# Dietary Compound Isoliquiritigenin, an Antioxidant from Licorice, Suppresses Triple-Negative Breast Tumor Growth via Apoptotic Death Program Activation in Cell and Xenograft Animal Models

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# These authors equal contribution as the first author in this study.

## Supplemental Materials and Methods

*Immunohistochemistry staining*

After deparaffinization, antigen retrieval, and blocking of peroxidase activity, the tumor tissue sections were incubated with anti-VEGF antibody (1:200; Abcam, Cambridge, United Kingdom) at 4°C overnight. Slides were further incubated for 30 minutes with Super Enhance and polymer horseradish peroxidase (HRP). The bound antibody was elected by a species-specific secondary antibody, and then developed with 3-Amino-9-ethylcarbazole (AEC) substrate. Images for IHC staining were captured with The EVOS® microscope (Thermo Fisher Scientific).

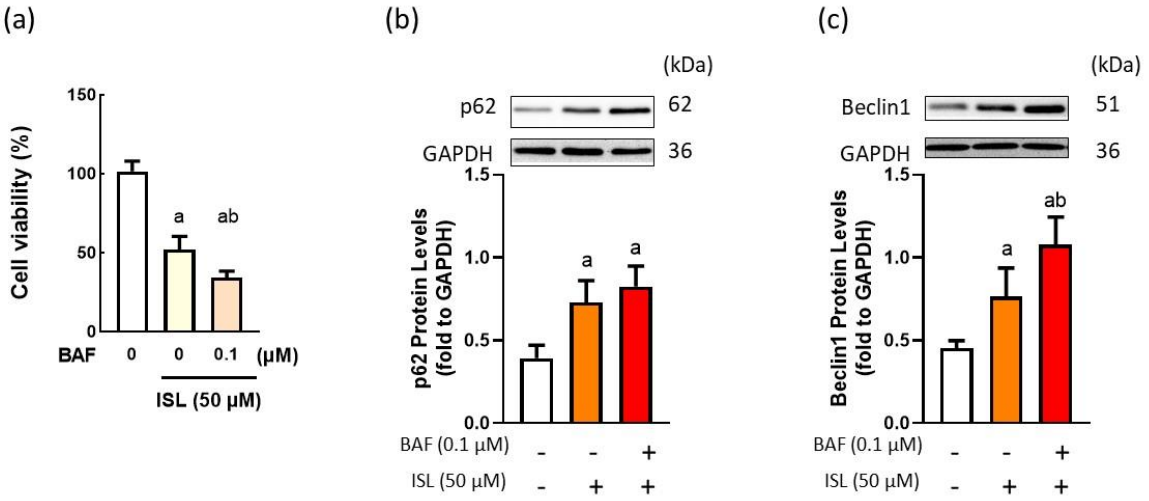
*Enzyme-linked immunosorbent assay (ELISA) of VEGF*

At the end of experiment, mice were euthanized and bloods were collected from the orbital venous plexus. Serum was separated by centrifugation and the VEGF concentration was analyzed by using the Human Quantikine VEGF ELISA Kit (R&D Systems, Inc.; Minneapolis, MN, USA). All procedures were performed according to the manufacturer's protocols. Inter- and intra-assay coefficients of variance given by the manufacturer for cell culture supernatant assays are 6.2–8.8 and 4.5–6.7 %, respectively.

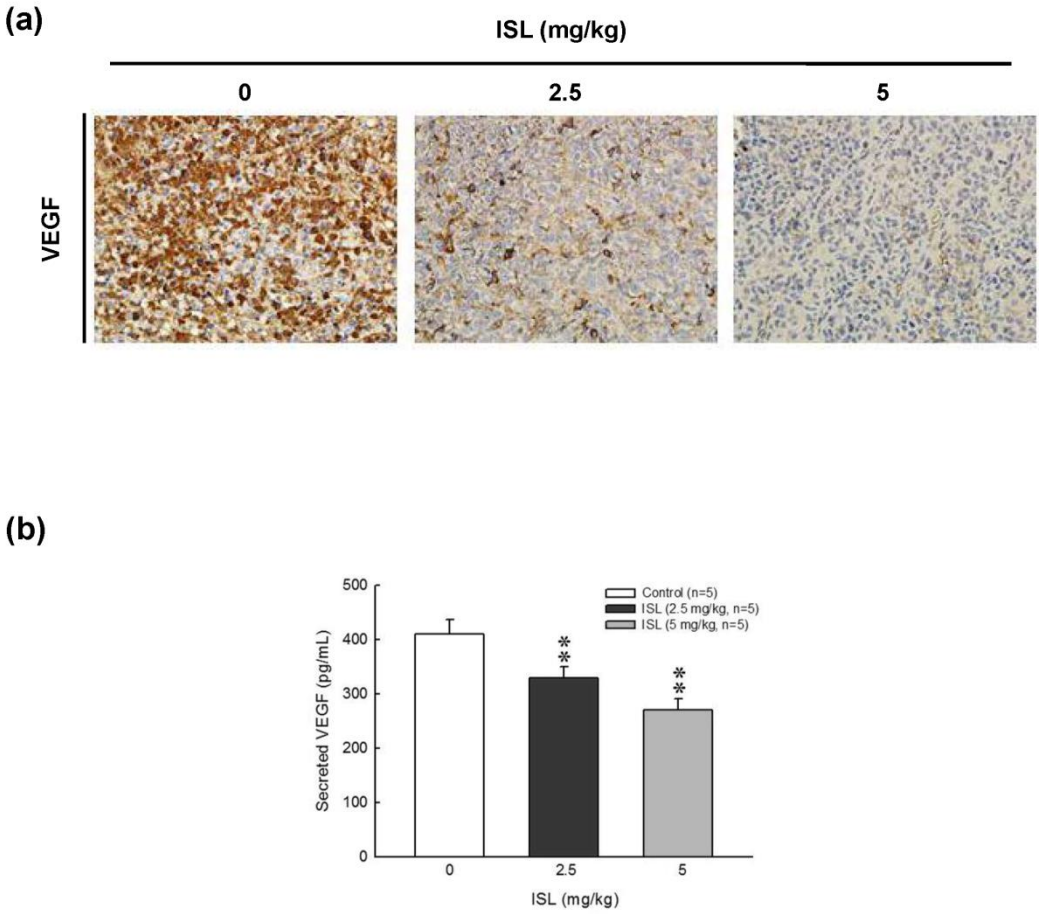
*Tube formation assay*

Mouse endothelial cells SVEC4-10 cell line was purchased from Bioresource Collection and Research Center (BCRC: #60220; Hsinchu, Taiwan). SVEC4-10 cells were incubated according to a protocol modified from one used previously [1]. Briefly, conditional media were collected from the equivalent cells

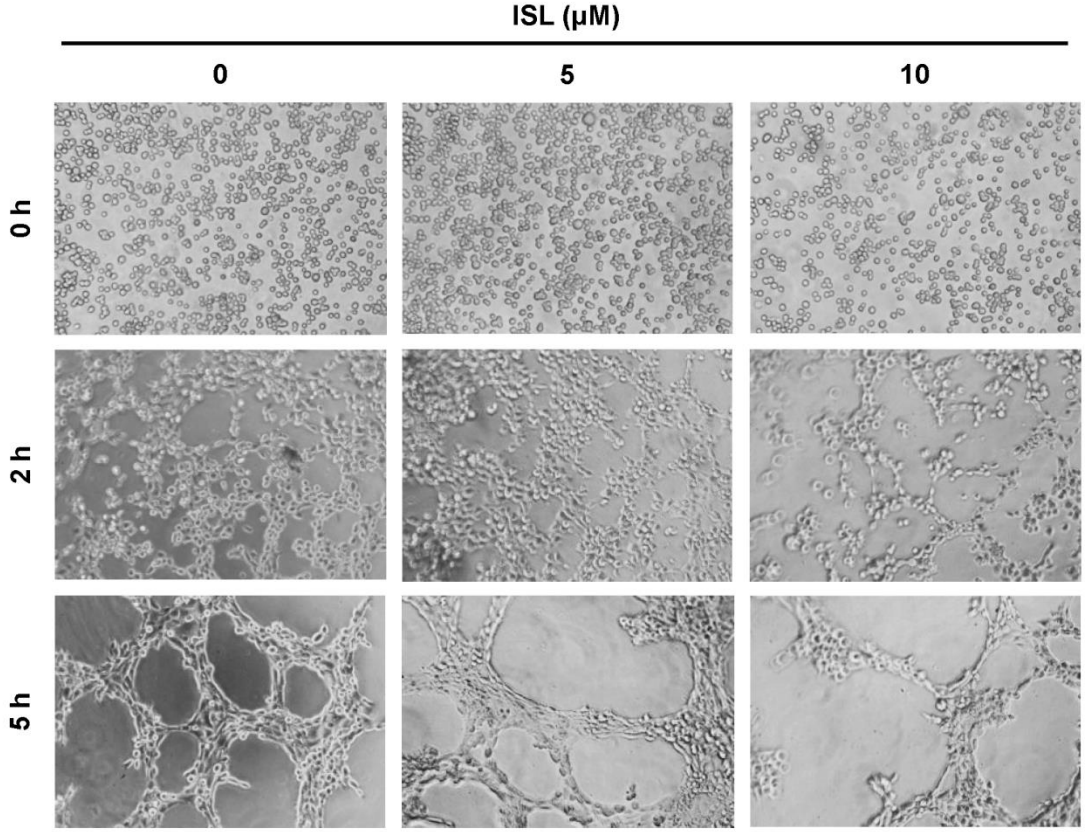
of MDA-MB-231 ($4x10^5$ cells) cultivated at 37°C for 24 hr. First, Matrigel (BD Biosciences; 354234) 50 μL/well was covered in 96-well plates and incubated at 37°C for approximately 1 hour for gel formation. SVEC4-10 cells ($4x10^5$) were resuspended in 100 μl fresh culture medium on the colloid Matrigel. Then, conditional media form MDA-MB-231 cells were diluted (volume ratio=1:1) with fresh media and added into wells for SVEC4-10 culture. Random photographs were taken of each well from 0 to 5 hr.




**Supplementary Figure S1. ISL and bafilomycin A1 (BAF) treatment induced the expression of autophagy-associated proteins and caused cell toxicity.** MDA-MB-231 cells were seeded in 96-well plates (3000 cells per well). Cell were pretreated with BAF for 3h, and combined with ISL for 48h. At the end of incubation, cell viability was measured by MTT assay. MDA-MB-231 cells were pretreated with BAF for 3h, and combined with ISL for 48h. The expression of (b) p62 and (c) Beclin1 protein were analyzed using western blotting. Data were represented as means ± SD. a, $p<0.05$, compared with control group. b, $p<0.05$, compared with ISL group.




**Supplementary Figure S2. Effects of ISL on the expression and secretion of VEGF.** MDA-MB-231 tumor cells (5 x $10^6$ cells per mouse) were implanted and mice were treated with ISL for 25 days. At the end of experiment, **(A)** tumor tissues were isolated and performed immunohistochemistry to analyze VEGF protein level. Images were photographed at 200x magnification. **(B)** Serum VEGF levels were measured using ELISA. Data represent as means ± SEM (n = 5 each group). $^{**}P < 0.01$ compared with the control group.




**Supplementary Figure S3. Effects of ISL on the capillary-like tube formation.** ISL inhibited capillary-like tube formation of mouse endothelial cells SVEC4-10 cells in matrigel. Images were photographed at 100x magnification.

**References:**

1. Shih, Y.H.; Chang, K.W.; Chen, M.Y.; Yu, C.C.; Lin, D.J.; Hsia, S.M.; Huang, H.L.; Shieh, T.M. Lysyl oxidase and enhancement of cell proliferation and angiogenesis in oral squamous cell carcinoma. *Head Neck* **2013**, *35*, 250-256, doi:10.1002/hed.22959.