Supplementary material

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

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Abstract

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.

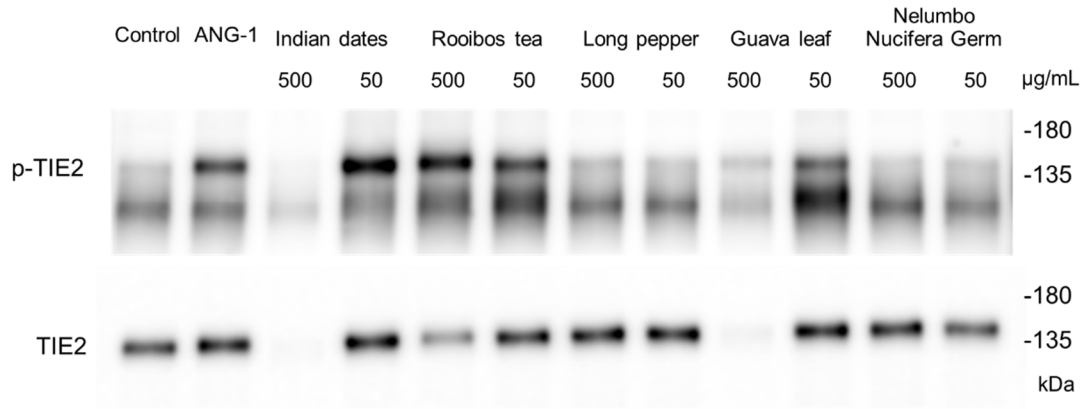

Figure S1. TIE2 phosphorylation activity of plant extracts. ANG-1 was tested at 500 ng/mL. The band above 135 kDa is the phosphorylated-TIE2 (p-TIE2).

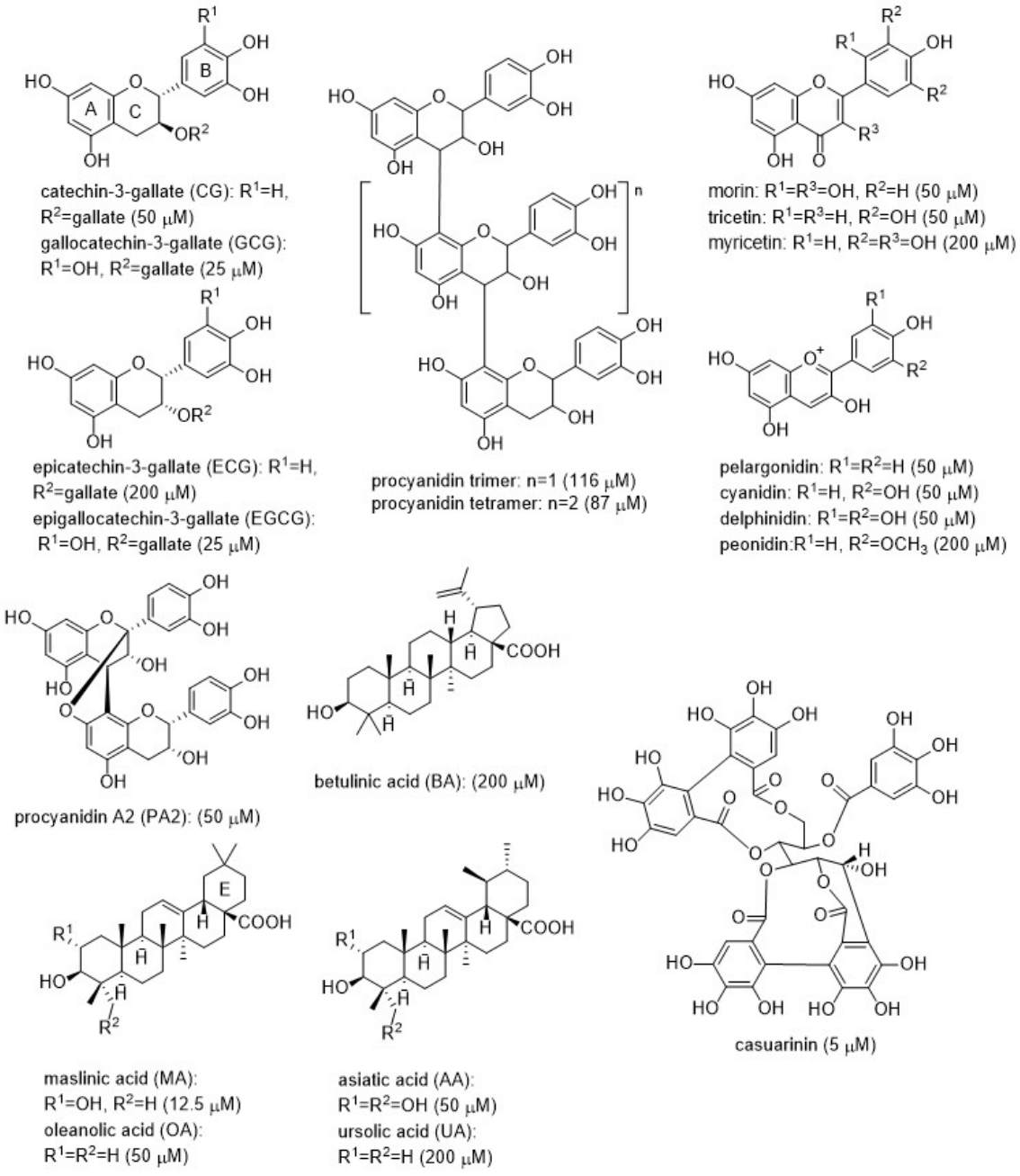

Figure S2. Structure of the compounds which induced TIE2 phosphorylation. Number in the bracket shows the minimal effective concentration to phosphorylate TIE2.

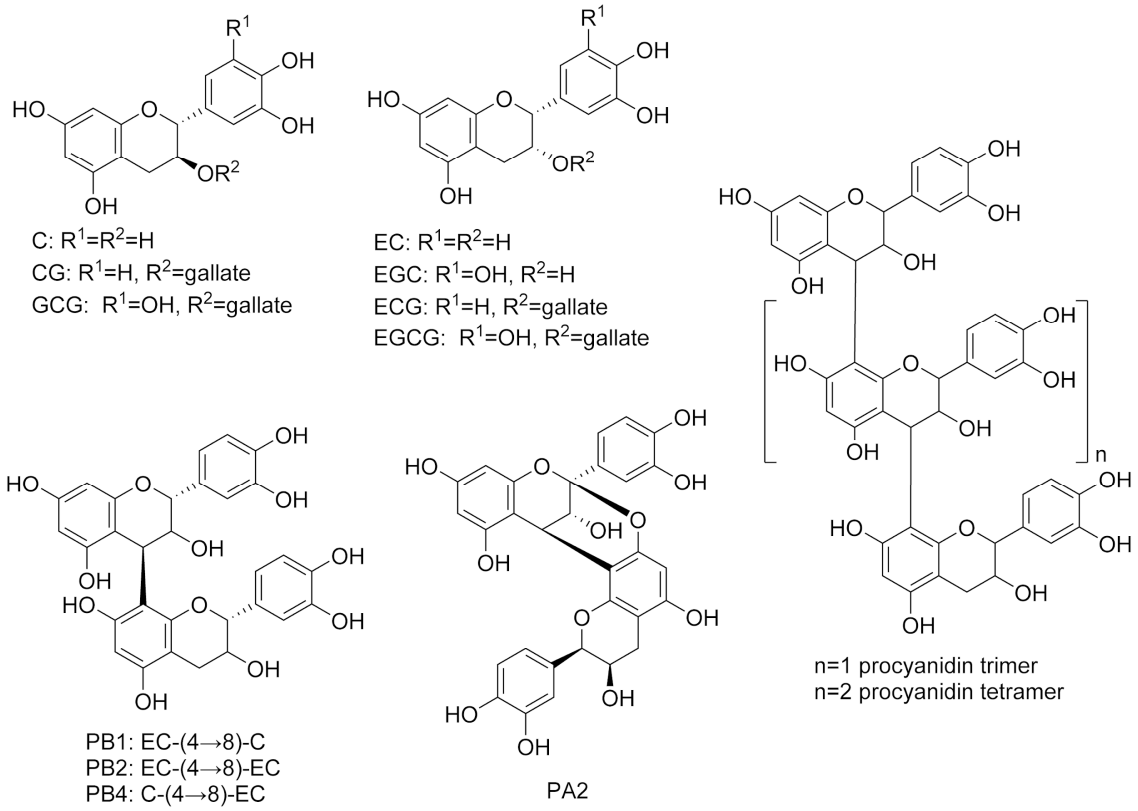

Figure S3. Flavan-3-ols selected for screening.

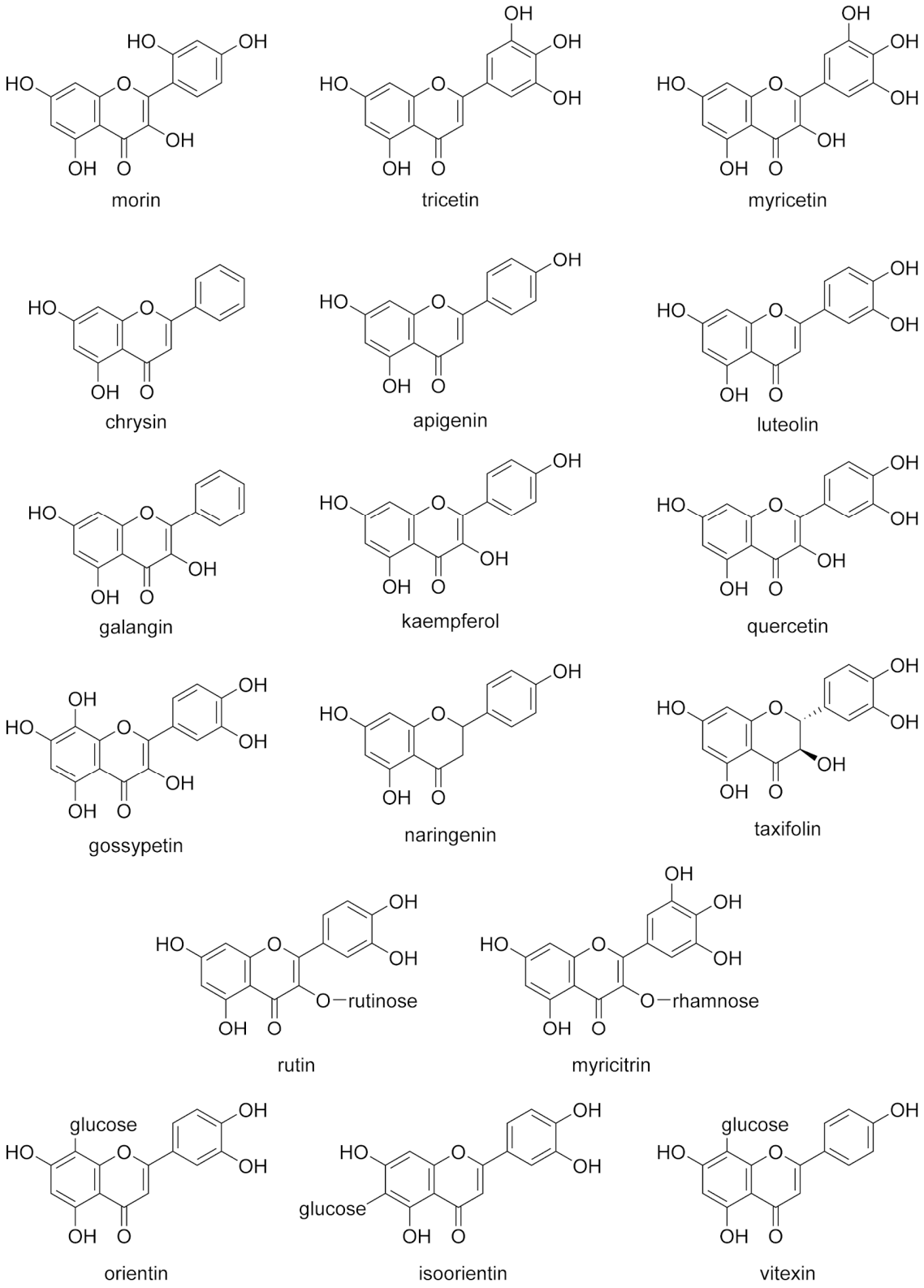

Figure S4. Flavonoids selected for screening.

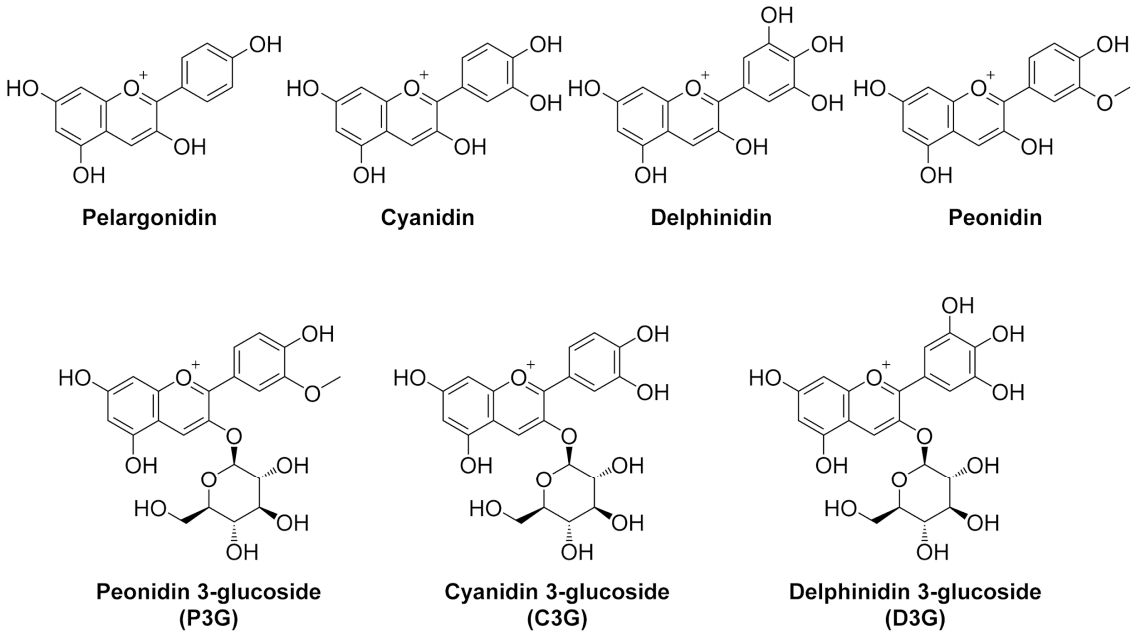

Figure S5. Anthocyanins and anthocyanidins selected for screening.

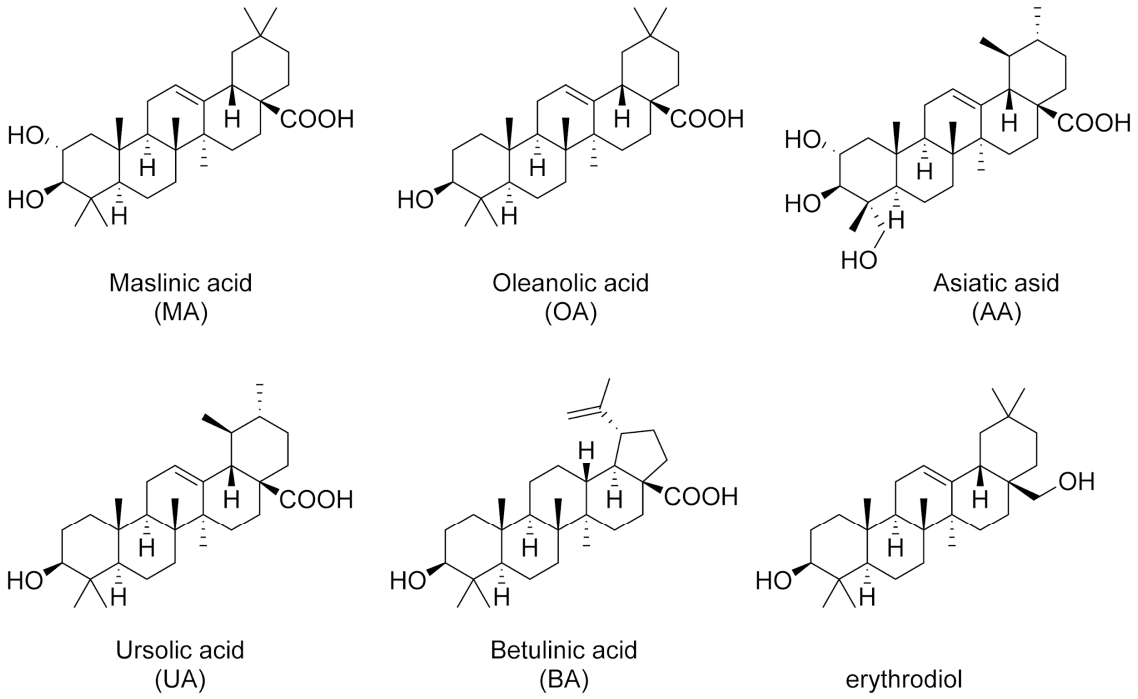

Figure S6. Triterpenoids selected for screening.

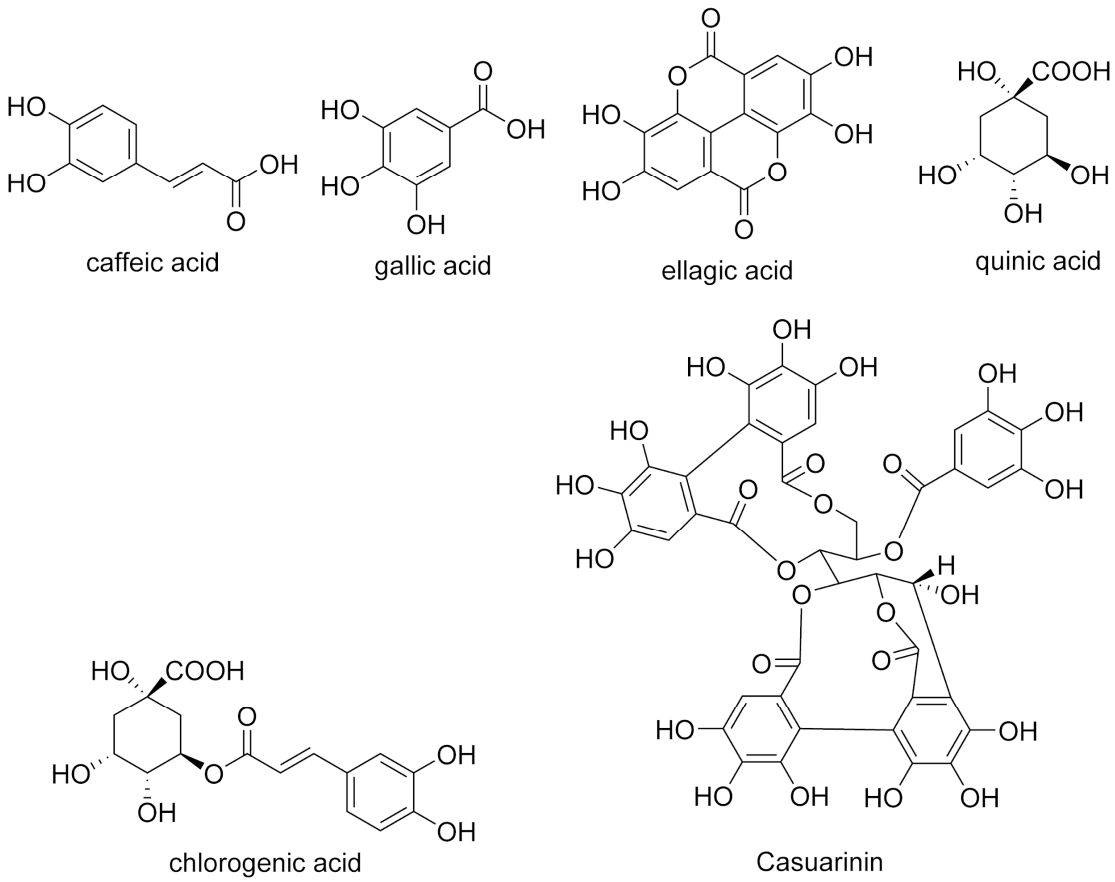

Figure S7. Other compounds selected for screening.

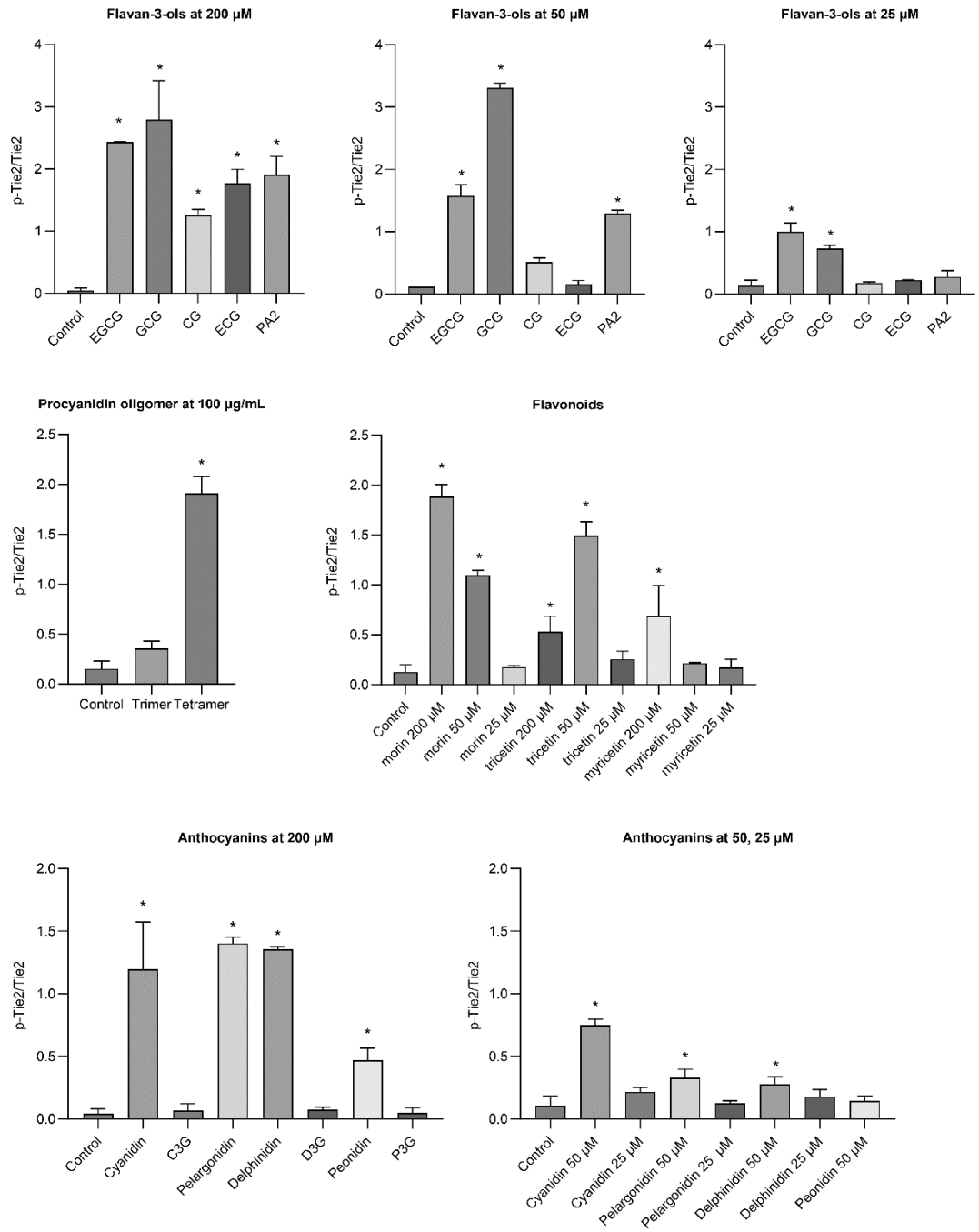

Figure S8. Quantitated p-TIE2/TIE2 after stimulation by compounds. Significantly higher p-TIE2/TIE2 values compared to controls were considered active (N=4).*$p<0.05$ (Tukey test)

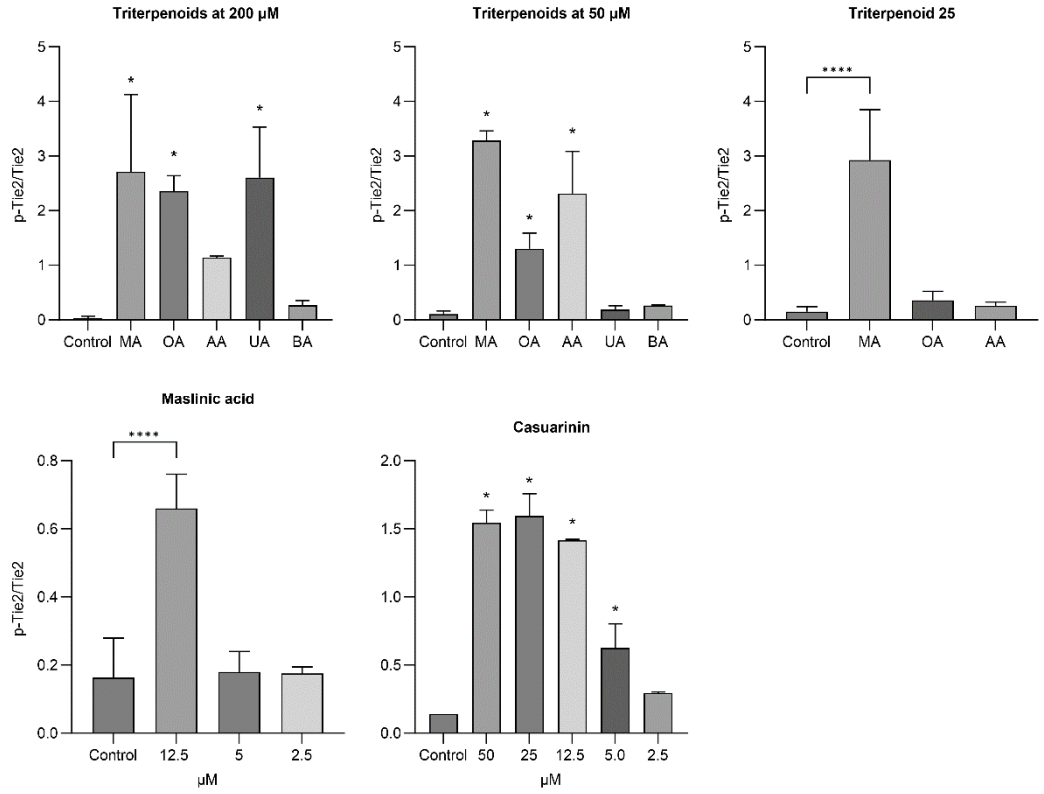

Figure S9. Quantitated p-TIE2/TIE2 after stimulation by compounds. Significantly higher p-TIE2/TIE2 values compared to controls were considered active (N=4).*$p<0.05$ (Tukey test)

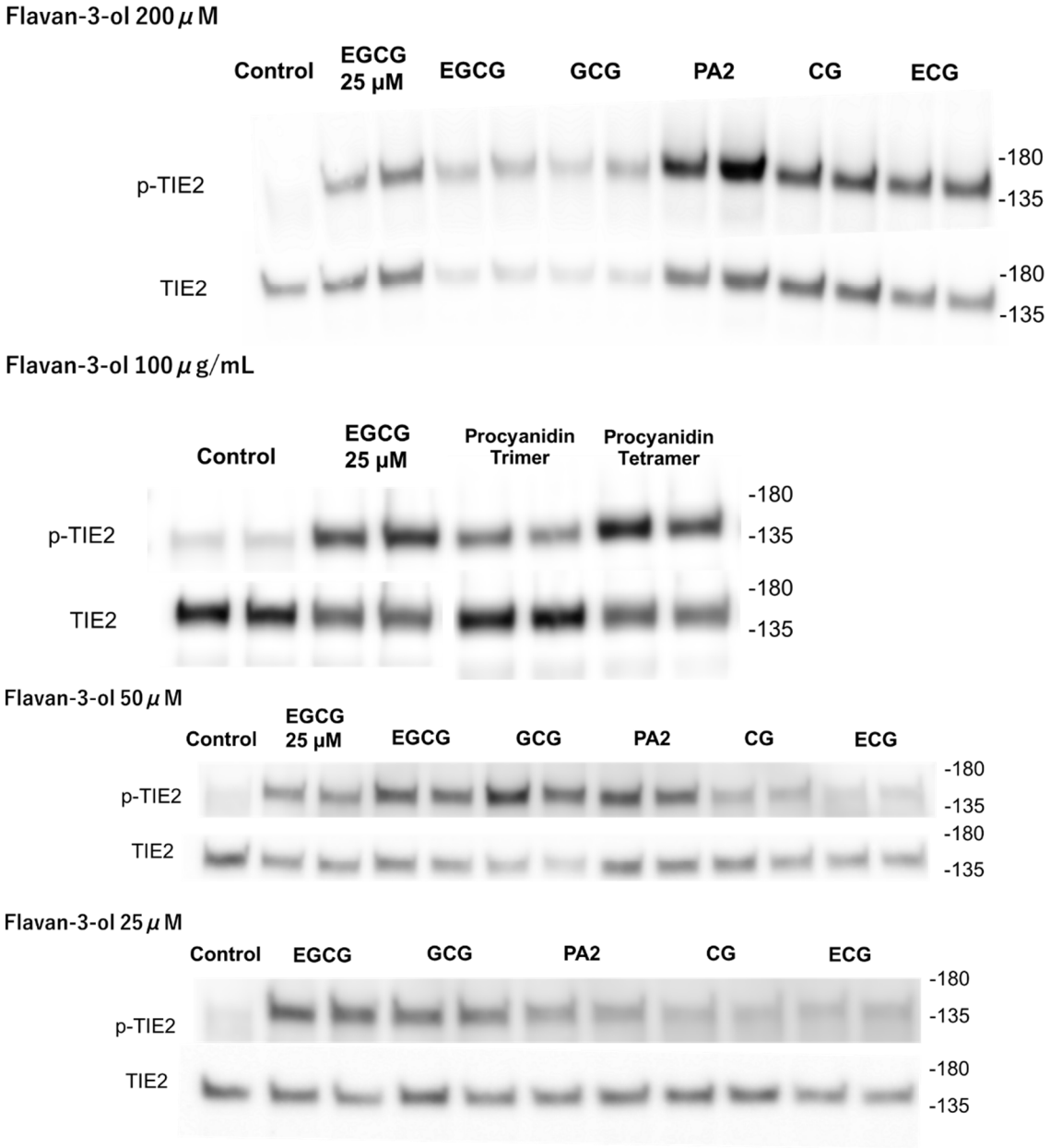

Figure S10. Western blotting analysis of p-TIE2 and TIE2 (flavan-3-ol)

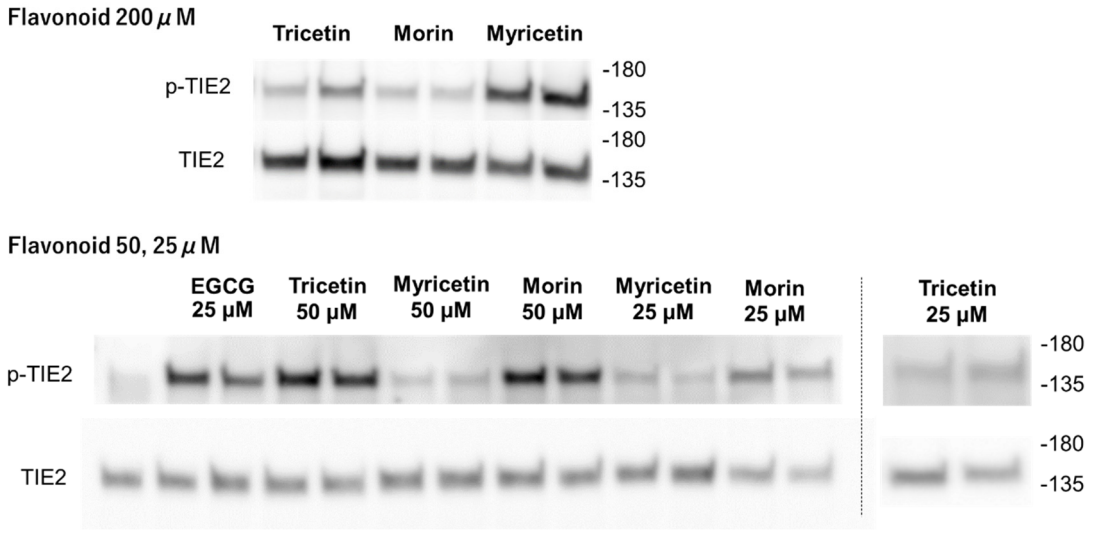

Figure S11. Western blotting analysis of p-TIE2 and TIE2 (flavonoids)

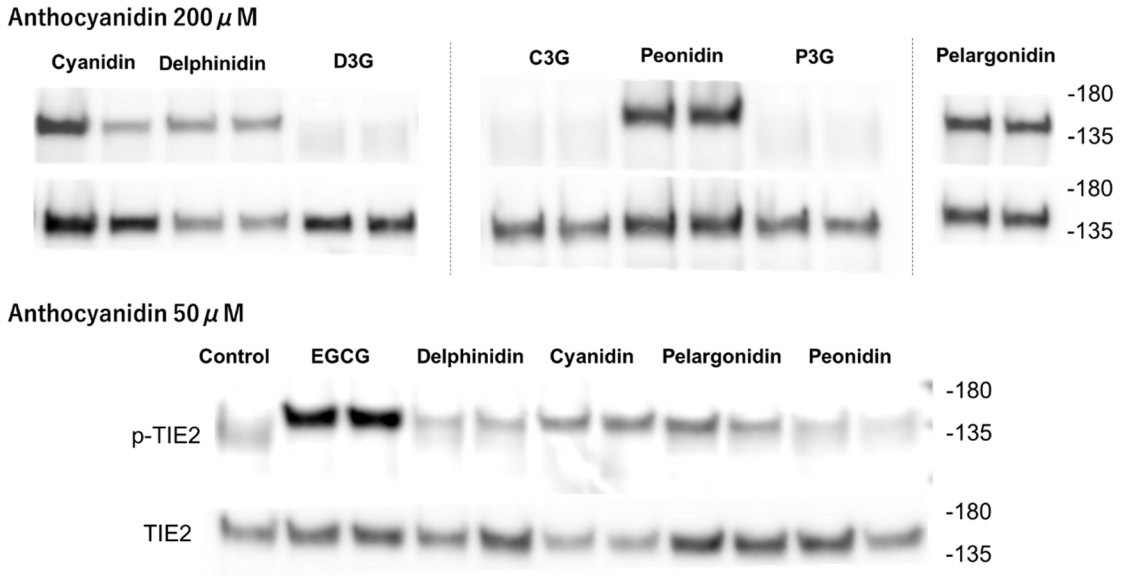

Figure S12. Western blotting analysis of p-TIE2 and TIE2 (anthocyanins)

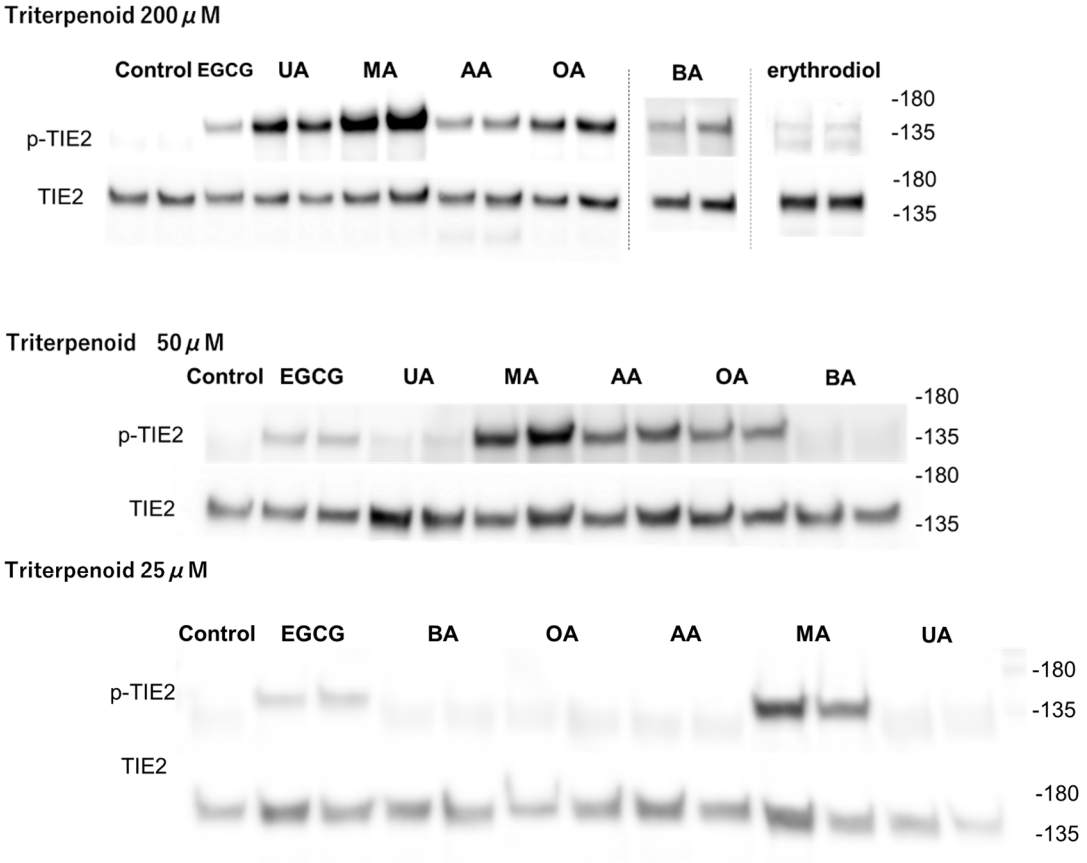

Figure S13. Western blotting analysis of p-TIE2 and TIE2 (triterpenoids)

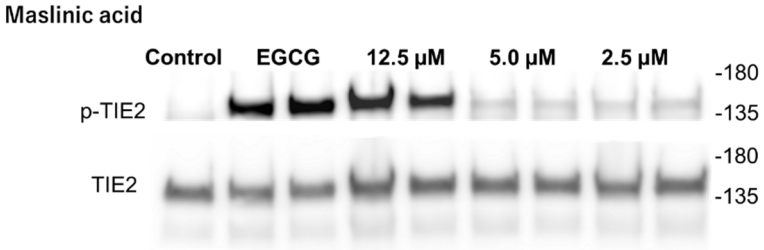

Figure S14. Western blotting analysis of p-TIE2 and TIE2 (maslinic acid)

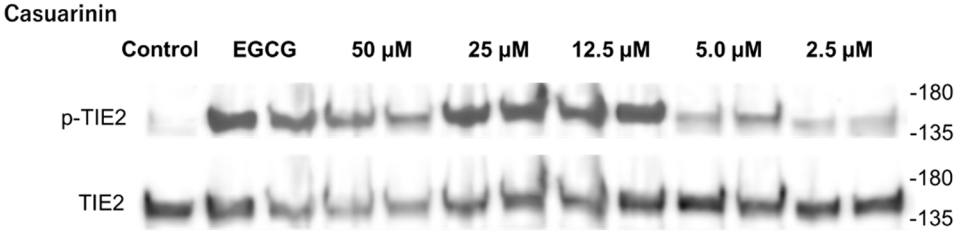

Figure S15. Western blotting analysis of p-TIE2 and TIE2 (casuarinin)

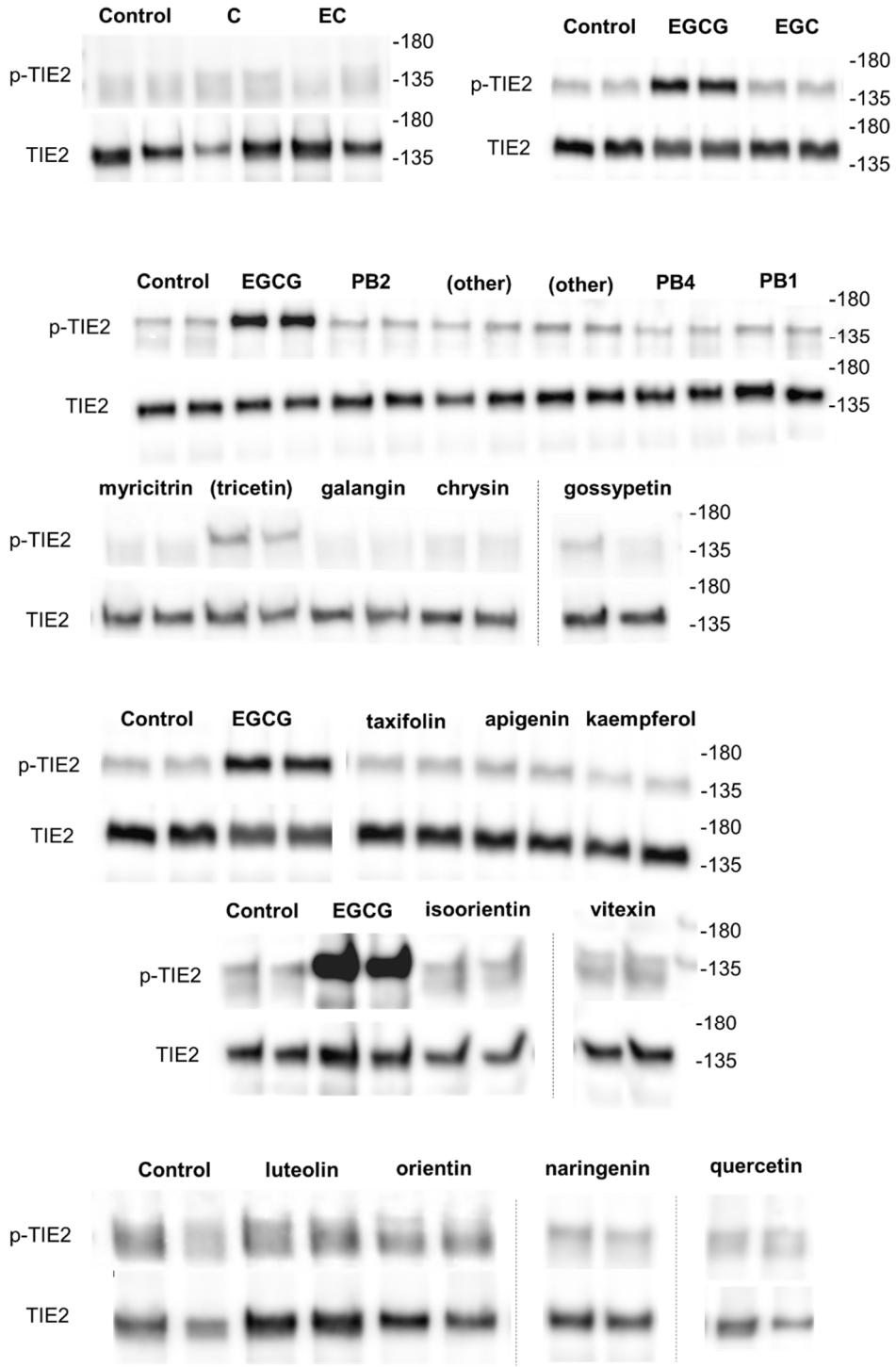

Figure S16. Western blotting analysis of p-TIE2 and TIE2 (inactive compounds)

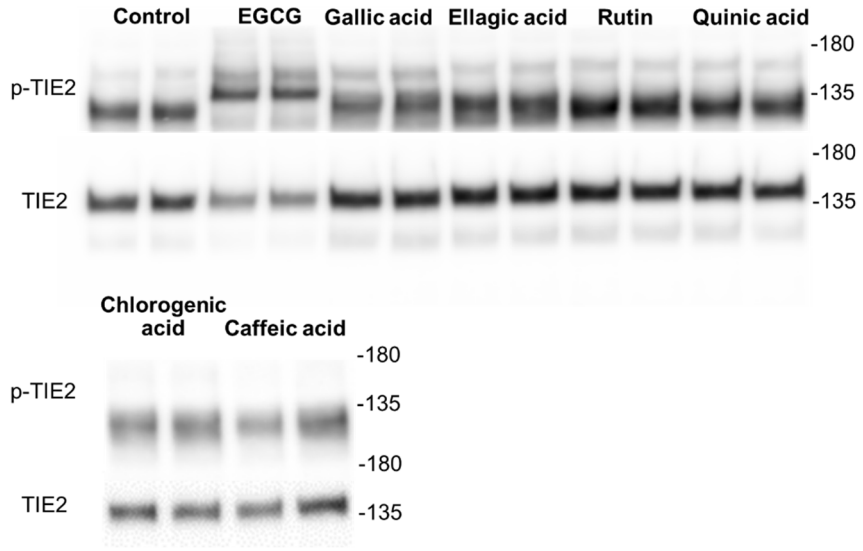

Figure S17. Western blotting analysis of p-TIE2 and TIE2 (inactive compounds)

Experimental procedures

Plant samples and chemicals

Powdered extracts of plant samples were purchased from Maruzen Pharmaceuticals Co., Ltd. (Hiroshima, Japan). Lot numbers: Indian dates (Lot No. 10125037), Rooibos tea (Lot No. 10227080), Long pepper (Lot No. 10527013), Guava leaf (Lot No. 00907034), Nelumbo nucifera germ (Lot No. 10311168). 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 LC-MS (Figure S18 and S19). The method of separating procyanidins according to their degree of polymerization (Kelm et al. 2006) and the m/z of the detected peaks (trimer m/z=867, tetramer m/z=1155, $[M+H]^{+}$) supported that the purified compounds were the trimer and tetramer of procyanidin.

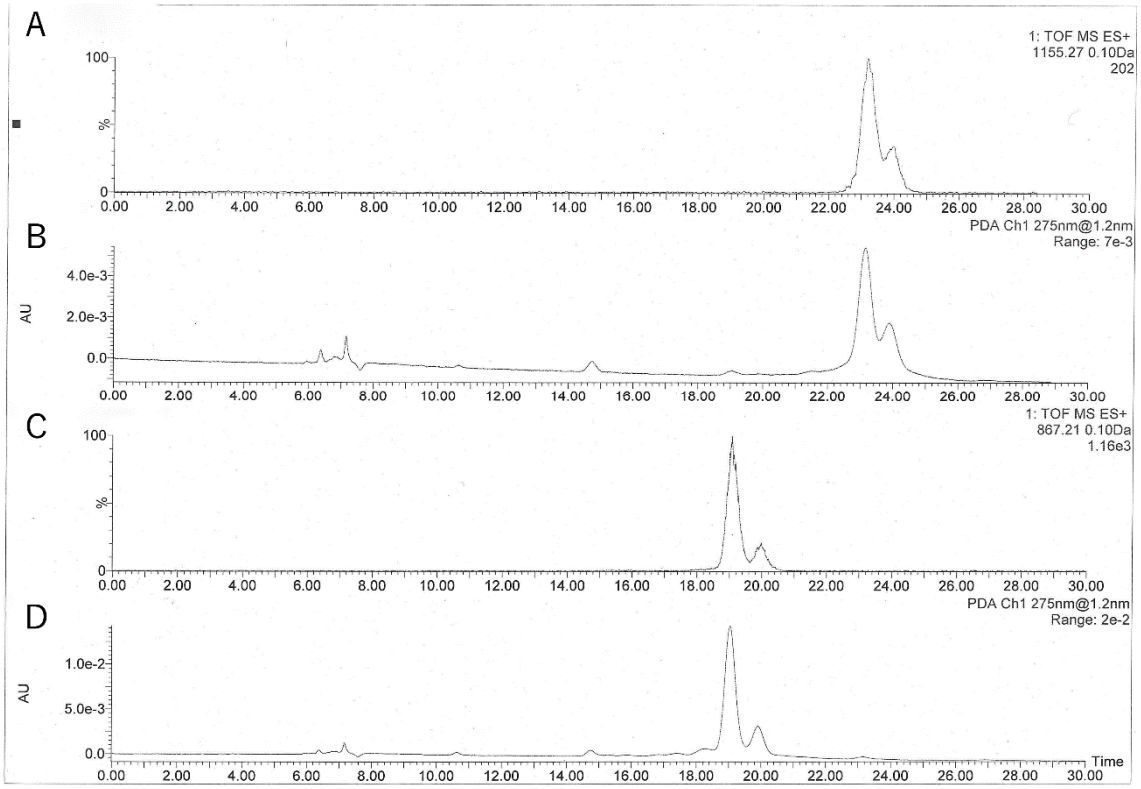

Figure S18. LC-MS analysis of the procyanidin trimer and tetramer. A,B) Extracted ion chromatogram (m/z=1155) and UV chromatogram (275 nm) of procyanidin tetramer. C,D) Extracted ion chromatogram (m/z=867) and UV chromatogram (275 nm) of procyanidin trimer. Column: Inertsil HILIC (GL Science Co., 4.6 mm×250 mm), Eluent: Solvent A/B = 8/2 (0 min) to 4/6 (60 min), Solvent A: acetonitrile with 0.1% formic acid, Solvent B: methanol with 3% water and 0.1% formic acid, Flow rate: 0.4 mL/min, Temperature: room temperature, Detection: 275 nm

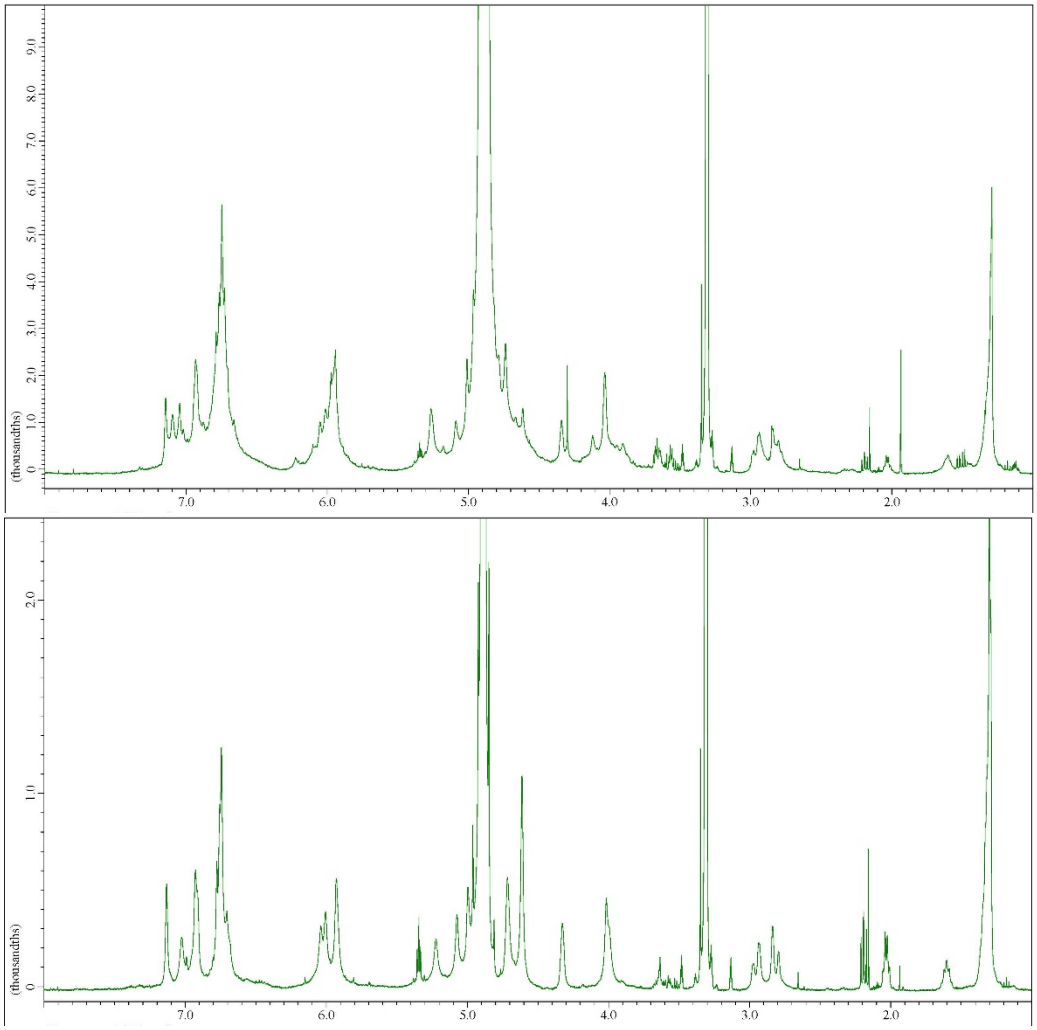

Figure S19. ^{1}H-NMR (400 MHz, CD_3OD, room temperature) spectrum of procyanidin trimer (bottom) and tetramer (top).

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 π NAP (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)(Figure S20). The NMR data was matched with the reported data (Figure S21)(He et al. 2017). HRMS data also matched with the structure (m/z=935.0802, $C_{41}H_{27}O_{26}$ calculated 935.0796 $[M-H]^-$) (Figure S22).

^{1}H-NMR (500 MHz, CD_3OD): 4.05 (1H, d, J=13.3 Hz), 4.69 (1H, dd, J= 2.0, 5.0 Hz), 4.90 (1H, dd, J= 3.4, 13.3 Hz), 5.30 (1H, dd, J= 3.4, 8.7 Hz), 5.37 (1H, dd, J= 1.9, 2.0 Hz), 5.44 (1H, dd, J= 1.9, 8.7 Hz), 5.51 (1H, d, J= 5.0 Hz), 6.37 (1H, s), 6.50 (1H, s), 6.81 (1H, s), 7.06 (2H, s) ppm; ^{13}C-NMR (125 MHz, CD_3OD): 65.1, 67.8, 70.6, 71.7, 74.8, 78.0, 105.3, 107.7, 109.2, 110.4, 116.0, 116.7, 116.9, 118.0, 120.2, 120.8, 125.2, 127.0, 127.6, 135.8, 137.0, 137.9, 140.1, 140.3, 144.4, 144.5, 144.9, 145.0, 145.96, 145.98, 146.5, 146.6, 146.9, 167.0, 167.2, 169.5, 170.4, 170.9 ppm.

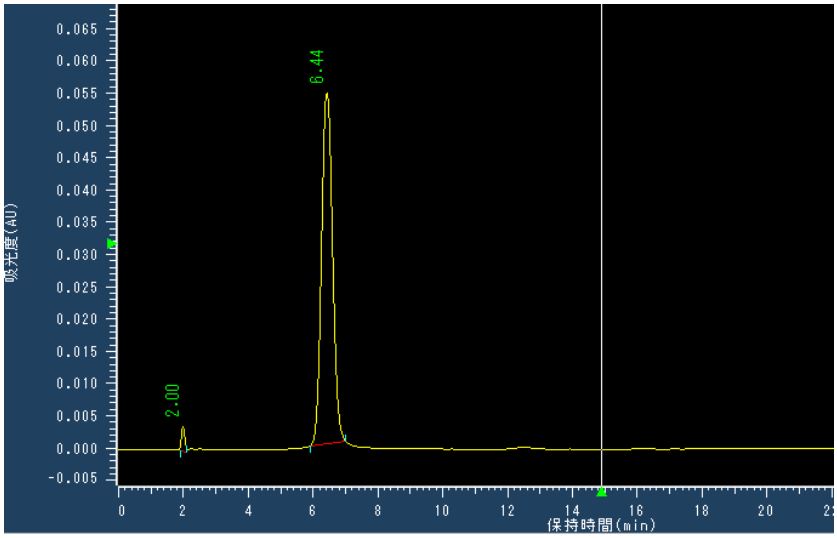

Figure S20. HPLC chromatogram of the isolated casuarinin (Rt=6.44 min).
Column: Cosmosil π NAP (4.6 mm × 150 mm), Eluent: 25% aq. methanol with 0.1% trifluoroacetic acid, Flow rate: 1 mL/min, Temperature: 40°C, Detection: 254 nm

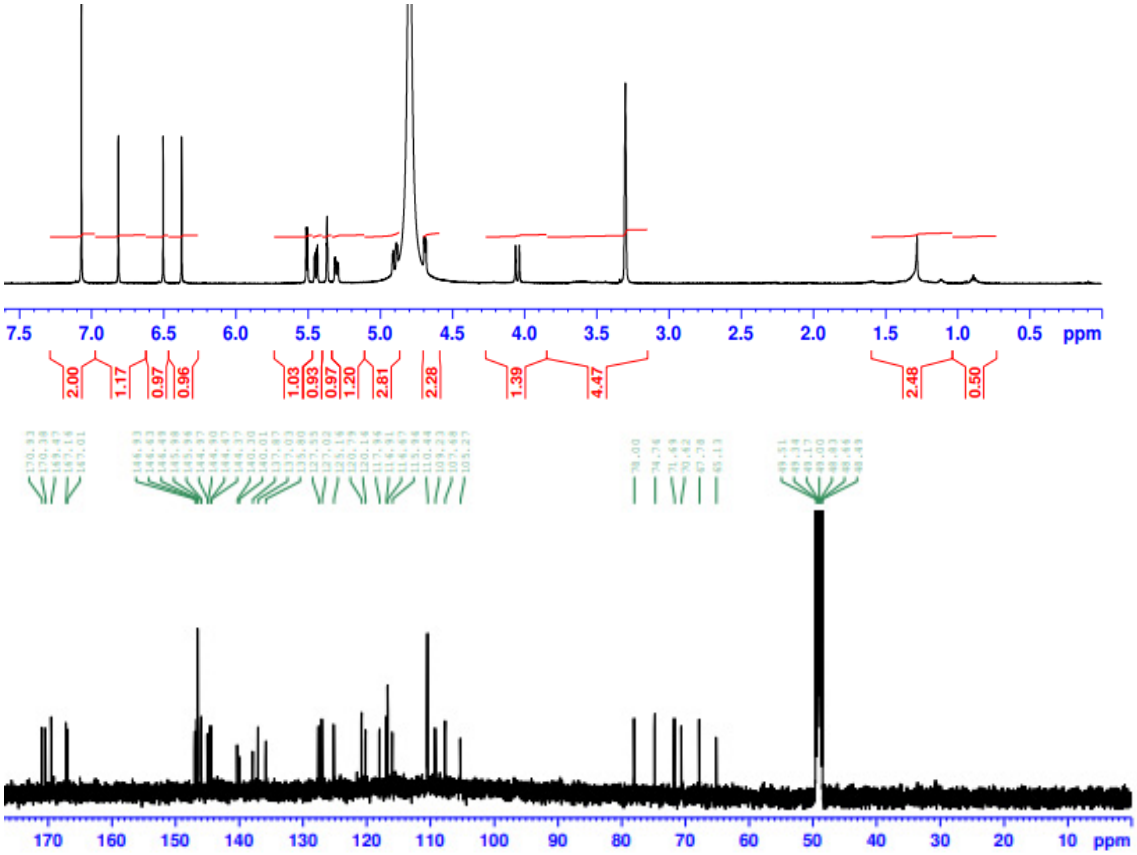

Figure S21.[1]H-NMR (500 MHz, CD_3OD) and [13]C-NMR (125 MHz, CD_3OD) spectrum of casuarinin

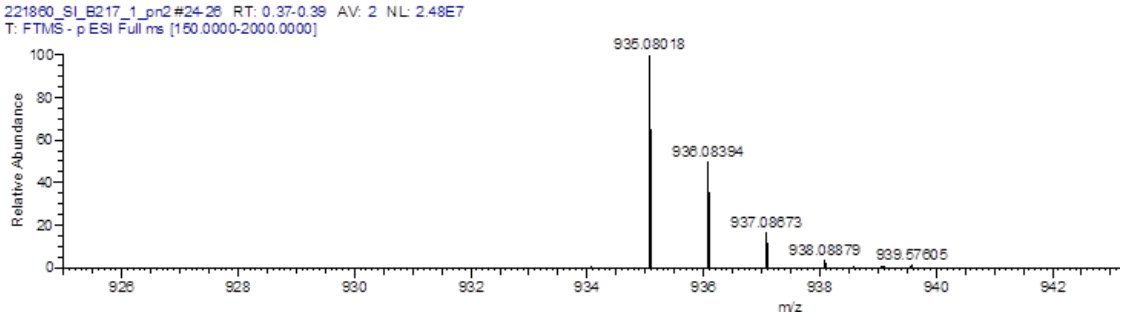

Figure S22. HRMS (negative) spectrum of casuarinin.

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

Kelm MA, Johnson JC, Robbins RJ, Hammerstone JF, Schmitz HH. 2006. High-Performance Liquid Chromatography Separation and Purification of Cacao (*Theobroma cacao* L.) Procyanidins According to Degree of Polymerization Using a Diol Stationary Phase. J Agric Food Chem. 54(5):1571–1576. https://doi.org/10.1021/jf0525941