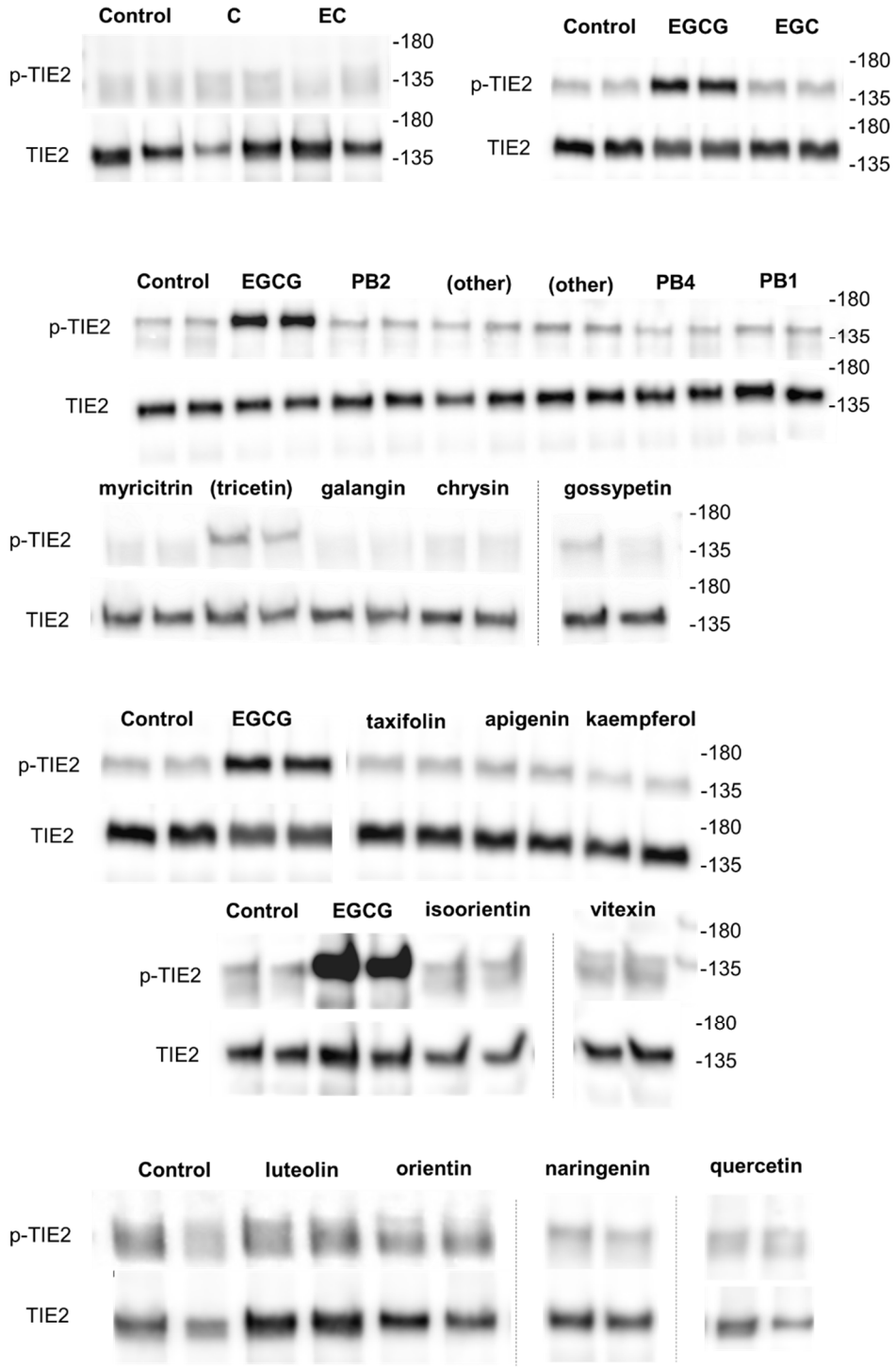


Figure S14. Western blotting analysis of p-TIE2 and TIE2 (inactive compounds)

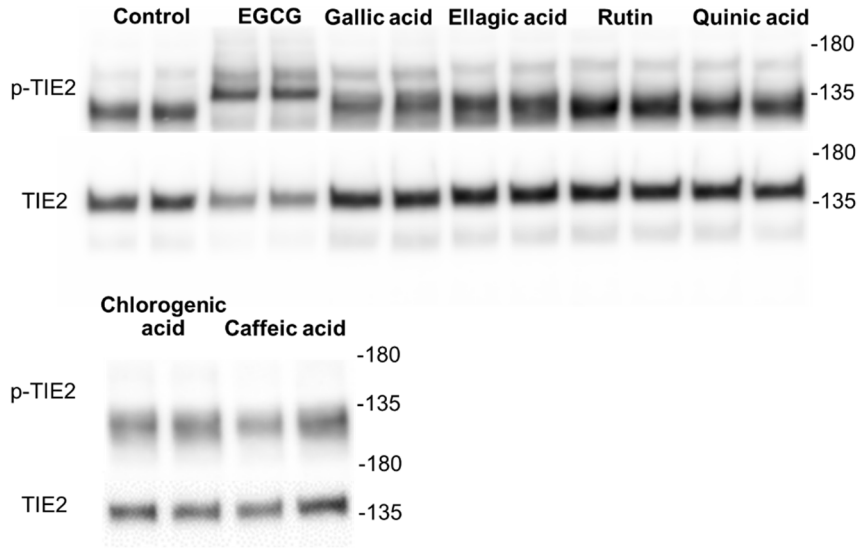


Figure S15. Western blotting analysis of p-TIE2 and TIE2 (inactive compounds)

Experimental procedures

Chemicals

Powdered extract of plant samples were purchased from Maruzen Pharmaceuticals Co., Ltd. (Hiroshima, Japan). Chemicals were purchased from FUJIFILM Wako Pure Chemical Co., Tokyo Chemical Industry Co., Cayman Chemical Co., Angene Chemical, Extrasynthese, Biosynth., Tokiwa Phytochemical Co., Ltd., Nagara Science Co. Ltd., or Sigma-Aldrich Co. Procyanidin trimer and tetramer were isolated from Indian dates. Casuarinin was isolated from Guava leaf.

Cell culture

HeLa cells were obtained from the Japanese Collection of Research Bioresources Cell Bank (JCRB9004). The cells were cultured at 37°C in a 10% CO_2 atmosphere in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, non-essential amino acids, and antibiotics (50 μg/mL gentamycin, 100 μg/mL streptomycin, and 100 unit/mL penicilin).

Transfection of TIE2

HeLa cells were seeded in 24 well plates (1×10^5 cell/well) and cultured for 24 h. TIE2 expression vector (1 μg/well, pcDNA3.1(+) inserted with TIE2 coding gene) was transfected with Xfect Transfection reagent (Clontech) following manufactures instruction. Cells were cultured for 48 h after transfection and were used for the experiment.

TIE2 phosphorylation assay

TIE2 transfected cells were serum starved for 2 h and stimulated with the sample for 1 h. Samples were dissolved in DMSO and diluted by DMEM. Control cells were treated with the same concentration of DMSO. Angiopoietin-1 (500 ng/mL) or EGCG (25 μM) were used as positive control. After stimulation, cells were washed with phosphate-buffered saline and lysed by EzRIPA Lysis kit (Atto Co.). The lysate was separated by SDS-PAGE (7.5%) and transferred to PVDF membrane. The membrane was blocked by EveryBlot Blocking Buffer (Bio-rad Co.) and phosphorylated TIE2 was detected using Human/Mouse Phospho-Tie-2 (Y992) Antibody (1:500, AF2720, R&D Systems), Anti-rabbit IgG HPR-linked Antibody (1:2000, 7074S, Cell signaling technology, Inc.) and ImmunoStar Zeta detection reagent (FUJIFILM Wako Pure Chemical Co.). The membrane was stripped and TIE2 was detected using Tie2 (D9D10) Rabbit mAb (1:8000, #7403, Cell Signaling Technology), Anti-rabbit IgG HPR-linked Antibody and EzWestLumi One (Atto Co.). The obtained image was analyzed by Image-J software for evaluation of p-TIE2/TIE2 ratio.

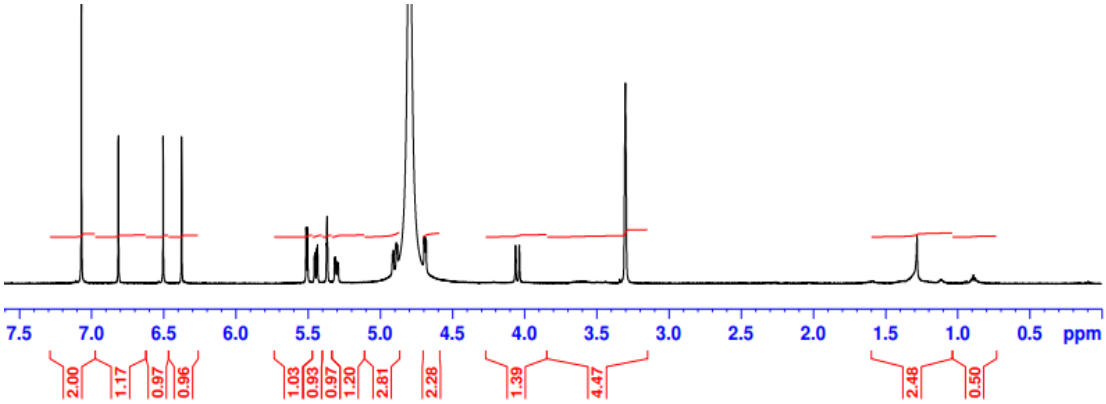
Isolation of procyanidin trimer and tetramer

Powdered extract of Indian dates (11.9 g) was immersed in water and extracted by ethyl acetate and 1-butanol. The 1-butanol extract (4.20 g) was adsorbed on Diaion HP-20 (Mitsubishi Chemical Co.) and eluted by water, 50% aq. methanol and methanol. The 50% aq. methanol eluted fraction (2.08 g) was separated by Cosmosil 75C18-OPN (Nacalai Tesque Inc.) column chromatography with 10-90% aq. methanol as an eluent. The 20-90% aq. methanol eluted fraction (48 mg) was adsorbed on Sephadex LH-20 (Cytiva) and eluted with methanol and then 70% aq. acetone. The 70% aq. Acetone fraction was separated with HPLC using Inertsil HILIC (GL Science Co.) as a column. The mobile phase was composed of (A) acetonitrile containing 0.1% formic acid and (B) methanol containing 3% water and 0.1% formic acid. Separation was achieved using a linear gradient from 80% to 40% of component A over a period of 60 minutes. The obtained trimer and tetramer were analyzed by ^{1}H-NMR and MS (Trimer m/z=867, Tetramer m/z=1172, $[M+H]^{+}$).

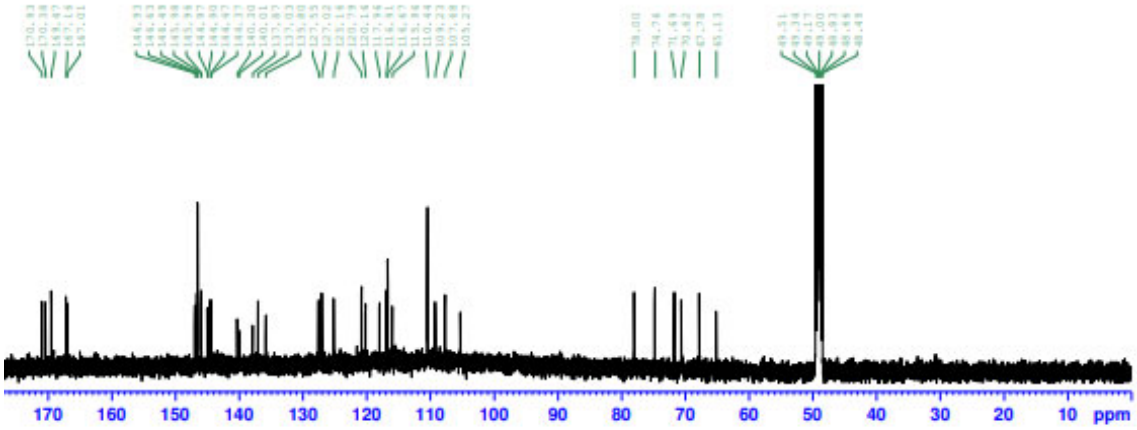
Isolation of casuarinin

Powdered extract of Guava leaf (10 g) was immersed in water and extracted by ethyl acetate and 1-butanol. The 1-butanol extract (1.60 g) was adsorbed on Diaion HP-20 (Mitsubishi Chemical Co.) and eluted by 10% aq. methanol, 50% aq. methanol and methanol. The 50% aq. methanol eluted fraction (742 mg) was separated by Silicagel 120 RP-18 (Kanto Chemical Co., Inc.) column chromatography with 10-90% aq. methanol as an eluent. The 10% aq. methanol eluted fraction (223 mg) was separated with HPLC using InertSustain C18 (GL Science Co.) as a column and 25-75% aq. methanol containing 0.1% trifluoroacetic acid as a mobile phase. The obtained fraction was again separated with HPLC using Cosmosil πNAP (Nacalai Tesque Inc.) as a column and 25% aq. methanol containing 0.1% trifluoroacetic acid as a mobile phase to obtain casuarinin (2.0 mg). The NMR data was matched with the reported data (He et al. 2017). HRMS data also matched with the structure (m/z=935.0801, calculated 935.0796 $[M-H]^{-}$).

NMR Spectrum of casuarinin



^{1}H-NMR (500 MHz, CD_3OD)



^{13}C-NMR (125 MHz, CD_3OD)

Reference

He Z, Lian W, Liu J, Zheng R, Xu H, Du G, Liu A. 2017. Isolation, structural characterization and neuraminidase inhibitory activities of polyphenolic constituents from Flos caryophylli. Phytochem Lett. 19:160–167. https://doi.org/10.1016/j.phytol.2016.12.031