Electronic Supplementary Material (ESI) for Food & Function.

Supporting Information

(*R,S*)-Equol 7-β-D-glucuronide, but not other circulating isoflavone metabolites, modulates migration and tubulogenesis in human aortic endothelial cells targeting the VEGFR2 pathway

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Experimental procedures

Western blot analysis of the bFGF pathway activation. The analysis of this pathway implied two steps: (i) Time-course activation (measured as ERK activation) of the bFGF pathway in HAEC cells. HAEC cells at ~80% confluence were incubated in basal medium for 7 h and then treated with 100 ng/mL bFGF for 2, 5, 15, and 30 min before extracting the cellular protein using RIPA buffer. (ii) Effect of the isoflavones on FGFR1 level and ERK activation in bFGF-treated cells. The cells were incubated in basal medium for 3 h before the treatment with 10 μM GEN, DAZ, Eq, and Eq 7-glur for 4 h. The cells were then stimulated with 100 ng/mL bFGF for 5 min (time point determined in the first step), and the protein was extracted using RIPA buffer. In both experiments, an equal amount of cell lysates (20 μg) were loaded into acrylamide gels (10 – 12%) and transferred to nitrocellulose membranes. To determine the activation of the bFGF pathway, we analyzed phosphorylated (p)- and total (t)- ERK via incubation of the membranes with p-ERK (Thr202/Tyr204, 20G11; #4377) and t-ERK (137F5; #4695) using a dilution 1:1000. The FGFR1 level was determined through the incubation of the membrane with total (t)-FGF receptor 1 (D8E4, #9740) using a dilution 1:1000. GAPDH (D4C6R, #97166), at a dilution 1:2500, was used as a loading control. Anti-rabbit and anti-mouse (1:5000 dilution) were used as secondary antibodies. The membranes were incubated for 5 min with SuperSignal West Pico PLUS Chemiluminescent Substrate detection system (ThermoFisher, Barcelona, Spain) and scanned using Amersham Imager 600 (Chicago, IL, USA). The analysis of the images obtained was determined using the ImageJ software v 1.53K (NIH, USA).

Western blot analysis of VEGFR1 and p38 in VEGF-treated HAECs. HAECs at ~80% confluence were incubated in basal medium for 3 h before being treated with 10 μM GEN, DAZ, Eq, and Eq 7-glur for 4 h. The cells were then stimulated with 100 ng/mL $VEGF_{165}$ for 5 min, and the lysates were obtained as described above. We used p-VEGFR1 (Y1213; AF4170), t-VEGFR1 (E7T9H, #64094), p-p38 (Thr180/Tyr182; D3F9, #4511), t-p38 (D13E1, #8690) as primary antibodies. GAPDH (1:2500) was used as the loading control. The development of the membranes was performed as described above.

Supplementary Figures

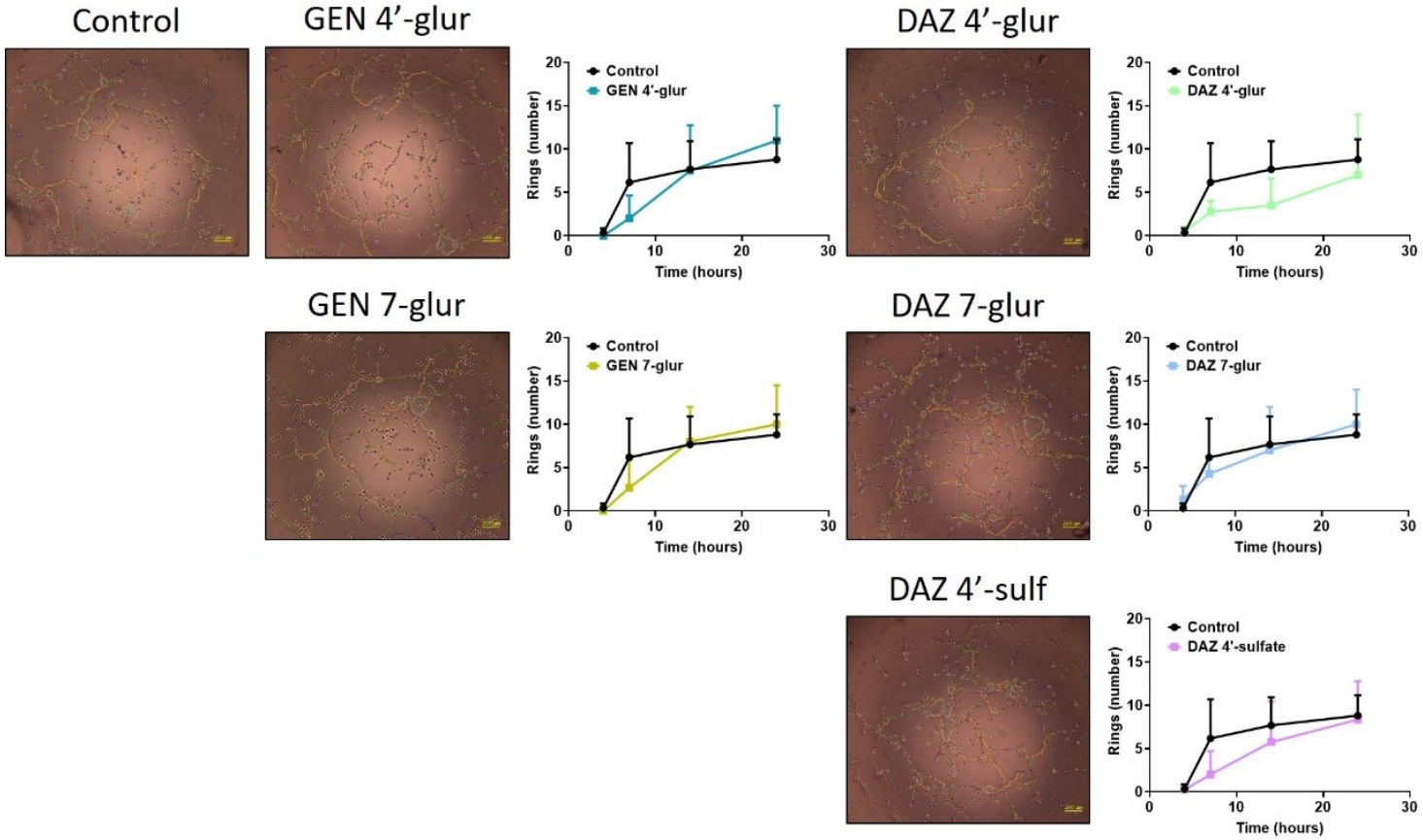

Figure S1. Time-course effect of isoflavones on tubulogenesis. Endothelial cells treated with 10 μM GEN 4'-glur, GEN 7-glur, DAZ 4'-glur, DAZ 7-glur, and DAZ 4'-sulf for 4 h, followed by the stimulation with 1.5% GS (/v) were photographed at different time points and analysed/quantified to determine the effect of isoflavones on cellular network formation. Illustrative images of the tube-like formation, taken at 24 h after isoflavone treatments, are accompanied by graphics that show the number of rings formed in the presence or absence of isoflavones. The results are expressed as the mean ± SD of three independent assays (n = 3).

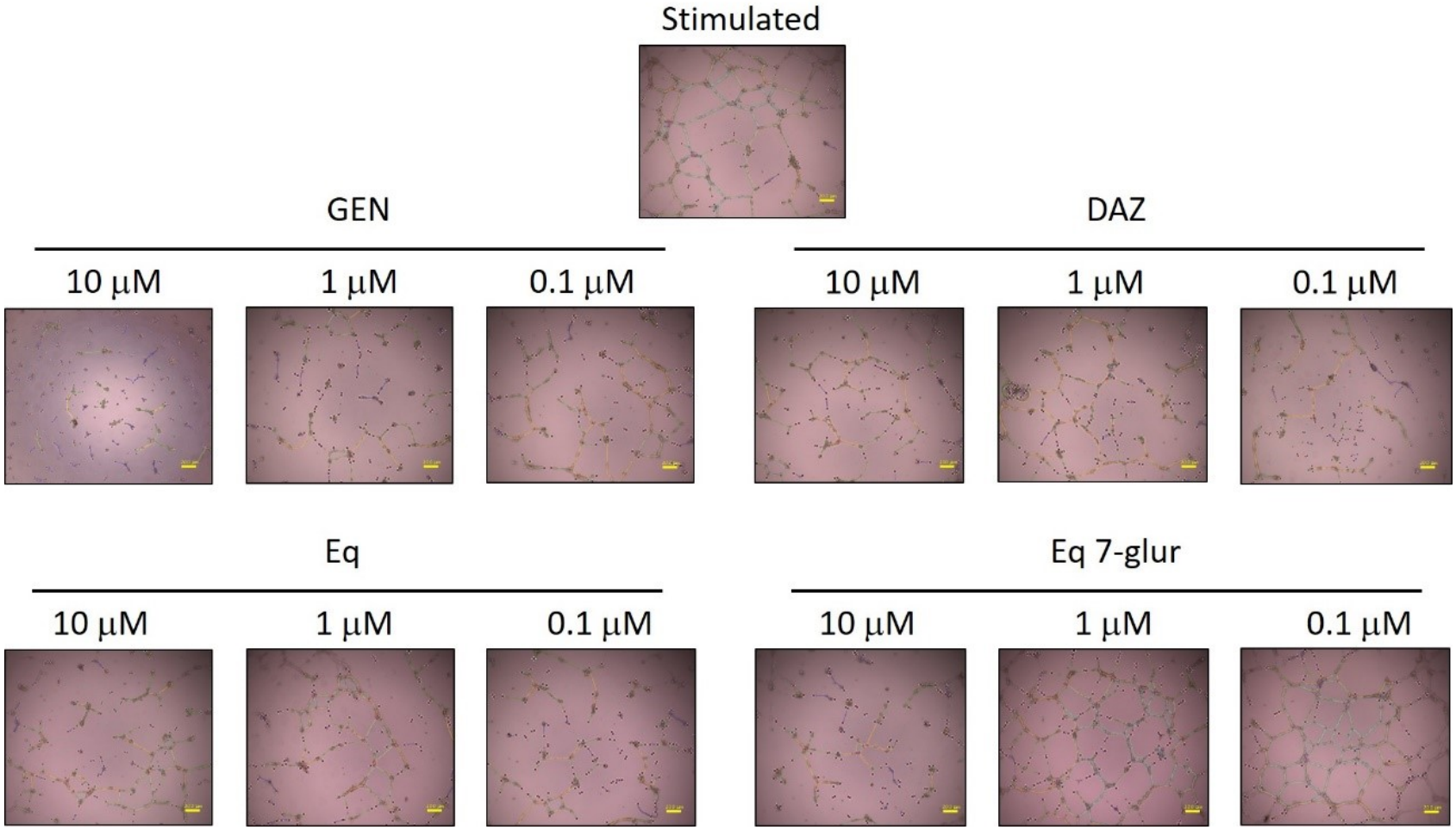

Figure S2. Illustrative images of the dose-dependent effect of isoflavones on tubulogenesis. Endothelial cells seeded on matrigel-coated wells and treated with GEN, DAZ, Eq, and Eq 7-glur (10 – 0.1 μM) for 4 h were stimulated with 1.5% GS (v/v). The cells were photographed at 5x magnification using a microscope (Zeiss fluorescence microscope) after 24 h treatment.

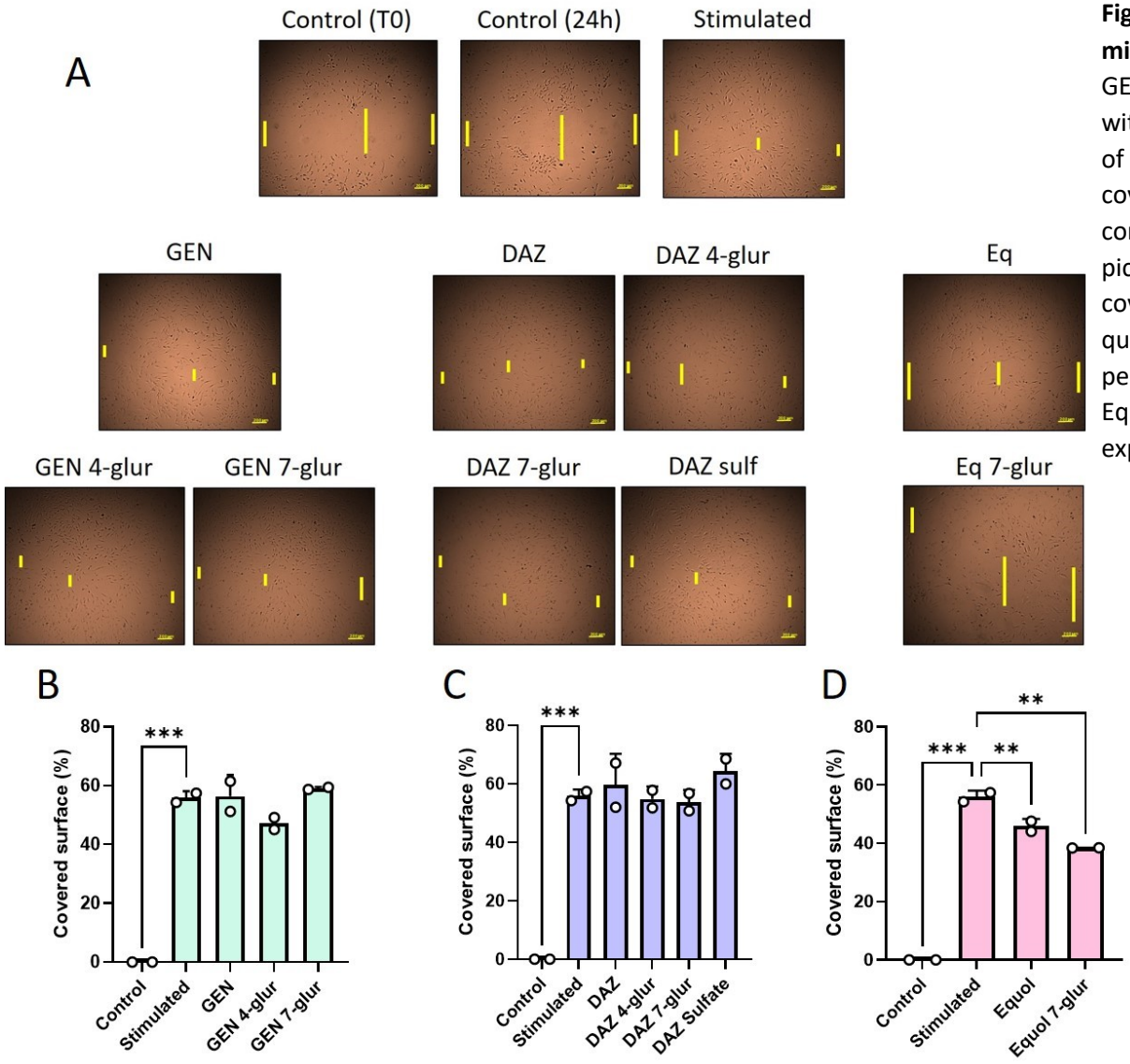

Figure S3. Effect of isoflavones on endothelial cell migration. Scratched cells were pretreated with 10 µM GEN, DAZ, Eq, and Eq 7-glur for 4 h before the stimulation with 1.5% GS (v/v). Representative images of the effects of isoflavones on the capacity of the endothelial cells to cover the gap formed are shown. Vertical yellow lines connecting the cells at the top and the bottom of the picture are used to illustrate the percentage of surface covered in the presence or absence of isoflavones (A). The quantification of the surface covered, expressed as a percentage (%), in the presence of GEN (B), DAZ (C) and Eq (D) is showed as the mean ± SD of two independent experiments (n = 2). **p<0.01, ***p<0.001.

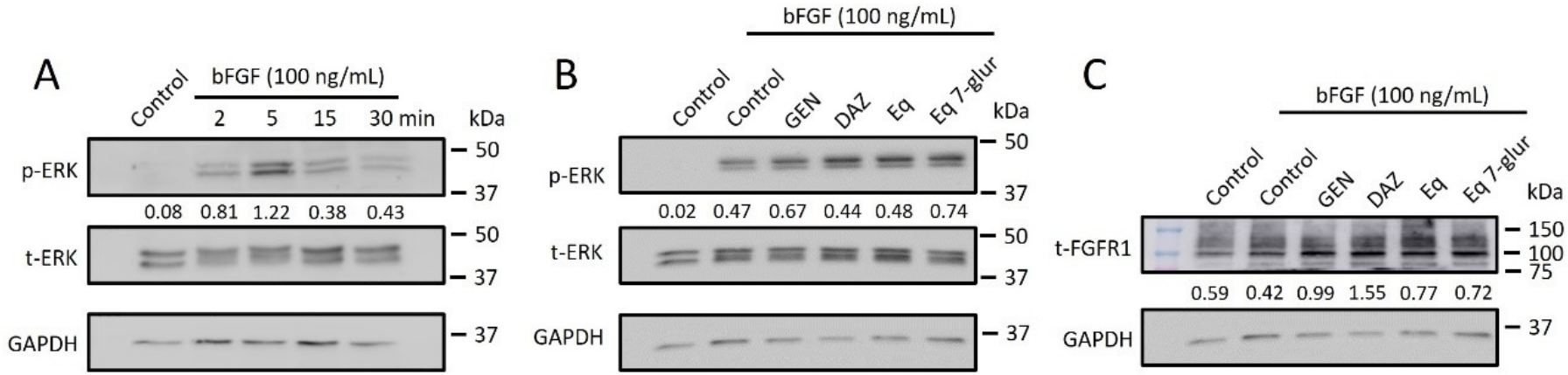

Figure S4. Western blot analysis of HAEC cells response to the stimulation with 100 ng/mL of bFGF. Time-course activation of the ERK pathway in bFGF-treated HAECs (A). Effect of 10 μM GEN, DAZ, Eq, and Eq 7-glur on the activation of the ERK pathway (B) and t-FGFR1 level (C) in HAEC cells stimulated with bFGF for 5 min (time points determined in A). The numbers between the phosphorylated and corresponding total (in A and B) or total receptor and loading control bands (C) show relative intensity (GAPDH was used as a loading control in A, B, and C).

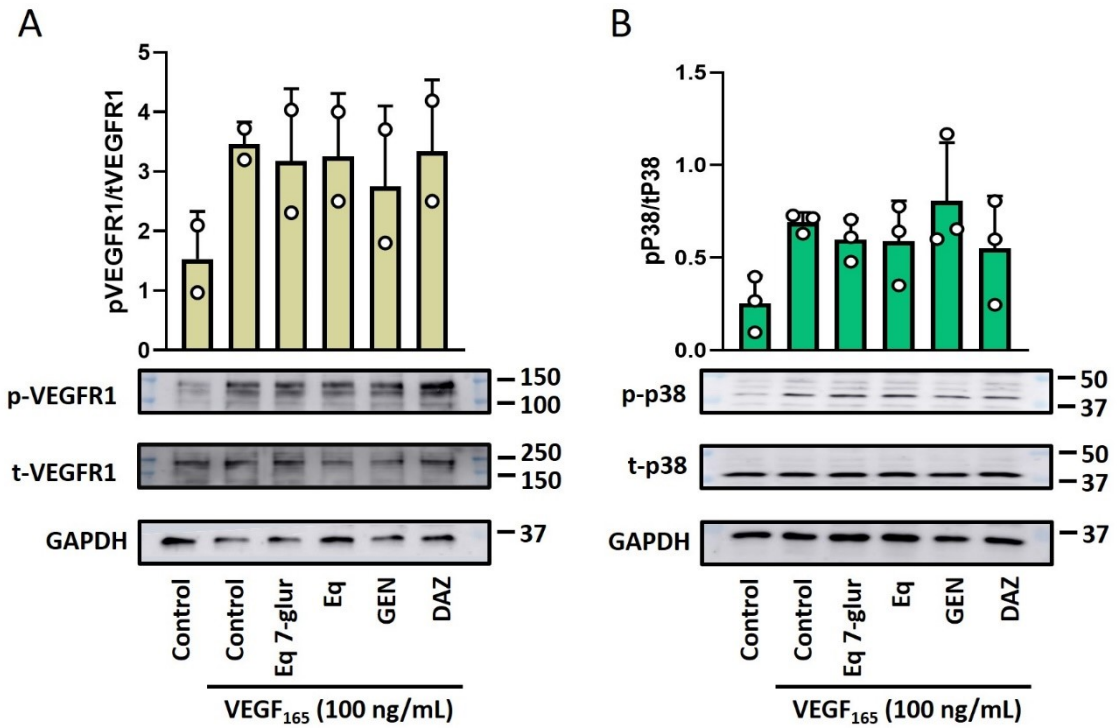

Figure S5. Phosphorylation of VEGFR1 and downstream p38 kinase in $VEGF_{165}$-treated HAECs. Endothelial cells were treated with 10 μM GEN, DAZ, Eq, and Eq 7-glur for 4 h, followed by 100 ng/mL $VEGF_{165}$ stimulation for 5 min. Western blots for phosphorylated and total VEGFR1 (A) and p38 (B) together with GAPDH as loading control are shown. The relative quantification of the phosphorylation is shown as bar graphs obtained from 2 to 3 biological replicates (n = 2 – 3). *Abbreviations:* VEGFR, vascular endothelial growth factor receptor.

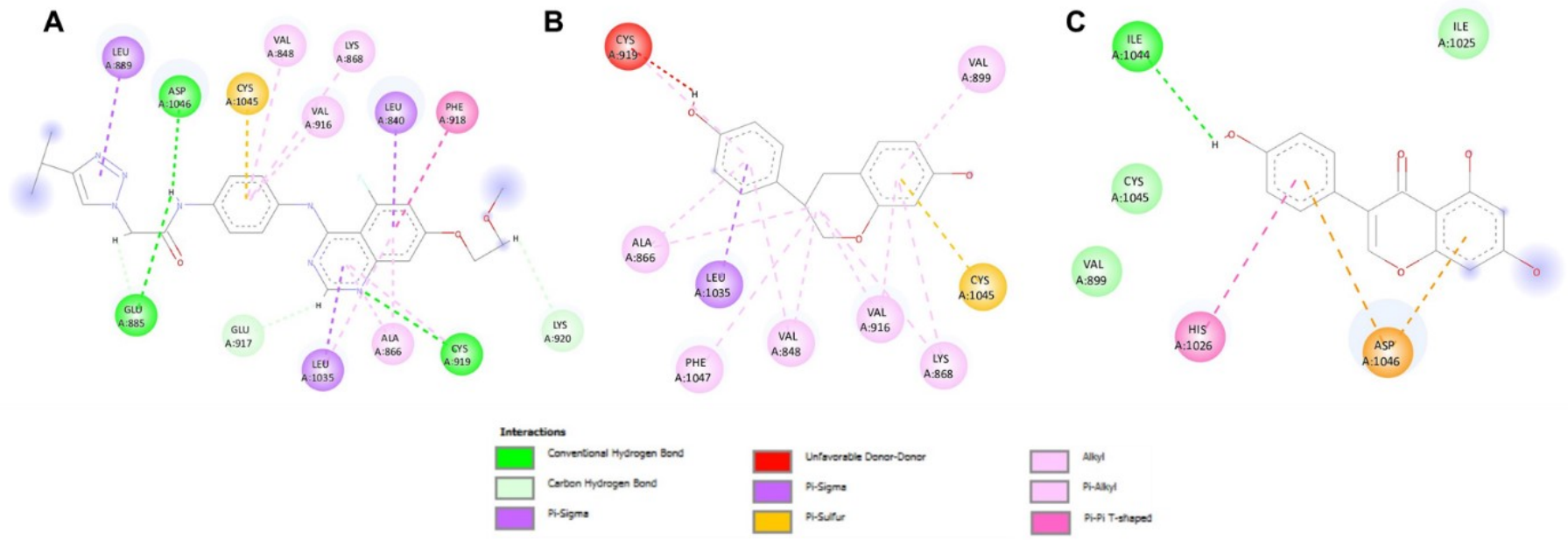

Figure S6. Illustration of the binding hotspots in 2D. **A**) 2D graph of the specific inhibitor AZD2329 in the pocket of VEGFR2, **B)** 2D graph of Eq in the pocket of VEGFR2, and **C)** 2D graph of GEN in the pocket of VEGFR2.

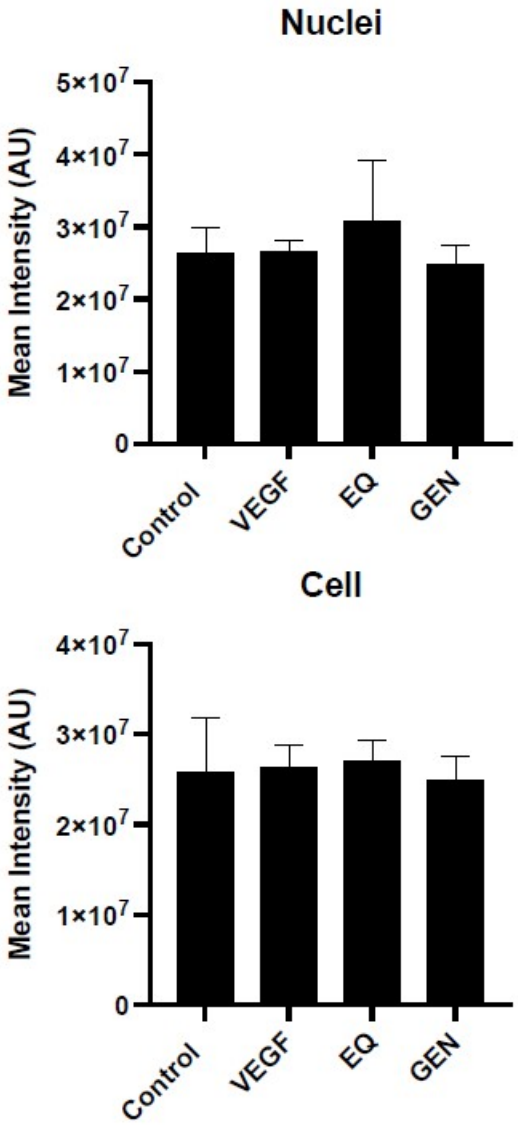

Figure S7. Quantification of the fluorescence intensity corresponding to VEGR2 in nuclear and cytoplasmic fractions of HAEC cells pretreated with 1% (v/v) DMSO 10 μM Eq and GEN followed by the stimulation with 100 ng/mL $VEGF_{165}$. Unstimulated cells treated with 1% (v/v) DMSO were used as controls.

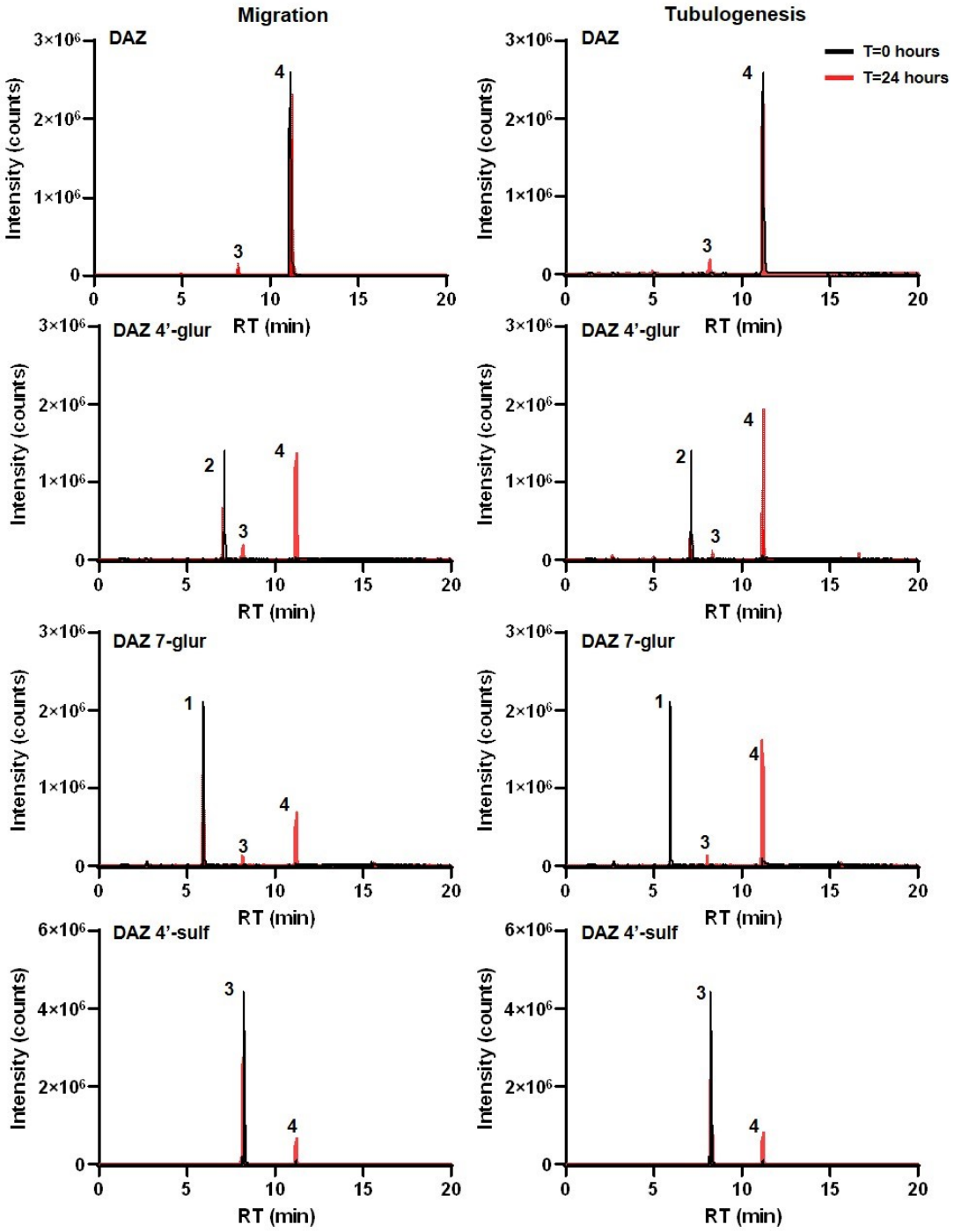

Figure S8. Extracted ion chromatograms (EICs) of daidzein (DAZ) and DAZ metabolites. EICs were obtained from HAEC cell tubulogenesis and migration assays at the initial (0 h; black chromatograms) and final time points (red chromatograms). Peaks: 1, DAZ 7-glur; 2, DAZ 4'-glur; 3, DAZ 4'-sulf; 4, DAZ.

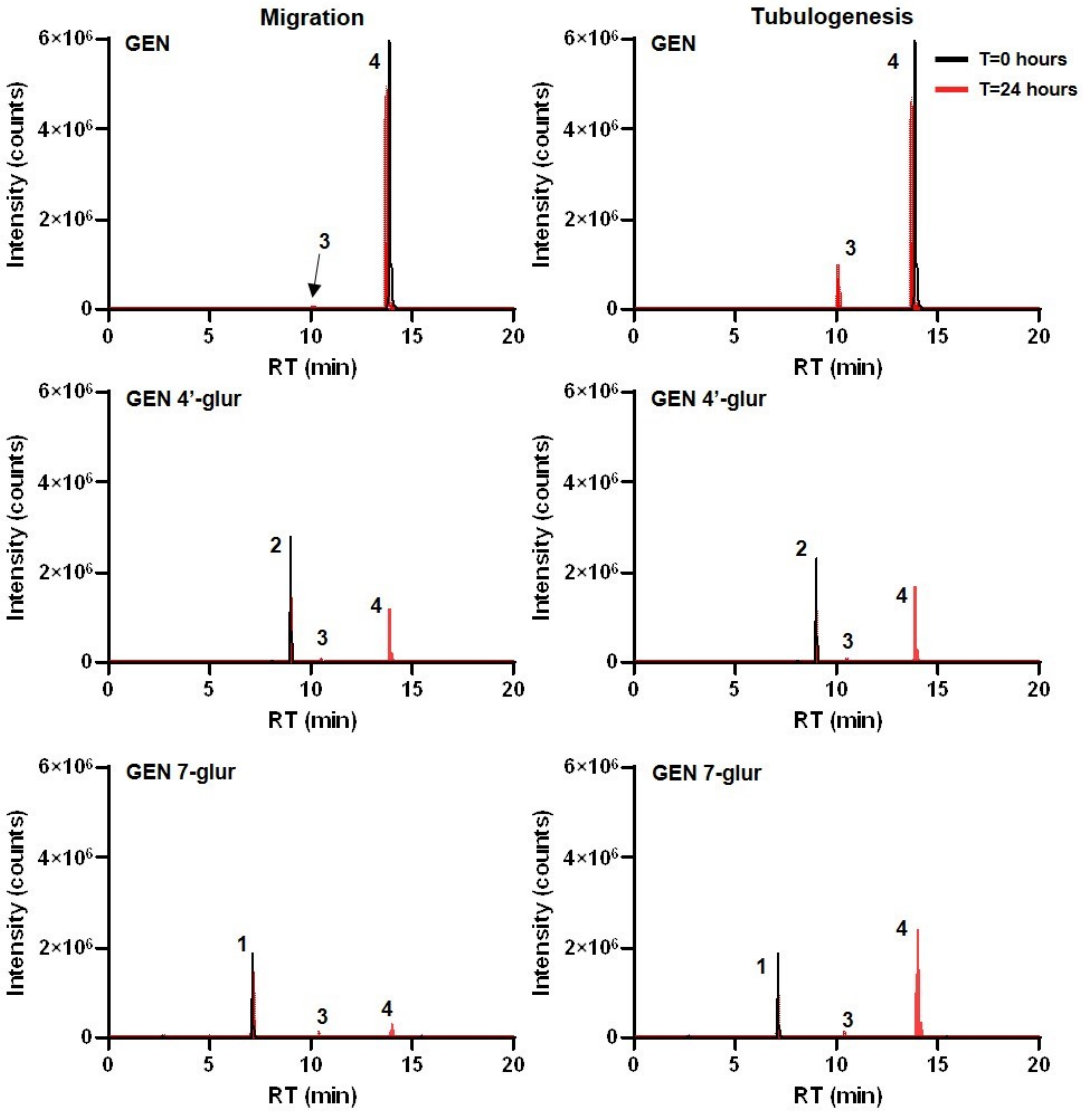

Figure S9. Extracted ion chromatograms (EICs) of genistein (GEN) and GEN metabolites. EICs were obtained from HAEC cell tubulogenesis and migration assays at the initial (0 h; black chromatograms) and final time points (red chromatograms). Peaks: 1, GEN 7-glur; 2, GEN 4'-glur; 3, GEN-sulf; 4, GEN.

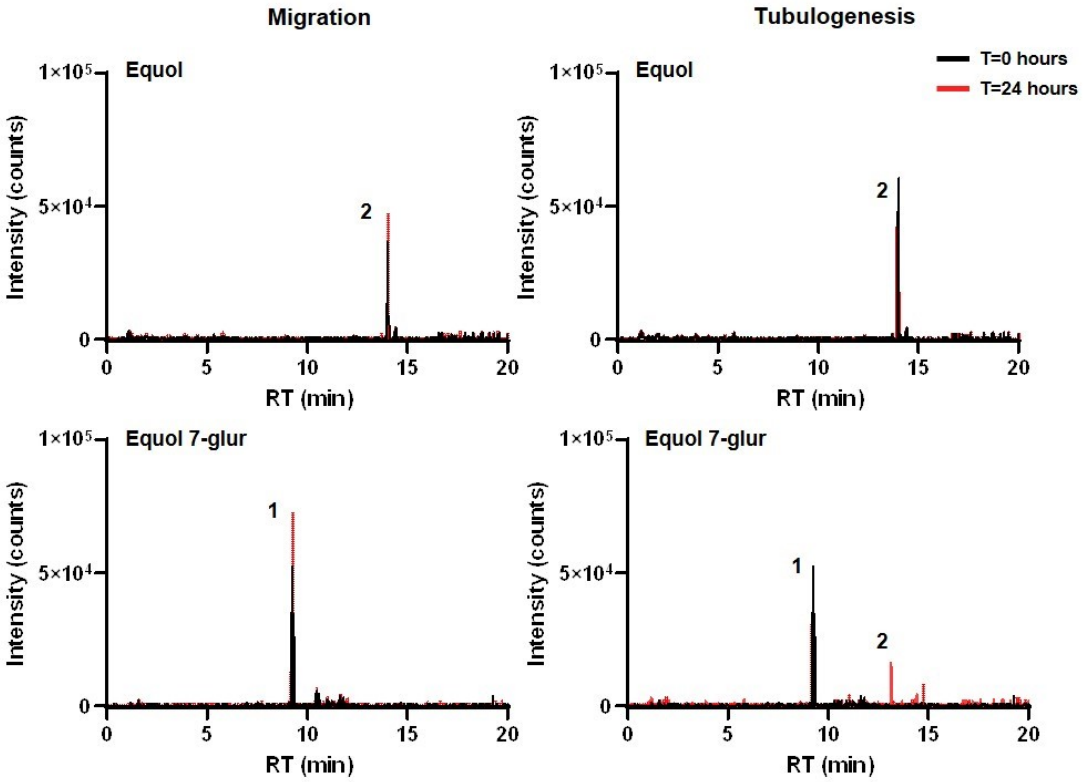

Figure S10. Extracted ion chromatograms (EICs) of Equol and Equol 7-glur. EICs were obtained from HAEC cell tubulogenesis and migration assays at the initial (0 h; black chromatograms) and final time points (red chromatograms). Peaks: 1, Equol 7-glur; 2, Equol.