**Functional evaluation of TIE2 activators identified in a model system using human umbilical vein endothelial cells**

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# Functional evaluation of TIE2 activators identified in a model system using human umbilical vein endothelial cells

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**Abstract**

TIE2 (or TEK) receptor is a tyrosine kinase receptor important for maintaining vascular system function. Decreased TIE2 signalling associates with various diseases and deletion of TIE2 increases the risk of vascular diseases like atherosclerosis. Therefore, activators of TIE2 are suggested and shown to be efficient for maintaining healthy vascular systems and protect from diseases. We have previously explored several plant derived compounds as TIE2 activator using transiently expressed TIE2 in HeLa cells. In this study, we evaluated if the identified TIE2 activators, casuarinin, procyanidin tetramer (PC4), and morin, are capable to activate TIE2 signalling in human umbilical vein endothelial cells. We showed that casuarinin and PC4 activates AKT and ERK, the two kinase downstream of TIE2, and enhances tube formation in human umbilical vein endothelial cells. These results support the idea that the TIE2 activators identified in the TIE2-expressing HeLa cells are candidates for vascular protection.

**Keywords**

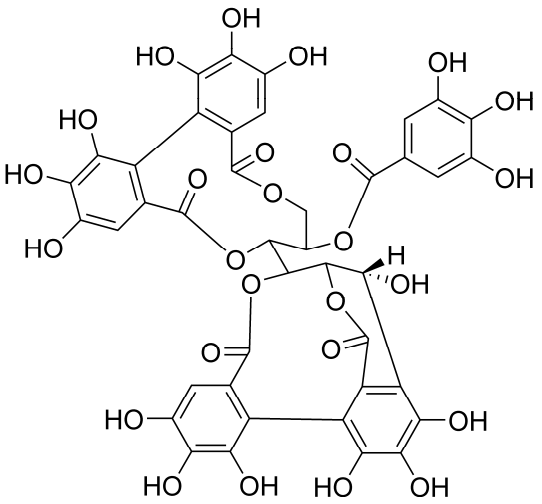
TIE2/TEK receptor, vascular system, HUVEC, tube formation, angiogenesis, casuarinin, procyanidin

TIE2 (or TEK) receptor is a tyrosine kinase receptor expressed in vascular endothelial cells that maintains vascular functions.[1] The receptor's endogenous ligand is angiopoietin(ANG)-1, which supports the formation and maintenance of a healthy vascular systems.[1]

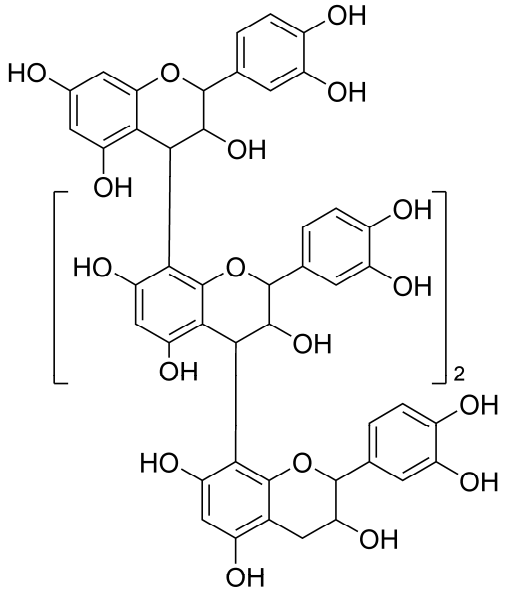
TIE2 signalling associates with variety of diseases. Increased ANG-2, a context-dependent TIE2 antagonist, is associated with cancer, inflammation, and diabetic retinopathy.[2] Deletion of TIE2 in the arteries increases atherosclerosis in mice.[3] Conversely, activation of TIE2 by either AKB-9778 or COMP-ANG1 delayed tumour growth and enhanced the response to cytotoxic drugs,[4] suppressed inflammation,[5] and ameliorated diabetic retinopathy,[6] suggesting that TIE2 activators are efficient to maintain healthy vascular systems and protect from diseases.

We have recently identified natural compounds such as triterpenes, flavonoids, procyanidins and hydrolysable tannins as activators of TIE2 from Indian dates (*Tamarindus indica*) and guava leaves (*Psidium guajava*).[7] This suggests that the identified compounds have a potency to protect vascular systems through activation of TIE2. However, the above TIE2 activators were evaluated in the model system using HeLa cells transiently expressing TIE2. Therefore, it is not yet known whether these compounds activate TIE2 and induce beneficial functions in human vascular endothelial cells.

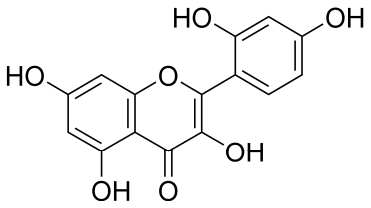
In this study, casuarinin which was isolated from Indian dates; procyanidin tetramer (PC4) which was isolated from guava leaves; and morin which was identified from screening of flavonoids were selected (Fig. 1). Among the previously identified compounds, these compounds activate TIE2 at relatively low concentrations in the HeLa model system.[7] Thus, their effects on human umbilical vein endothelial cells (HUVEC) were evaluated.




casuarinin




procyanidin tetramer




morin

Figure 1. Compound structures

Due to the moderate expression levels of TIE2 in HUVECs and the difficulty in detecting its phosphorylation, downstream factors were selected to assess the effects of the compounds. AKT and ERK are the two kinases downstream of TIE2 in HUVECs.[8]

The activity of the compounds against these kinases was assessed and the involvement of TIE2 was also investigated by co-incubation with TIE2 inhibitor (CAS No. 948557-43-5) (Figure 2,3 and S1-4).

Casuarinin was found to act as a TIE2 activator in HUVECs. The compound phosphorylated AKT and addition of TIE2 inhibitor diminished the effect. However, casuarinin did not activate ERK. The absence of ERK phosphorylation is probably due to its inhibitory activity against ERK. Casuarinin is reported to inhibit the glutamate induced phosphorylation of ERK in HT22 cells (mouse hippocampal neuronal cell line) and TNF-α induced phosphorylation of ERK in HaCaT cells (immortalized human keratinocyte cell line).[9, 10] The mechanism of ERK inhibition was shown to be by preventing the accumulation of glutamine-generated reactive oxygen species (ROS) in HT22 cells.

PC4 is also found to act as a TIE2 activator in HUVECs from the phosphorylation of ERK. The compound also activated AKT, but was not diminished by TIE2 inhibitor, suggesting the involvement of other mechanism in the activation of AKT. The activation of AKT is likely to involve ROS, as procyanidins have been reported to induce AKT activation in HUVECs via the production of ROS.[11, 12]

Morin does not activate both AKT and ERK, suggesting that it does not act as a TIE2 activator in HUVECs. However, in immortalized murine BV2 microglial cells studies, morin is shown to inhibit both AKT and ERK,[13] and other studies also show the inhibition of either AKT or ERK.[14, 15] It is therefore possible that the TIE2-mediated function of morin may have been abolished by the effect of morin on inhibiting AKT and ERK.

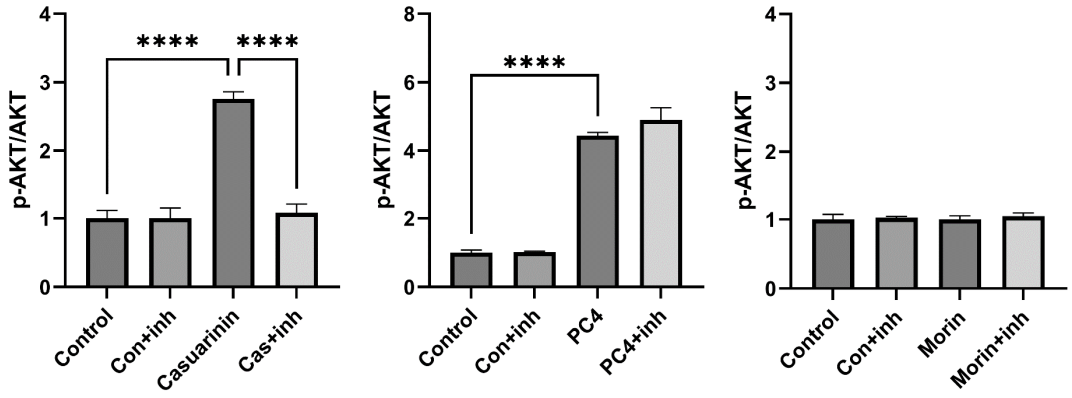



Figure 2. Effect of compounds on phosphorylation of AKT.

HUVECs were stimulated by casuarinin (cas, 5 μM), procyanidin tetramer (PC4, 5 μM), or morin (100 μM) with or without TIE2 inhibitor (inh, 500 nM) for 60 min. **p<0.01, ****p<0.0001, ns: no significance (n=4, Tukey's test).

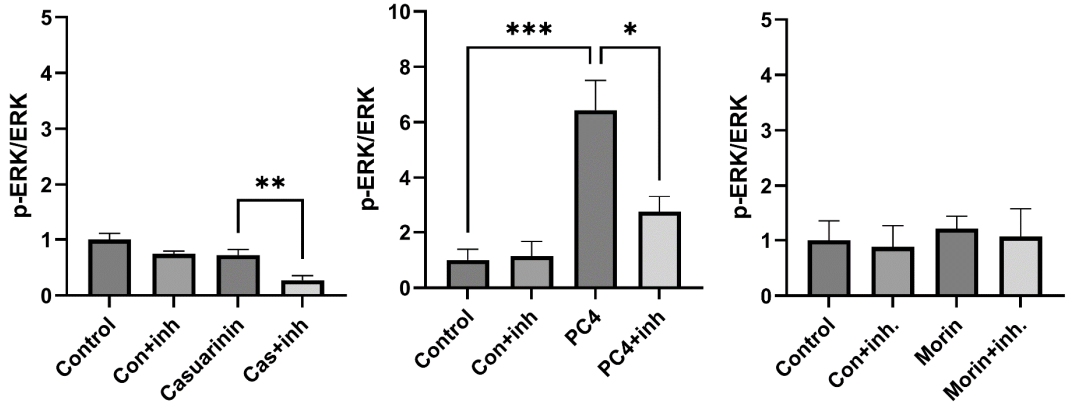



Figure 3. Effect of compounds on phosphorylation of ERK.

HUVECs were stimulated by casuarinin (Cas, 5 μM), procyanidin tetramer (PC4, 10 μM), or morin (100 μM) with or without TIE2 inhibitor (inh, 500 nM) for 15 min. *p<0.05, **p<0.01, ***p<0.001, ns: no significance (n=4, Tukey's test).

The results so far show that casuarinin and PC4 activate TIE2 in HUVECs. Therefore, these compounds are expected to induce similar effects to ANG-1 in HUVECs. One of the actions of ANG-1 is an angiogenesis-promoting effect, which can be measured by a tube formation assay.

HUVECs were seeded on Matrigel® matrix and stimulated by the compounds. The tube formation was evaluated by Angiogenesis Analyzer for ImageJ.[16] Number of junctions, total length, and total meshes area clearly indicated the efficacy of casuarinin and PC4. These parameters showed apparent increase in the values compared to control, and significant decrease by co-incubation with TIE2 inhibitor (Figure 4 and S5). Therefore, casuarinin and PC4 have been proved to mediate at least one of the beneficial effects of ANG-1 in HUVECs.

In conclusion, casuarinin and PC4, the TIE2 activators identified by TIE2-expressing HeLa cell, were proved to be effective in modulating the function of human vascular endothelial model cells HUVECs. However, multiple signalling pathways are involved in some cases, and those pathways may interfere with part of TIE2 signalling pathway. This study showed that even part of the kinase activation is interrupted, enhancement of tube formation, an indicator of angiogenesis capacity, is still apparently shown by the TIE2 activators. TIE2 has multiple functions including anti-inflammatory activity. The remaining functions still need to be explored to fully understand the efficacy of the identified TIE2 activators in protecting vascular endothelial cells.

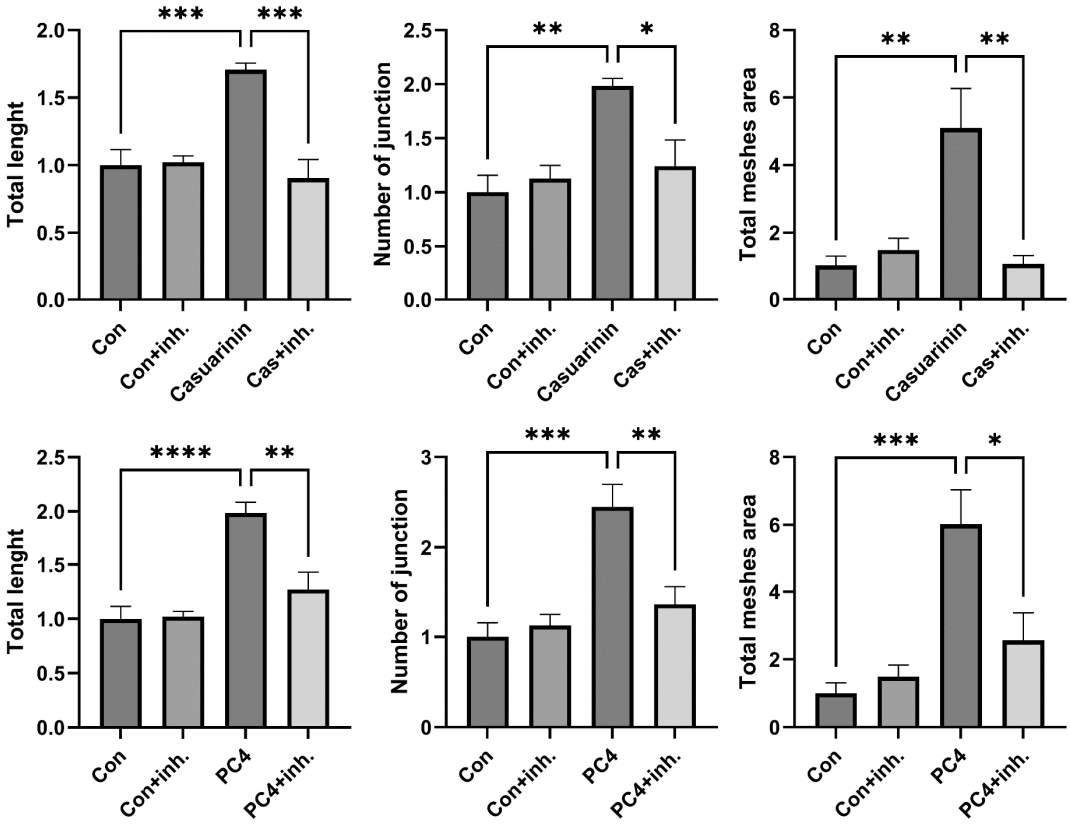



Figure 4. Enhancement of tube formation by the compounds.

Casuarinin (5 μM) or procyanidin tetramer (PC4, 5 μM) were added to HUVECs with or without TIE2 inhibitor (500 nM) and the tube formation was analyzed at 18-24 hr. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (n=5, Tukey's test).

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Supplementary material

# Functional evaluation of TIE2 activators identified in a model system using human umbilical vein endothelial cells

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## Content

# Supplementary Figures

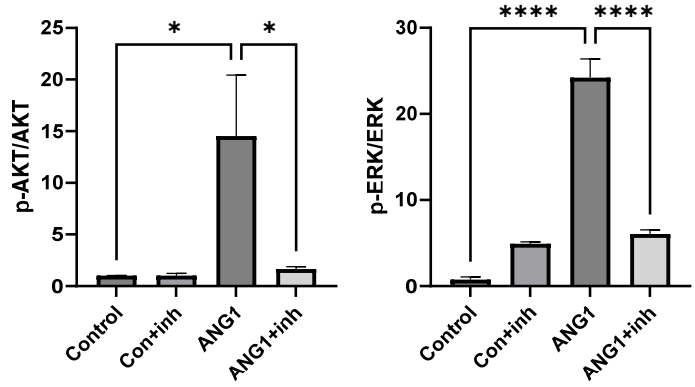


Fig. S1. AKT and ERK activation by Angiopoietin-1 (positive control)

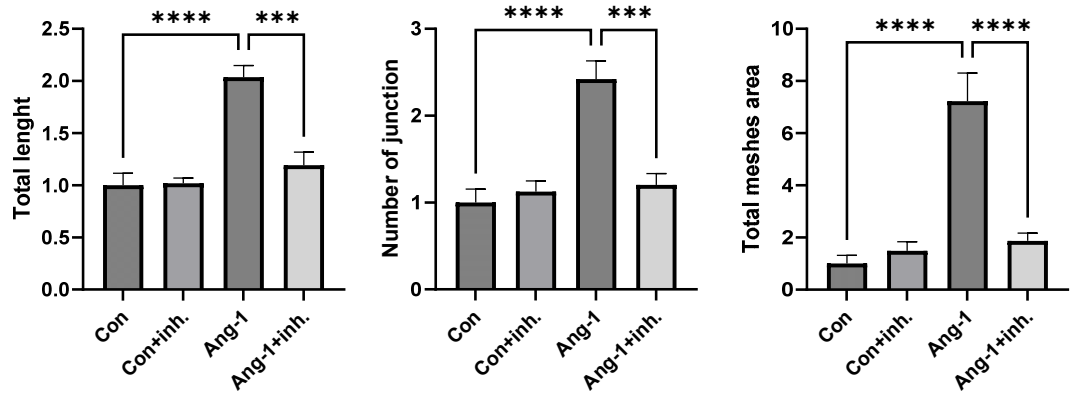


Fig. S2. Enhancement of tube formation by Angiopoietin-1 (positive control)

# Western Blotting Photos

- AKT

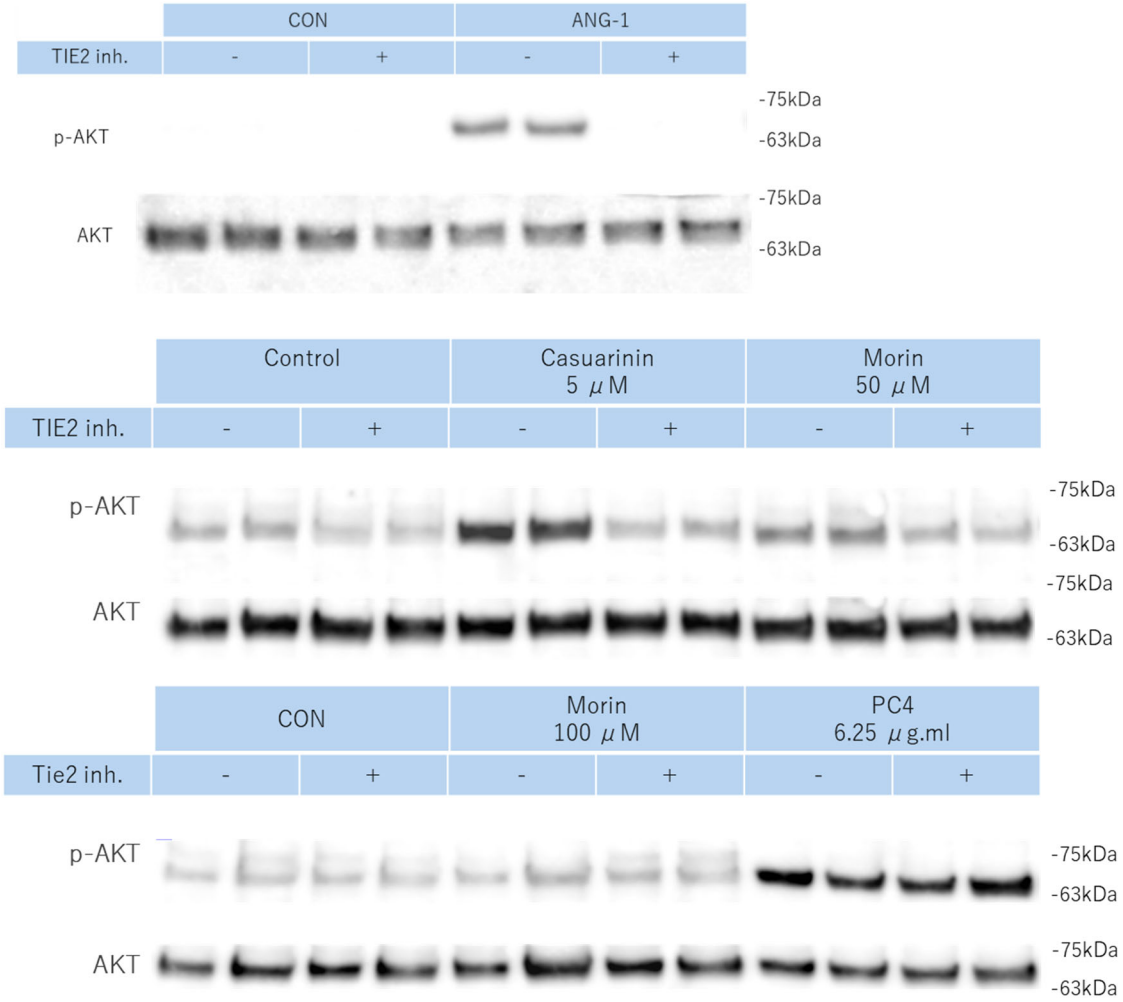


Figure S3. Western blotting photo for the evaluation of AKT activation.
HUVECs were serum starved for 90 min, preincubated with or without TIE2 inhibitor (500 nM) for 30 min, and stimulated by angiopoietin-1 (ANG-1), casuarinin, procyanidin tetramer (PC4), or morin at the indicated concentration with or without TIE2 inhibitor (500 nM) for 60 min.

- ERK

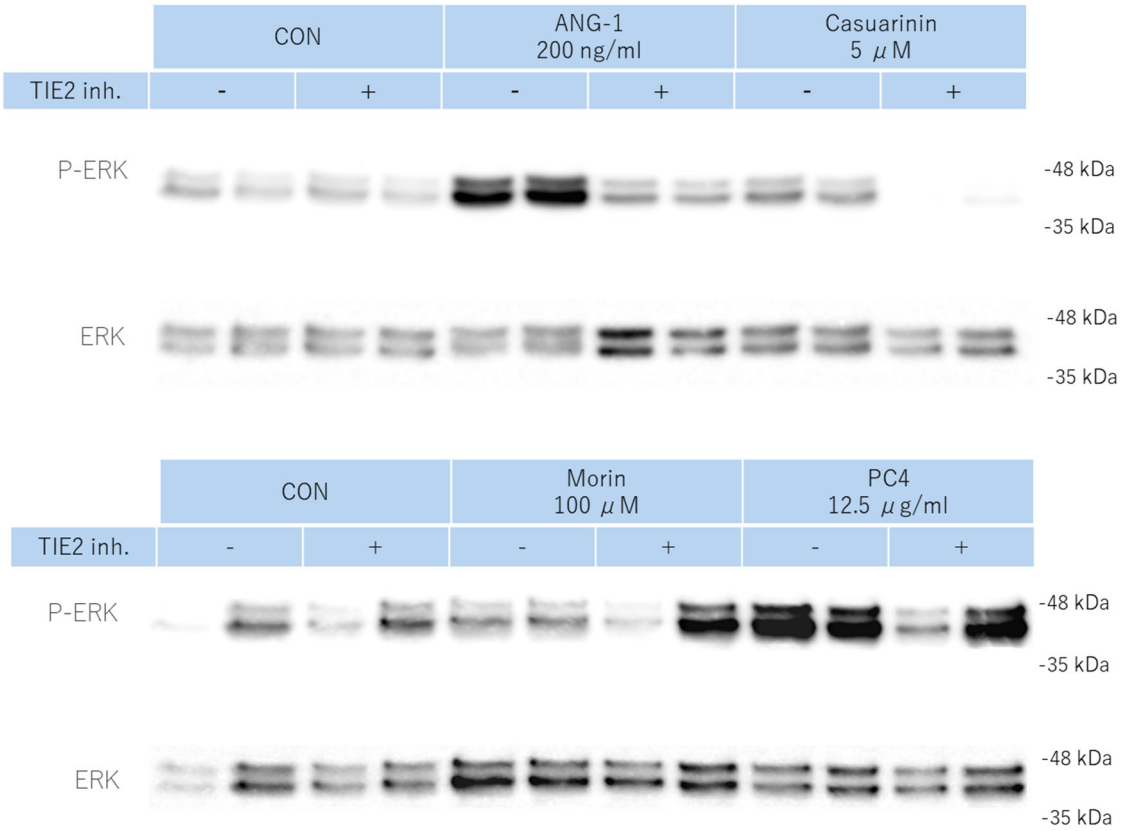


Figure S4. Western blotting photo for the evaluation of ERK activation.

HUVECs were serum starved for 18 hr, preincubated with or without TIE2 inhibitor (500 nM) for 30 min, and stimulated by angiopoietin-1 (ANG-1), casuarinin, procyanidin tetramer (PC4), or morin at the indicated concentration with or without TIE2 inhibitor (500 nM) for 60 min.

# Photo of tube formation assay

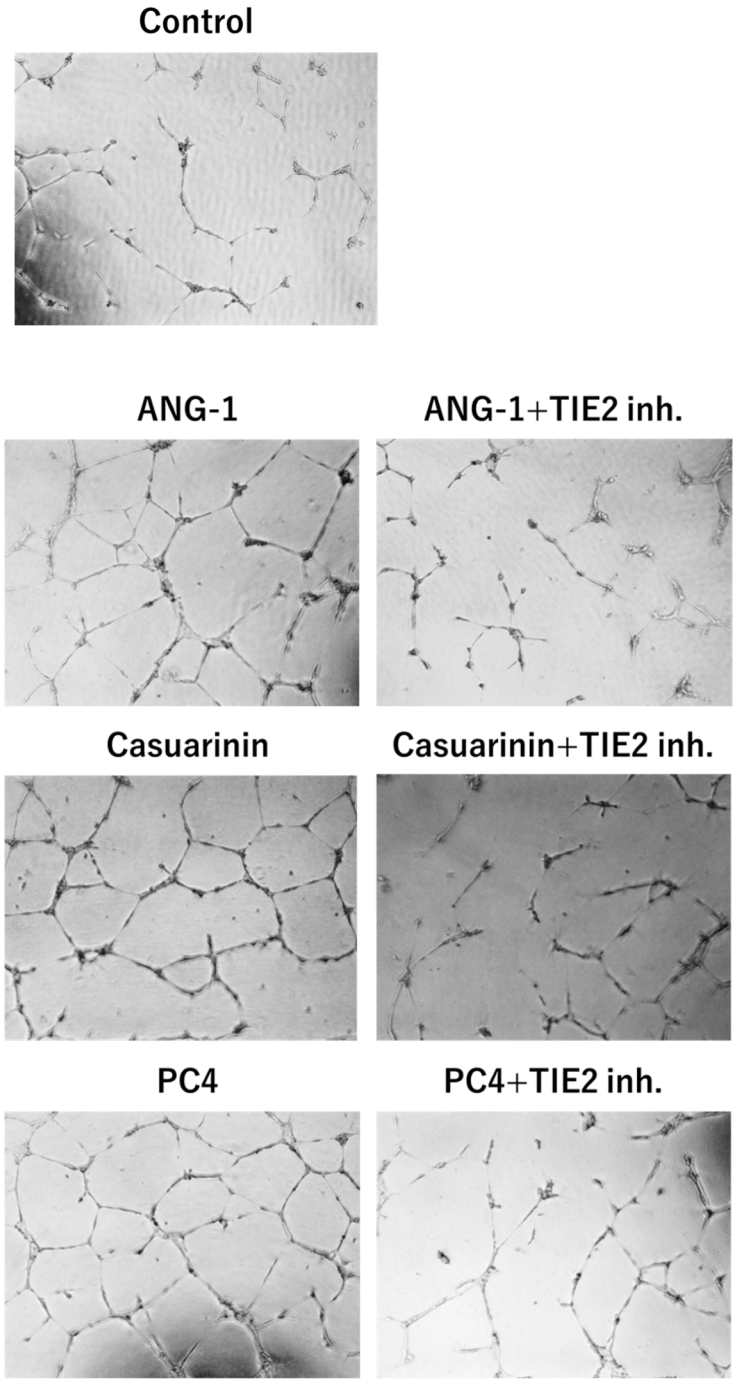


Figure S5. Representative photo for the evaluation of the enhancement of tube formation by compounds.

Casuarinin (5 μM) or procyanidin tetramer (PC4, 5 μM) were added to HUVECs with or without TIE2 inhibitor (500 nM) and seeded on Matrigel matrix and the photo was taken at 18-24 hr.

# Methods

**Compounds**

Casuarinin and procyanidin tetramers were isolated from Guava leaf and Indian dates as described previously.[1] Morin was purchased from Tokyo Chemical Industry Co., Ltd. TIE2 inhibitor (Item No. 17858) was purchased from Cayman Chemical. Recombinant Human Angiopoietin-1 Protein (Catalog #923-AN-025) was purchased from R&D systems.

**Cell culture**

HUVECs were obtained from Takara Bio Inc. The cells were cultured in collagen I coated cell culture dish at 37°C in a 10% $CO_2$ atmosphere in MCDB131 Medium (Gibco) supplemented with 10% fetal bovine serum (FBS), endothelial growth supplement, L-glutamine, heparin and antibiotics.

**Analysis of AKT phosphorylation**

HUVECs were seeded in collagen I coated 24 well plates at $5\times10^4$ cell/well and cultured for 48 hr. The medium was changed to MCDB131 with L-glutamine and serum starved for 90 min. In case of casuarinin 0.1% FBS and heparin was added to the medium. Then the cells were treated with TIE2 inhibitor for 30 min followed by sample compounds together with TIE2 inhibitor for 60 min. The cells were washed with phosphate buffered saline (PBS), lysed using EzRIPA Lysis buffer containing protease and phosphatase inhibitors (Atto Co.) and analyzed by western blotting.

**Analysis of ERK phosphorylation**

HUVECs were seeded in collagen I coated 24 well plates at $5\times10^3$ cell/well and cultured for 24 hr. The medium was changed to MCDB131 with L-glutamine and 1% bovine serum albumin and serum starved for 18 hr. Then the cells were treated with TIE2 inhibitor for 30 min followed by sample compounds together with TIE2 inhibitor for 15 min. The cells were washed with phosphate buffered saline, lysed using EzRIPA Lysis buffer containing protease and phosphatase inhibitors (Atto Co.) and analyzed by western blotting.

**Western blotting**

The lysate was separated by SDS-PAGE (10%) and transferred to PVDF membrane. The membrane was blocked by 3% non-fat dry milk in TBS-T (Tris buffered saline with tween 20). The membrane was treated with primary antibody for overnight at 4°C, secondary antibody (Anti-rabbit IgG, HRP-linked Antibody, 1:2000, cell signaling technology, 7074) for 1 hr at room temperature and then detected by either ImmunoStar® LD or Zeta (Fujifilm Wako Pure Chemical Co.). After detection, the membrane was soaked in stripping solution for 10-20 min and used to detect another protein.

Following antibodies and detection reagents were used.

| Target | Primary antibody | dilution |
|---|---|---|
| p-AKT | AKT (phospho Ser473) antibody (genetex, 128414) | 1:500 |
| AKT | AKT antibody [N3C2], Internal (genetex, 121937) | 1:1000 |
| p-ERK | Phospho-p44/42 MAPK (Erk1/2)(Thr202/Tyr204) Antibody (CST, 9101) | 1:8000 |
| ERK | p44/42 MAPK (Erk1/2)(137F5) Rabbit mAb (CST, 4695) | 1:2000 |

**Tube formation assay**

Matrigel matrix (Corning, Product Number 354234, 100 µL/well) was added to a cooled 48-well plate and allowed to stand at 37°C for 1 hour. HUVECs (2.5 x $10^4$ cells/well) were seeded on the prepared gel and the test compound was added and incubated for 18-24 hr. The medium was aspirated, washed with 250 µl of PBS and replaced with 250 µl of serum starved medium (MCDB131+L-glutamine). Six random photos were taken from one well and analysed using the Angiogenesis Analyzer in ImageJ.[2]


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