

Supplementary Data of Western blotting

Natural Extracts of *Alnus japonica* Induce BAK-Dependent Autophagy to Inhibit Liver Cancer Stem Cell Tumorigenesis

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5. Materials and Methods

5.1. Animals and reagents

Mouse embryonic fibroblasts (MEFs), HepG2, SNL76/7, 293, and 293T cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and the RIKEN Cell Bank (Tsukuba, Ibaraki, Japan). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA) with or without high glucose. All media were supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin–streptomycin (P/S; Gibco). Animal experiments were approved by the National Laboratory Animal Center (Taiwan), Kaohsiung Medical University (Taiwan), and the Animal Care Committee of the RIKEN BioResource Research Center (Japan). All procedures complied with the animal welfare guidelines of the National Laboratory Animal Center (protocol no. 106022), Kaohsiung Medical University (protocol nos. 106189, 107128, and 108244), and the RIKEN BioResource Research Center (Kiteisv.intra.riken.jp/JoureiV5HTMLContents/act/print/print110000514.htm).

Experiments were conducted in accordance with these guidelines approved. Hirsutanone (Hir) and Oregonin (Ore) were purchased from Sigma-Aldrich, Inc. (SMB00096 and SMB00088; St. Louis, MO, USA; Merck KGaA, Darmstadt, Germany). Apigenin (cat. no. 010-18914) was obtained from Fujifilm Wako Pure Chemicals (Osaka,

Japan). Luteolin (cat. no. 491-70-3) was purchased from Sigma-Aldrich, Inc. Kaempferol (cat. no. 520-18-3) and quercetin (cat. no. 849061-97-8) were obtained from Tokyo Kasei Co. (Tokyo, Japan). Chloroquine diphosphate (C6628) was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA; Merck KGaA, Darmstadt, Germany). Torin 1 (CAS 1222998-36-8) was obtained from NJ, USA, and Bafilomycin A (CAS 88899-55-2) was purchased from AdipoGen Life Sciences (San Diego, CA, USA).

5.2. Generation of unfermented and fermented tree bark extracts of *A. japonica*

A. japonica has been cultivated in the Yanbaru district of Okinawa since its introduction from Taiwan (*Alnus formosana*) in 1910. Gushiken et al. reported the preparation of fermented bark extracts from *A. japonica* roots coexisting with the symbiotic fungus *Frankia*, yielding fermented tree extracts that were developed as agricultural antibacterial materials [31]. A voucher specimen of *A. japonica* stem bark was deposited at the University of the Ryukyus, Okinawa, Japan [32]. Stems, leaves, and rhizomes of *A. japonica* were gently washed to remove *Frankia* fungi, then mixed and cut into small pieces using an electric cutter (Hitachi Inc., Ibaraki, Japan). The pieces were fermented at room temperature for 7–10 days, dried, and ground into powder. During fermentation, the materials were stirred occasionally to ensure uniform processing, allowing the surfaces to be covered with white mycelium and promoting bacterial growth. For preparation of unfermented extracts, the fermentation step was omitted. For extraction, water was added at a ratio of 100 L per 1 m³ of plant material, followed by boiling and distillation. Hirsutanone (Hir) and oregonin (Ore) were used as positive controls.

5.3. Isolation of Polyphenols, Flavonoids, and Other Secondary Metabolites

Previous phytochemical studies of *A. japonica* identified diarylheptanoids, triterpenoids, and flavonoids [2–4,19]. To extend these findings, boiled extracts of fermented and unfermented tree bark (1.0 kg) were prepared with 50% aqueous EtOH (2.0 L, three times) at 40 °C under sonication. After solvent removal, the residue was resuspended in 1.0 L of water and partitioned successively with CH₂Cl₂ and EtOAc (1.0 L each, three times), yielding CH₂Cl₂-soluble (35.0 g) and EtOAc-soluble (53.5 g) fractions.

A portion of the EtOAc fraction (40.0 g) was fractionated on silica gel using a CHCl₃–MeOH gradient (15:1 to 0:1, v/v), producing six fractions (F1–F6). Fraction 2 (2.6 g) was further chromatographed as described [33], with silica gel (CHCl₃–MeOH, 10:1), followed by RP-C18 chromatography (MeOH–H₂O, 1:1), yielding compound 5 (hirsutanone, 60 mg). Fraction 4 (4.5 g) was subjected to RP chromatography (MeOH–H₂O, 6:5), generating seven subfractions (F2.1–F2.7). Subfraction 2.1 (1.8 g) was re-chromatographed on silica gel (CHCl₃–MeOH, 6:1), affording compound 4 (oregonine, 1.6 g). Fraction 6 (80 mg) was chromatographed with EtOAc–MeOH (15:1), yielding compounds 1 (18 mg) and 3 (15 mg). Compound 2 (11 mg) was purified from subfraction 2.7 (350 mg) using silica gel chromatography (CHCl₃–MeOH, 5:1). The lignan [(–) - (2R,3R)-1,4-O-diferuloylsecoisolariciresinol, DFS] was extracted with methanol [20]. Concentrated extracts (50 g) were partitioned with CHCl₃, and the soluble fraction (4.8 g) was chromatographed on silica gel using hexane/EtOAc, yielding three fractions. Fraction 2 (300 mg) was subjected to RP chromatography (gradient 30–80% MeOH), affording DFS (27.5 mg). Purity was confirmed by HPLC Major flavonoids (quercetin, kaempferol,

myricetin, apigenin, luteolin) were extracted by refluxing boiled bark extract with aqueous methanol (62.5%) containing tertiary butylhydroquinone (2 g/L) and HCl (final concentration 1.2 M in 50% aqueous methanol) at 90 °C for 2 h [33]. The cooled extract was filtered, adjusted to 50 mL with methanol, and passed through a 0.45 µm membrane filter prior to HPLC analysis. Extracts were stored in amber bottles at –20 °C until use.

Flavonoid aglycones were quantified by RP-HPLC (Nova-Pak C18, 3.9 × 150 mm, 4 µm; Waters, Milford, MA) using MeOH/H₂O (50:50, v/v, pH 2.5 with trifluoroacetic acid) as mobile phase, UV detection at 365 nm, and a flow rate of 1 mL/min. Chromatograms were compared with authentic standards. All determinations were performed duplicate. Structures were confirmed by LC-MS, 1D/2D NMR, and comparison with reported data [34]. In general, NMR spectra were recorded at 100 and 270 MHz (¹H NMR), and 25.05 MHz (¹³C NMR). Chemical shifts are given in δ (ppm) with TMS as int. std. Negative FAB-MS were measured at 1.5 kV (accelerating voltage) with Me & O-glycerol as matrix. High-resolution techniques including HPLC, UHPLC-QqQ-MS/MS (Agilent Technologies, Wilmington, DE, USA), LC-ESI-MS/MS (Agilent Technologies, Wilmington, DE, USA, coupled with a hybrid quadrupole linear ion trap mass spectrometer QTRAP® 5500 (AB Sciex, Foster City, CA, USA) equipped with an electrospray ionization source (ESI), were employed for separation, identification, and quantification of phenolic compounds [35]. Coupling HPLC with UV-visible or diode array detectors (HPLC-UV, HPLC-DAD; Shimadzu Corp. Kyoto, Japan), as well as GC-MS (Shimadzu Corp. Kyoto, Japan), enabled sensitive detection of flavonoids and phenolic acids. QTOF-MS (Shimadzu Corp. Kyoto, Japan), provided accurate molecular mass determination, facilitating identification of novel bioactive compounds in tree bark extracts.

5.4. Generation of rG2-DC-1C cells

Recombinant lentiviruses encoding human OSKM (RDB08323, RDB08324, RDB12904 in RIKEN BRC) C-JUN (RDB06254 in RIKEN BRC), and shBAK (TRCN000033464; siRNA Core Facility at Academia Sinica (Taipei, Taiwan) were produced in 293T cells co-transfected with *pCAG-HIVgp* and *pCMV-VSV-G-RSV-Rev* by the Lentivirus preparation protocol in RIKEN BioResource Center, Japan as described elsewhere [30]. HepG2 cells and the iPSHep FB/Ng/gfp-103C-1 cell line were infected with lentiviruses at a multiplicity of infection (MOI) of 50 and incubated for 1 week in DMEM [30]. Subsequently, cells were transferred onto mitomycin C (Sigma-Aldrich)-treated MEFs in DMEM supplemented with ESGRO (Merck KGaA, Darmstadt, Germany; 10 ng/mL) to generate iPSC-like cells from HepG2 cells. A single colony (approximately 200 cells) of HepG2-derived iPSC-like cells was injected into severe combined immunodeficiency (SCID) mice. Tumor formation was observed approximately 9 weeks later. The tumor was resected, and a primary cancer cell line was established, designated “reprogrammed HepG2-derived cancer cells from one colony” (rG2-DC-1C). This cell line was used for subsequent experiments.

5.5. Cell viability assays using MTT and trypan blue dye exclusion

Cell viability was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay as described previously [36]. Cells (3×10^4) were treated with unfermented or fermented tree bark extracts, as well as the indicated concentrations of Hir and Ore, at 37 °C for indicated time periods. Following treatment, 10 μ L of MTT solution (10 mg/mL) was added to each well and incubated for 2 h at 37 °C.

After centrifugation at $412 \times g$ for 5 min, the medium was removed, and 100 μL of dimethyl sulfoxide (DMSO; Sigma) was added to dissolve the formazan crystals. Absorbance was measured at 570 nm using a microplate reader (Immuno Mini NJ-2300, Nihon InterMed, Tokyo, Japan) during the culture periods until 8 days. Cell viability was expressed relative to the absorbance of untreated control cultures. Cell viability was also determined using the trypan blue dye exclusion assay as described elsewhere [37]. Briefly, cells were stained with trypan blue and counted using a hemocytometer to determine the number of viable cells in suspension. Proliferation rates were calculated based on viable cell counts.

5.6. Cell proliferation assay and apoptosis

For the cell proliferation assay, cells were plated and cultured for 3~8 days under environmental oxygen conditions (20%), followed by treatment with 10 μM 5-ethynyl-2-deoxyuridine (EdU) for 6 h. Growing cells were stained with Alexa Fluor azide, and total cells were counterstained with Hoechst using the Click-iT EdU Assay Kit (C10337, Thermo Fisher Scientific, Waltham, MA, USA). Fluorescence microscopy was used to image stained cells. The percentage of proliferating cells was calculated by dividing the number of EdU-positive cells by the total number of Hoechst-stained cells. Cell counts were obtained from five randomly selected fields using Cell Count software (version 1.1.7). For apoptosis analysis, cells were incubated for 24 h at 37 °C. Caspase-3 and -7 activities, key indicators of apoptotic pathways, were measured using the Caspase-Glo 3/7 Assay Kit (Promega). Cell viability was additionally assessed using the Trypan blue dye exclusion assay, in which viable cells were quantified by hemocytometer counting

5.7. Isolation of RNA and quantitative PCR (qPCR)

Total RNA was extracted using the PureLink™ RNA Mini Kit (Invitrogen). RNA was reverse transcribed into cDNA using SuperScript III Reverse Transcriptase (Invitrogen) [39]. Quantitative real-time PCR was performed on a StepOne or ABI7500 instrument (Applied Biosystems, Foster City, CA, USA) using Fast SYBR® Green Master Mix (Applied Biosystems) in 20 µL reaction volumes. Threshold cycle (Ct) values were averaged from technical duplicates. Transcript levels of target genes were normalized to Gapdh expression. Relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method, with expression levels normalized to DMSO-treated rG2-DC-1C cells (set as 1.0). Data are presented as mean $\pm$ SEM from three biological replicates.

The primer sequences used were as follows:

- OCT4: forward 5'- GGGTTTTTGGGATTAAGTTCTTCA-3',
reverse 5'-GCCCCCACCCTTTGTGTT-3'
- SOX2: forward 5'- GCTACAGCATGATGCAGGACCA-3',
reverse 5'- TCTGCGAGCTGGTCATGGAGTT-3'
- KLF4: forward 5'-CATCTCAAGGCACACCTGCGAA-3',
reverse 5'- TCGGTCGCATTTTTGGCACTGG-3'
- C-JUN: forward 5'-CCTTGAAAGCTCAGAACTCGGAG-3',
reverse 5'-TGTCTGCGTTAGCATGAGTTGGC-3'

5.8. Construction of AhR or c-Jun promoter luciferases and their corresponding cis-element mutant luciferases

AhR promoter regions [39,40] were cloned into the pGL4.1 plasmid (Promega, Madison, WI, USA) as described previously [40]. The orientation and integrity of the constructs were confirmed by restriction enzyme digestion and next-generation sequencing. Putative binding sites within the AhR promoter region were predicted using ALGGEN-PROMO (<http://alggen.lsi.upc.edu>). Site-directed mutagenesis of individual promoter sites,

including DRE2, was performed using the QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) [39,40].

Wild type and mutant M4 c-JUN promoter were generated and transfected into rG2-DC-1C cells as described previously [30]. Two days after transfection, cells were harvested and measured for luciferase. The WT c-JUN promoter and M1 to M4 mutants were cotransfected with various amounts (0–200 ng) of OCT4-expressing plasmids into rG2-DC-1C cells.

5.9. Transient transfection and luciferase reporter assay

rG2-DC-1C cells were seeded into 24-well plates (4×10^4 cells/well) and cultured for 24 h. Cells were cotransfected with 500 ng of the AhR luciferase plasmid and 10 ng of the pRL-CMV plasmid encoding *Renilla* luciferase using either Lipofectamine 2000 (Invitrogen) or polyethylenimine (linear, molecular weight 25,000; Polysciences, Warrington, PA, USA; Cat# 23,966). The total amount of transfected DNA was maintained at 1 μ g/well by supplementing with pBluescript II SK+ control plasmid (Addgene, Watertown, MA, USA). The human OCT 4 expression vector (pCEP4_WT_OCT4) was gift from James Thomson (Addgene Plasmid #40629; <http://n2t.net/addgene:40629>; RRID: Addgene_40629) and used for cotransfecting rG2-DC-1C cells.

Transfected cells were treated with DMSO for the indicated times and harvested 48 h post-transfection. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions, with detection performed using a GloMax 20/20 Luminometer (Promega). Reporter activity was

calculated as the ratio of Firefly luciferase to Renilla luciferase and expressed as fold induction relative to the empty vector in rG2-DC-1C cells [30]. All measurements were performed in duplicate, and values are presented as mean $\pm$ standard error of the mean (SEM) from at least three independent experiments.

5.10. shRNA lentivirus and autophagosome inhibitor

shRNA lentiviruses targeting human BAK1 (TRCN0000033464) were obtained from the siRNA Core Facility at Academia Sinica (Taipei, Taiwan). Predesigned ON-TARGETplus SMARTpool siRNA against human BAK1 and scrambled control siRNA were purchased from GE Dharmacon (Austin, TX, USA). Mouse embryonic fibroblasts (MEFs) were seeded into six-well plates (for Western blotting) or 24-well plates (for luciferase reporter assays) and transfected with 20–40 nM of either siRNA or control RNA in OPTI-MEM medium (0.5 mL for 24-well plates; 2 mL for six-well plates) using Lipofectamine RNAiMAX (Invitrogen). For rG2-DC-1C cells, shRNA was transduced at a multiplicity of infection (MOI) of 10. After 24 h, fresh culture medium containing 10% FBS was added, and cells were subsequently transfected with luciferase plasmids for reporter assays as described above. Knockdown efficiency was confirmed 48 h post-infection by immunoblotting and complementary analyses. To inhibit autophagosome formation, 10 μ M chloroquine was added during rG2-DC-1C cell culture and invasion assays involving *A. japonica* bark extracts. Following treatment, protein levels of BAK, Bax, Bad, Bcl-2, and p62 were examined by Western blotting.

5.11. ROS detection using CM-H2DCFDA fluorescence

Reactive oxygen species (ROS) levels were measured as described previously [40]. rG2-DC-1C cells were cultured in 0.1% gelatin-coated 12-well plates with or without tree bark extracts (unfermented or fermented), or with 20 μ M Hir or Ore for the indicated times. As positive controls, cells were treated with 150–200 μ M hydrogen peroxide for 15 min or with 50 μ M apigenin, luteolin, kaempferol, or quercetin for the indicated times before CM-H2DCFDA addition. Cells were rinsed with warm Hanks' balanced salt solution (HBSS; Gibco Invitrogen, Waltham, MA, USA) and incubated with 10 μ M CM-H2DCFDA (C-6827; Life Technologies) in complete growth medium for 30 min at 37 °C in the dark. After treatment, cells were washed twice with HBSS and examined using a Nikon inverted fluorescence microscope. Five randomly selected fields were imaged using a 10 $\times$ objective lens, and fluorescence intensity was quantified with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

5.12. *Cellular ROS accumulation*

The concentration of 8-oxo-dGuo was measured by liquid chromatography–mass spectrometry as described previously [39,40]. Reduced glutathione (GSH) and oxidized glutathione (GSSG) concentrations (mmol/mg protein) were determined using a GSH assay kit (703002; Cayman Chemical Co., Ann Arbor, MI, USA) and calculated from a standard curve, with values normalized to protein concentration. NQO1 activity was assessed using a 2,6-dichlorophenolindophenol reduction assay, as described previously [40]. Net intracellular ROS accumulation was measured using the ROS-Glo™ H₂O₂ assay (Promega). Briefly, cells were treated with antioxidants or H₂O₂ for 2 h, washed twice with Hanks' balanced salt solution (HBSS), and incubated with ROS-Glo™ Detection

Solution for 20 min. Fluorescence was detected using a GloMax® fluorometer (Promega) [42-44].

5.13. *RNA sequencing and gene clustering*

RNA sequencing was performed by Welgene Biotech (Taipei, Taiwan) following the manufacturer's protocol (Illumina, San Diego, CA, USA). cDNA libraries were prepared using TruSeq RNA Sample Prep Kits and sequenced on an Illumina GAIIx platform. Raw sequences were processed using the CASAVA Pipeline software, and low-quality reads were trimmed with ConDeTri. After filtering, qualified reads were analyzed using TopHat/Cuffdiff for gene expression estimation. Human Genome Build 19 and associated gene features were used for data processing. Gene expression levels were calculated as fragments per kilobase of transcript per million mapped reads (FPKM). Differentially expressed genes were identified using the following criteria: $\text{FPKM} \geq 0.3$, fold change ≥ 2 , and $p < 0.05$. Gene-level normalization was performed by transforming FPKM values to a $\log_2$ median-centered ratio. Clustering was conducted using Euclidean distance and complete linkage settings. Heatmaps were generated by coloring each gene according to its $\log_2$ median-centered ratio. Lists of liver cancer genes, oncogenes, tumor suppressor genes, stemness-related genes, and OCT4-signaling-associated genes were compiled from GSEA, KEGG, Gene Ontology, Life Technologies panels, and Qiagen panels. RNA sequencing data were deposited in the NCBI BioProject Database under accession number PRJNA273617 [30,41].

5.14. *Measurements of autophagy activity*

Autophagic activity was assessed using the Autophagy LC3 HiBiT Reporter Assay System [45]. U2OS HiBiT-HaloTag-LC3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with high glucose, supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin (8×10^3 cells per 80 μ L per well) in 96-well white clear-bottom plates. Cells were treated with unfermented or fermented *A. japonica* bark extracts, 20 μ M Hir, 20 μ M Ore, or 50 μ M apigenin, luteolin, kaempferol, or quercetin for the indicated times, and autophagic activity was measured using the screening kit as described above. Autophagy responses were further quantified using plasmid-based reporters [43,44]. Plasmids pMRX-IP-GFP-LC3-RFP-LC3 Δ G (#84572) and pMRX-IP-GFP-LC3-RFP (#84573) were obtained from the RIKEN DNA Bank (Tsukuba, Japan). rG2-DC-1C cells were transfected with these plasmids as described previously, and stable transfectants were established. Cells were then treated with the indicated compounds for 12 h in DMEM supplemented with 10% dialyzed fetal bovine serum. Autophagic flux was measured and calculated based on the GFP/RFP ratio as described [46,47].

5.15. *Autophagosome inhibitor*

Chloroquine (10 μ M; Sigma-Aldrich, C6628) was used as an autophagosome inhibitor [48] during rG2-DC-1C cell culture and invasion assays involving *A. japonica* bark extracts. Following treatment, protein levels of BAK and Bax were examined by Western blotting.

5.16. *Invasion assay*

Cells (1×10^4) were seeded onto Transwell inserts coated with Matrigel (1 mg/mL; Corning, NY, USA) in serum-free medium. The inserts were placed into wells containing DMEM supplemented with 10% FBS and incubated for 3 days in the presence or absence of 10 μ M chloroquine (Selleck Inc., Japan; S6999). Treatments included unfermented or fermented *A. japonica* bark extracts, 20 μ M Hir, 20 μ M Ore, or 50 μ M quercetin for the indicated times. Invaded cells on the lower surface of the membrane were fixed, stained, and counted under a microscope according to the manufacturer's instructions [40].

5.17. Tumor formation and immunohistochemistry

rG2-DC-1C cells (200–500) were injected subcutaneously into SCID mice, and teratomas were examined by immunohistochemistry as described previously [30]. Cells were fixed in 4% formaldehyde for 10 min, washed with PBS, and incubated with blocking solution containing 10% FBS and 0.1% Triton X-100 in PBS for 15 min. Cells were then incubated overnight with primary antibodies. After washing with PBS containing 0.05% Tween-20, cells were incubated for 1.5 h with the following secondary antibodies: Alexa Fluor® 594-labeled goat anti-rabbit IgG (Thermo Fisher Scientific; A-11037), Alexa Fluor® 488-conjugated rabbit anti-goat IgG (Thermo Fisher Scientific; A-11078), and Alexa Fluor® 647-labeled goat anti-rat IgG (H+L; Cell Signaling Technology; 4418). Nuclei were visualized using 4',6-diamidino-2-phenylindole (DAPI; 1:3000 dilution; 5 mg/mL stock in DMSO; Sigma-Aldrich). Cells were mounted with ProLong® Gold antifade reagent (Molecular Probes, Thermo Fisher Scientific; P36934), and immunofluorescence was observed using an Olympus FV1000 confocal laser scanning microscope.

5.18. *Ingenuity Pathway Analysis (IPA)*

Ingenuity Pathway Analysis (IPA; QIAGEN Inc., release date November 4, 2025) was used to identify direct and indirect network molecules targeted by apigenin, luteolin, kaempferol, and quercetin. Targeted molecules for each compound were compared with those in the autophagy signaling pathway. Molecules common to all four drug networks and the autophagy pathway were retained in the final network. These common molecules were highlighted in the autophagy canonical signaling pathway using distinct color coding.

5.19. *Statistical analysis*

Data are presented as mean $\pm$ standard error. Statistical comparisons between two groups were performed using Student's T-test (two-tailed, paired). For multiple group comparisons, one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was applied. All statistical analyses were conducted using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). Differences were considered statistically significant at $p < 0.05$.

Supplementary Figures

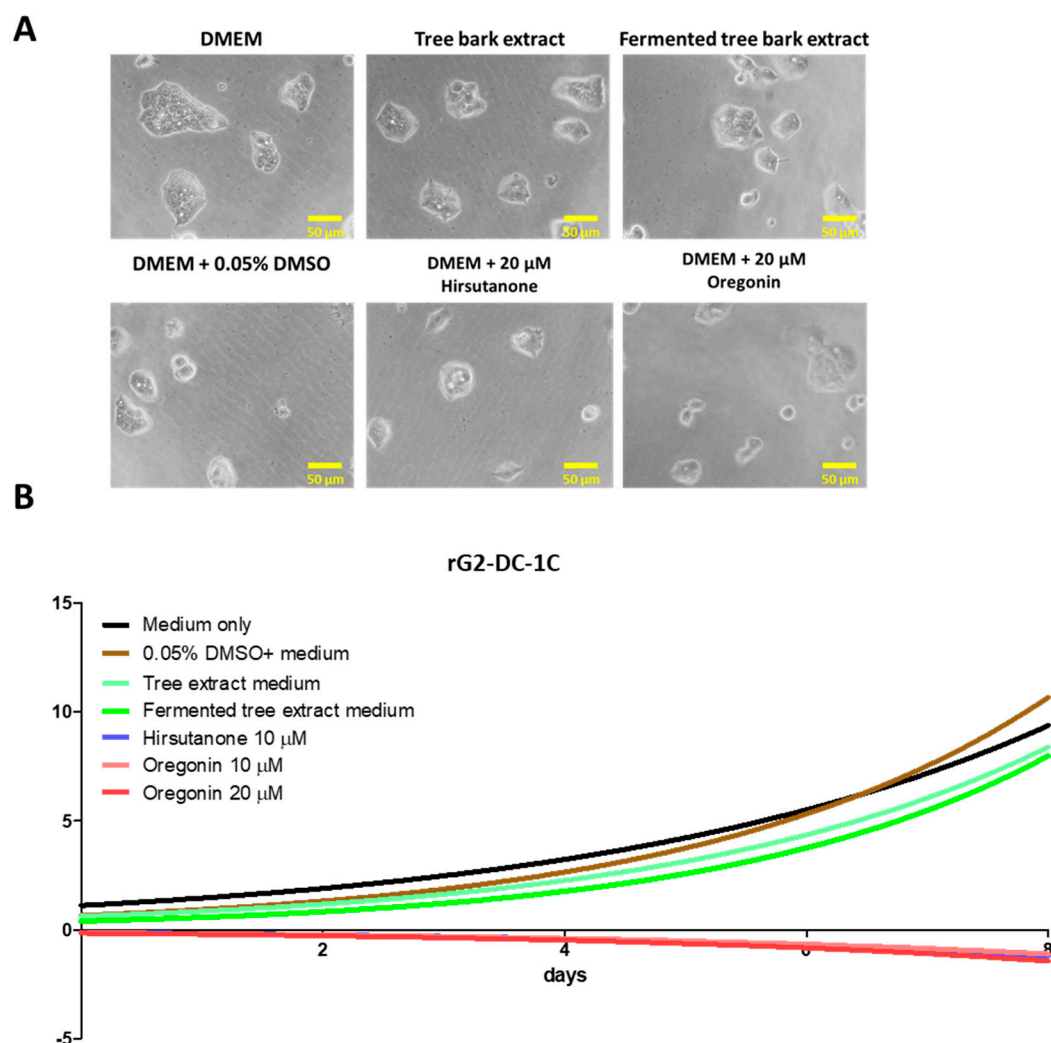


Figure S1. Microscopic morphology and cell proliferation/cytotoxicity of rG2-DC-1C cells after various treatments. (A) Microscopic morphologies of rG2-DC-1C cells after treatment with 0.05% DMSO, unfermented and fermented tree bark extracts, and 20 μ M Hir and 20 μ M Ore. **(B)** Cell viability was measured using MTT assay as described in Materials and methods. Cells (3×10^4) were exposed to unfermented and fermented tree bark extracts in culture medium and the indicated concentrations of Hir and Ore at 37 °C for the indicated time periods. The medium was then incubated with 10 μ L of 10 mg/mL MTT solution for 2 h at 37 °C. After centrifugation for 5 min, the medium was removed, and 100 μ L of DMSO was added to each well to dissolve the formazan. The

absorbance was then measured at 570 nm using a microplate reader. Cell viability over 8 days was determined relative to the control culture.

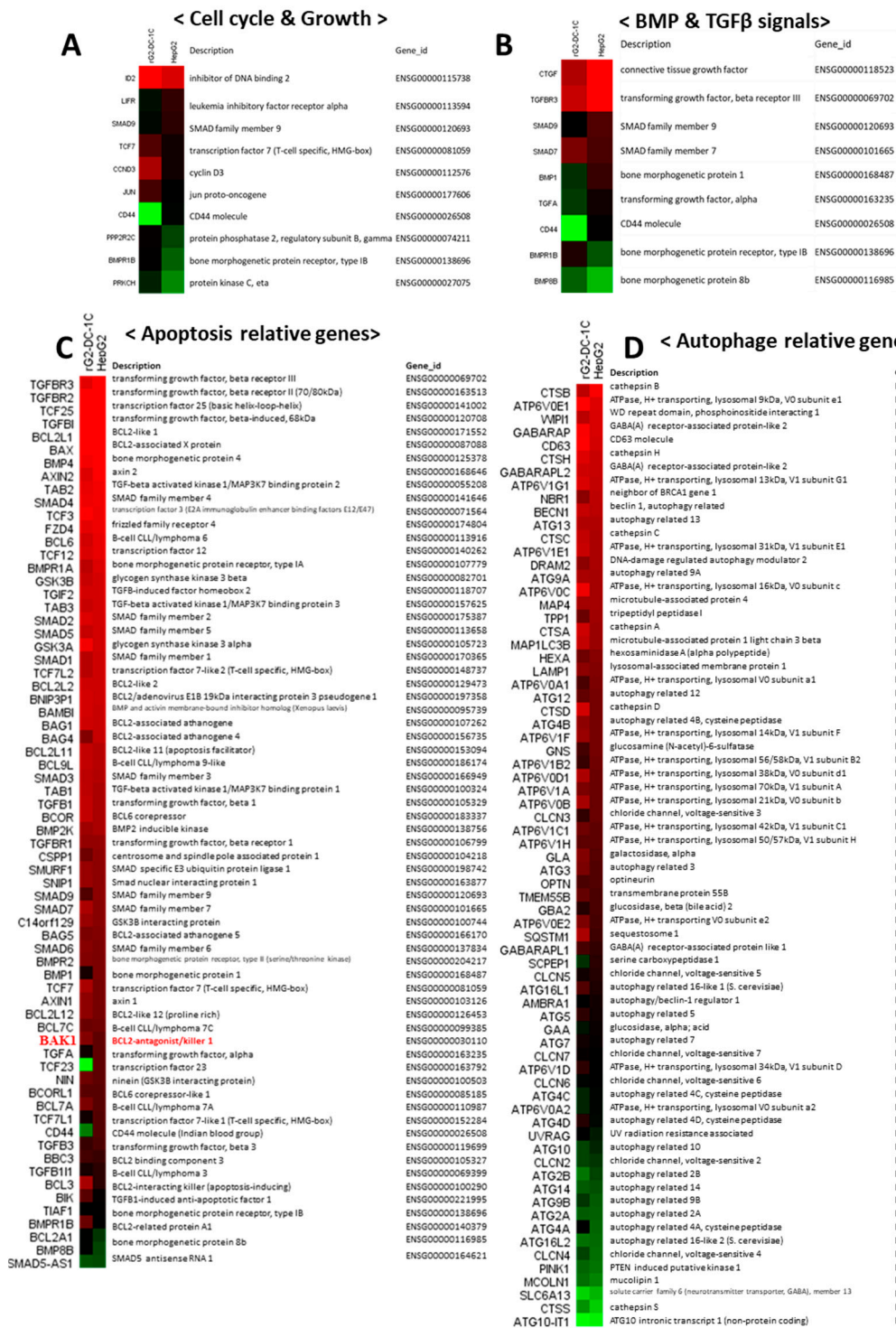


Figure S2. Comparative analysis between rG2-DC-1C and HepG2 cells. (A) Heatmap of the mRNA expression levels for select genes related to cell cycle and proliferation in rG2-DC-1C and HepG2 cells, as determined by RNA sequencing as described in Materials and methods. The number of upregulated (4.0-fold) and downregulated (0.25-fold) genes is indicated. (B) Heatmap of mRNA expression levels for BMP and TGF- β signaling genes in rG2-DC-1C and HepG2 cells, as determined by RNA sequencing. The number of upregulated (4.0-fold) and downregulated (0.25-fold) genes is indicated. (C) Comparison of mRNAs encoding apoptosis-related genes in rG2-DC-1C and HepG2 cells, as determined by RNA sequencing. (D) Comparison of mRNAs of autophagy-related genes in rG2-DC-1C and HepG2 cells, as determined by RNA sequencing. RNA sequencing data were deposited in the NCBI Bioproject Database (<http://www.ncbi.nlm.nih.gov/bioproject>) using the accession number PRJNA273617.

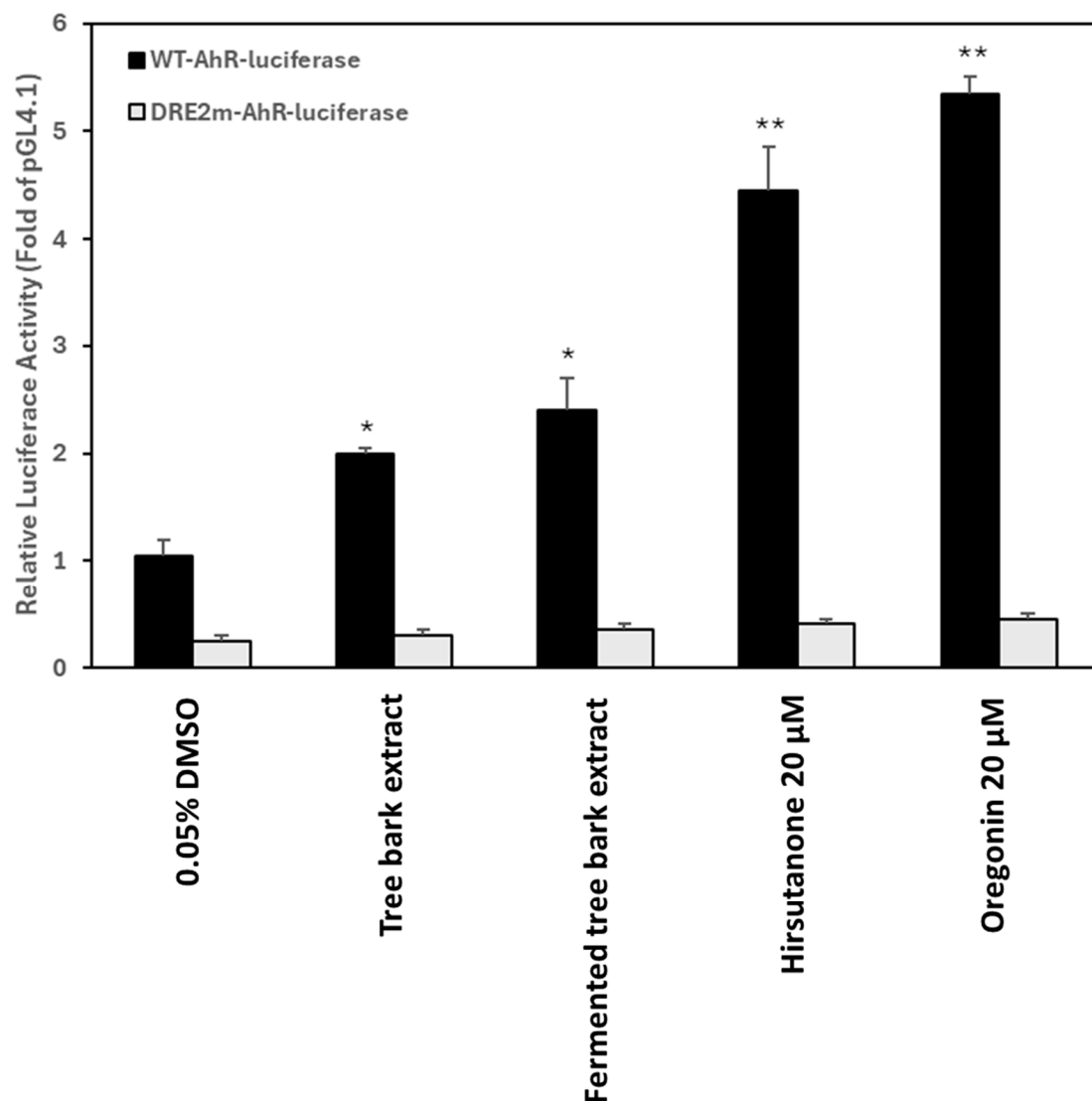
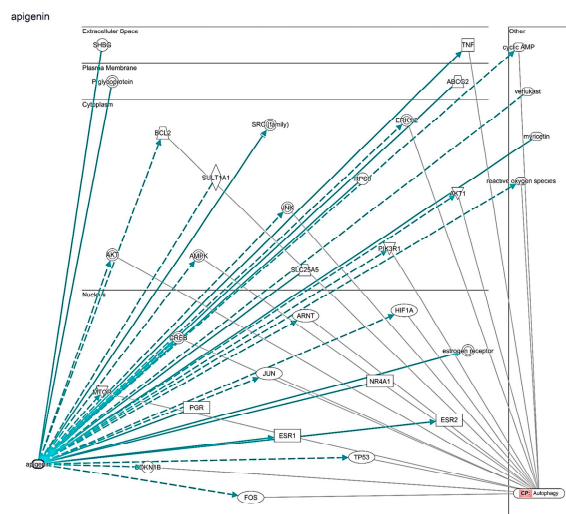
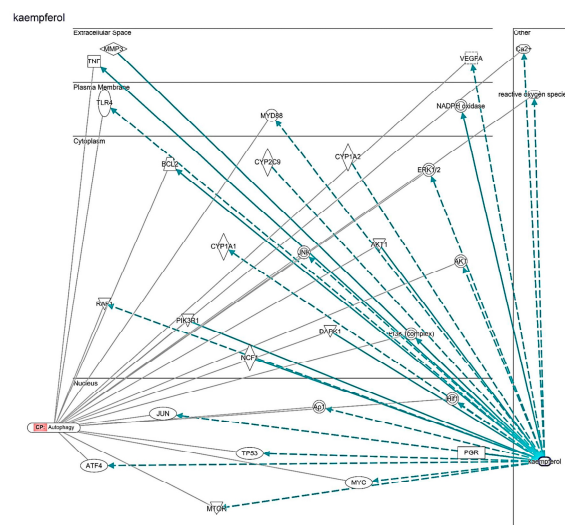


Figure S3. Relative AHR-luciferase activities in wild-type and BRD2 mutant constructs in rG2-DC-1C cells. Comparative luciferase activity of pGL4.1-AhR luciferase and DRE2 mutated pGL4.1 DRE2m-AhR luciferase was calculated in rG2-DC-1C cells. 0.05% DMSO addition was arbitrarily set at 1.0. Data were analyzed using one-way ANOVA with Tukey's post hoc test (* $p < 0.05$ and ** $p < 0.01$, $n = 5$).

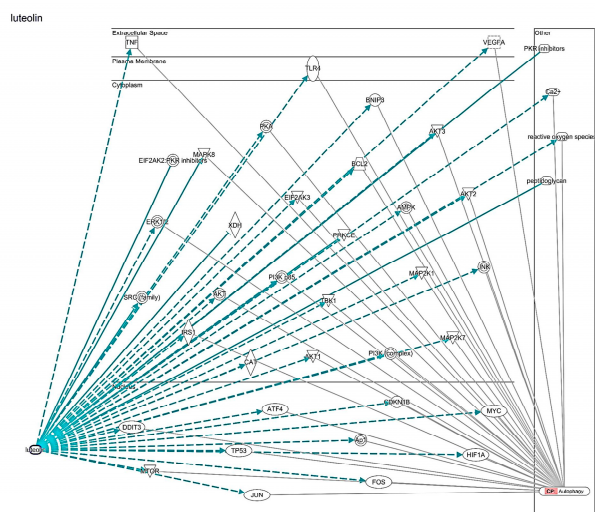
A



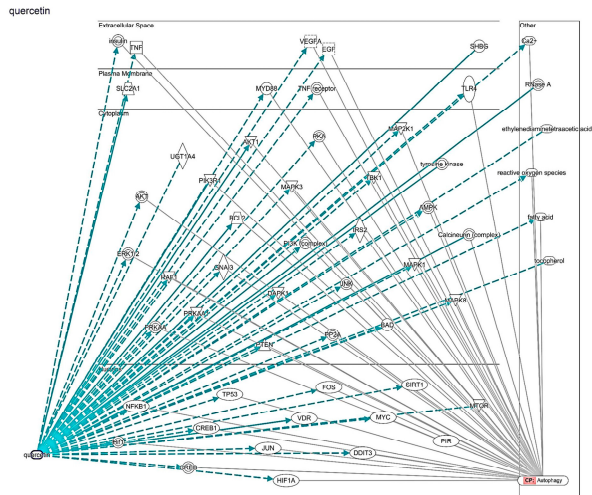
B



C



D



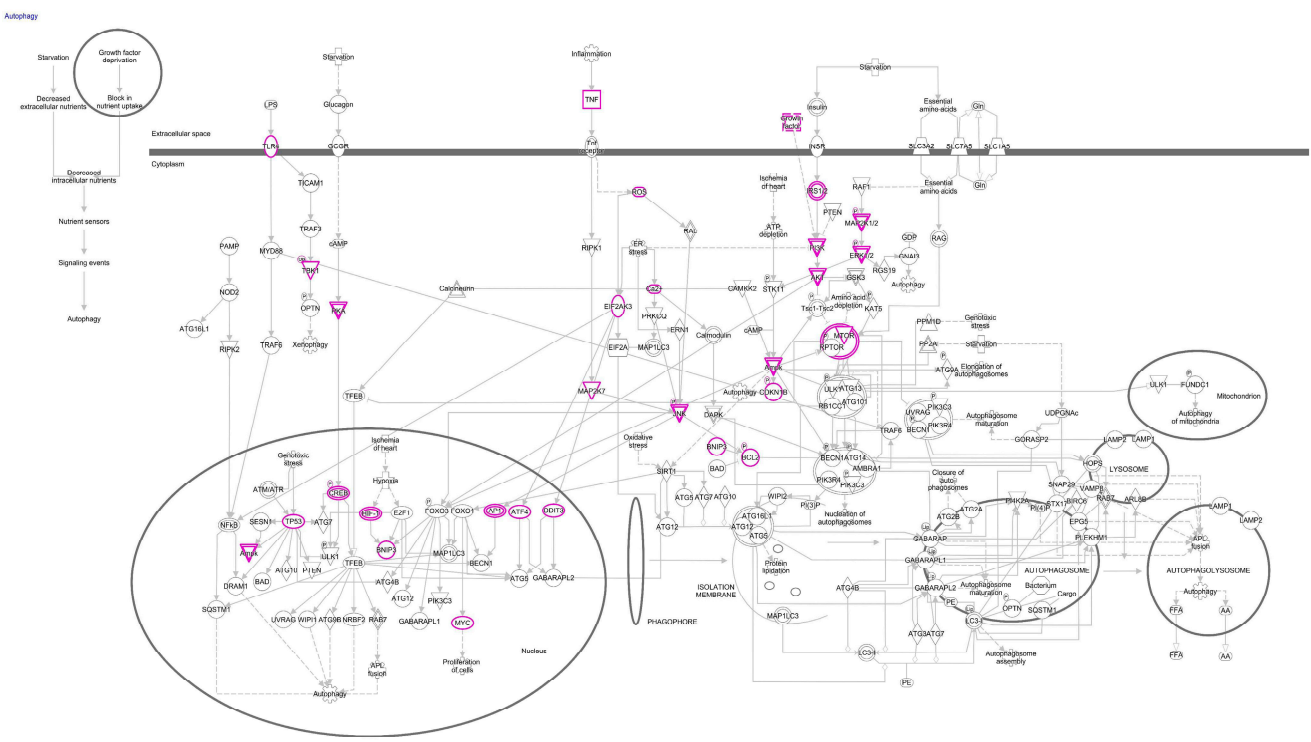
E



F



G



H

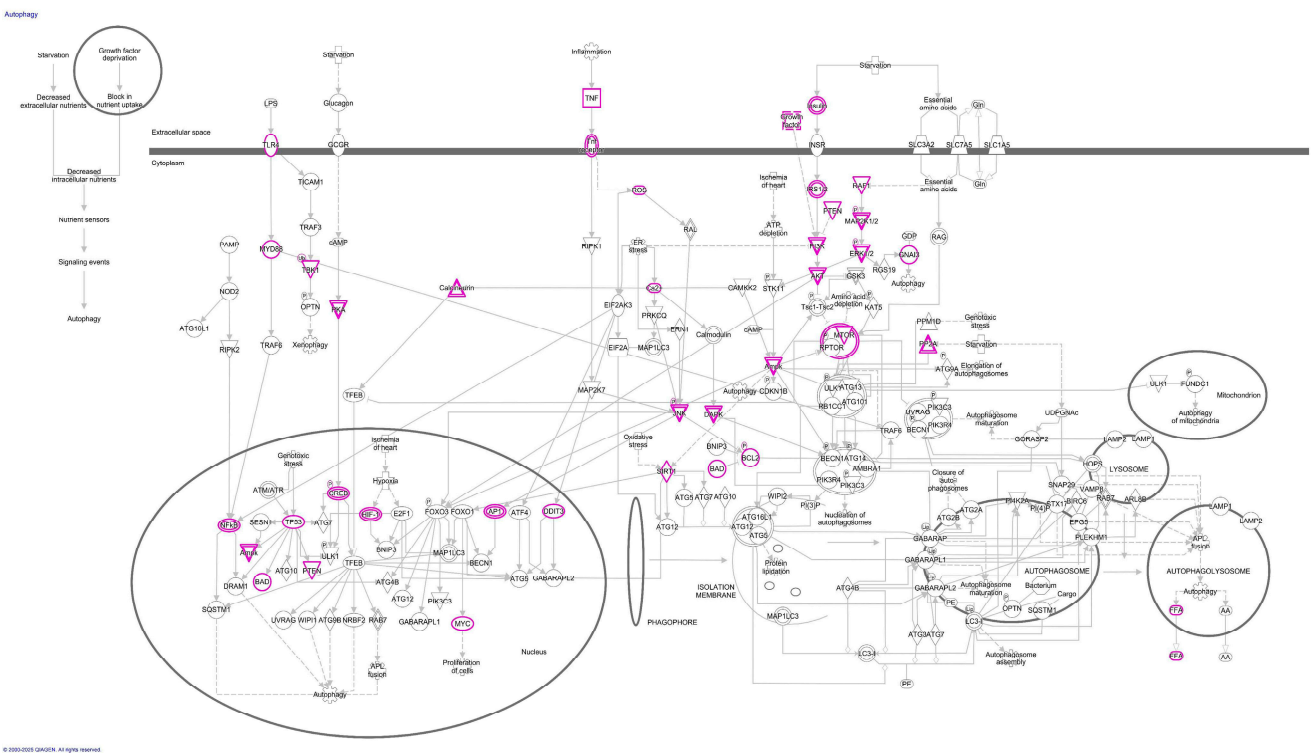
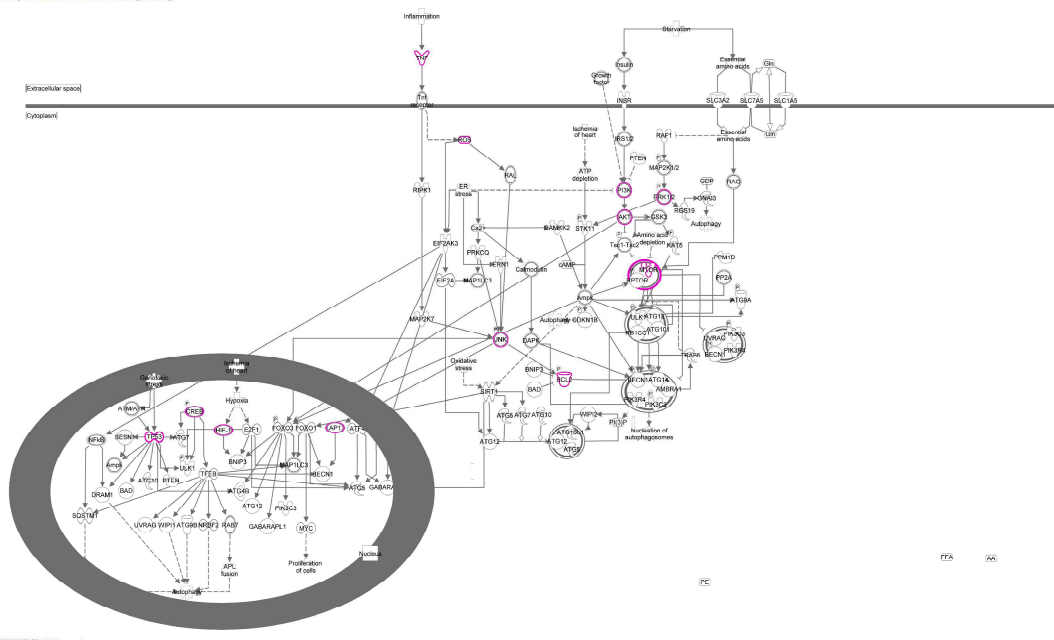


Figure S4. Ingenuity pathway analysis (QIAGEN Inc. released Nov. 4, 2025) was used to identify the network of extract components and the molecules of autophagy pathways. These targeted network molecules were matched to the molecules in the autophagy signaling pathway separately for each flavonoid using IPA. **(A)** Apigenin, **(B)** luteolin, **(C)** kaempferol, and **(D)** quercetin. After comparison, only the molecules common to these four networks and the autophagy signaling pathway were retained in the final network. Based on this result, these common molecules were also marked in the canonical autophagy signaling pathway using different colors. **(E)** Apigenin, **(F)** luteolin, **(G)** kaempferol, and **(H)** quercetin.

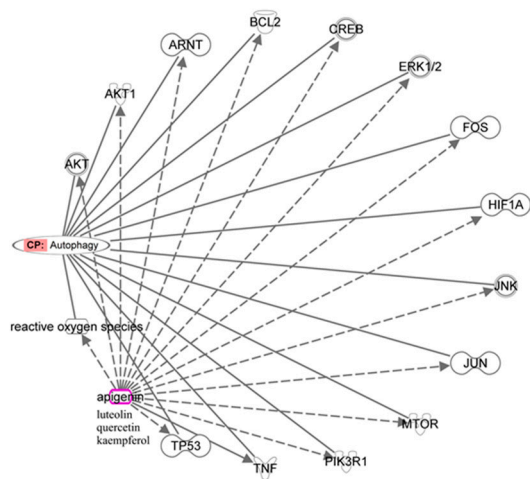
A

four drug common network molecules with autophagy Autophagy



B

Path Designer four drug network common molecules



C

Path Designer four drug common molecules in mTOR

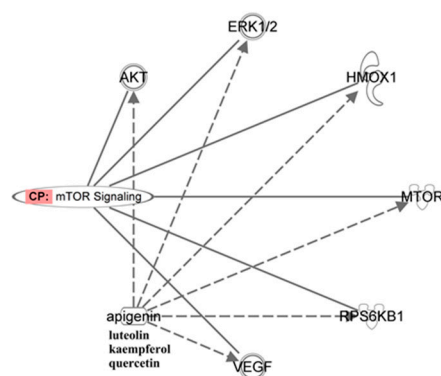


Figure S5. The proposed common network signaling of four flavonoids (apigenin, luteolin, kaempferol, and quercetin) and the autophagy pathway. The targeted network molecules identified by IPA were also linked to molecules in the autophagy signaling pathway, separately for each flavonoid. The common molecules were also

marked in the canonical autophagy signaling pathway. **(A)** Shared common pathway molecules in the signaling related to autophagy and the four flavonoids. **(B)** Schematic model of the signaling pathways common to these four flavonoids and autophagy. AKT, RNT, BCL2, CREB, ERK2, FOS, HIF1A, JNK, JUN, MTOR, PIK3R1, TNF, and TP53 were identified. **(C)** The common molecules identified across four networks were mapped to the mTOR signaling pathway.

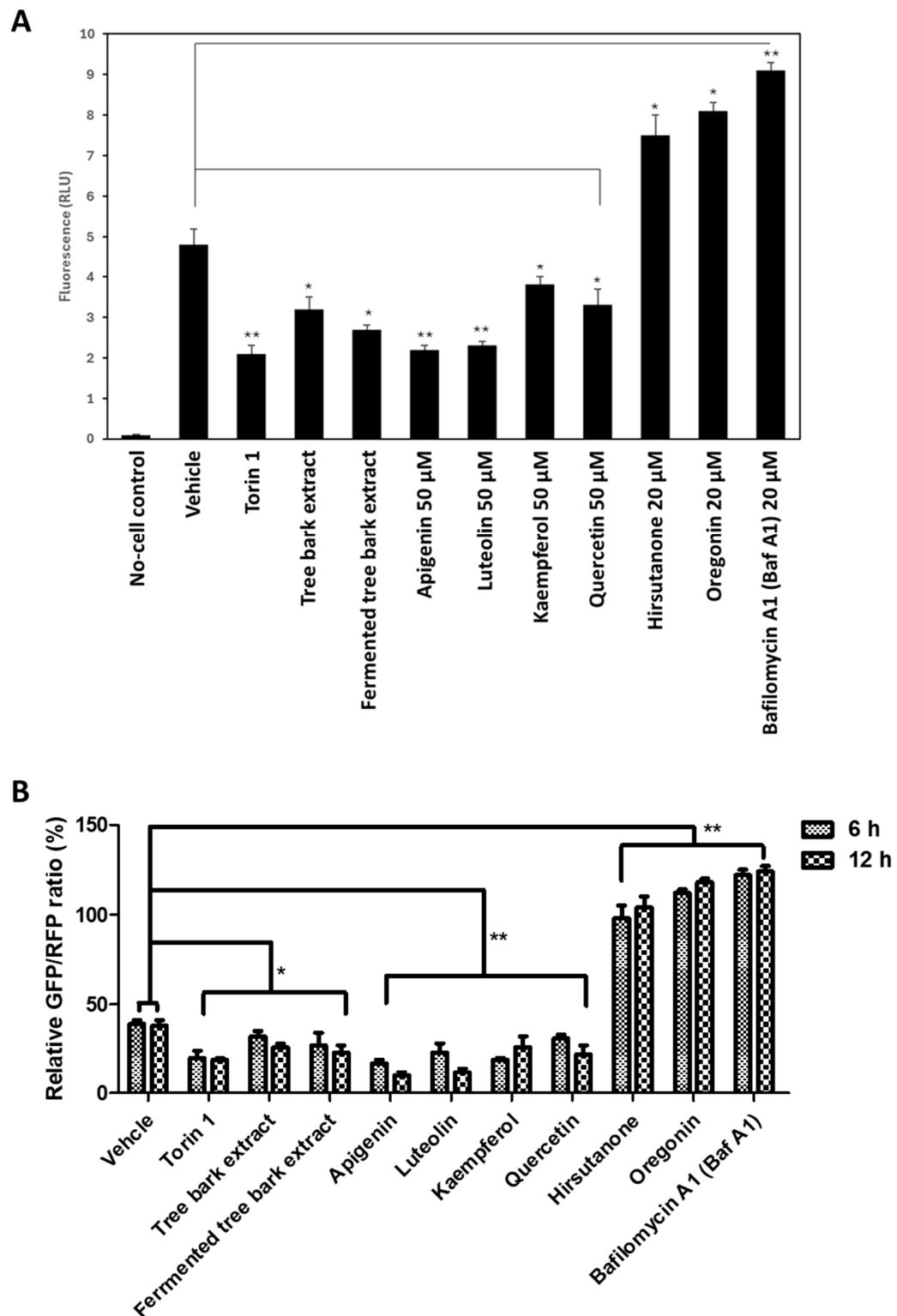


Figure S6. Light chain 3 HiBiT receptor assays and relative GFP/RFP ration value of autophagy reaction using apigenin, luteolin, kaempferol, and quercetin. Each

component was examined for autophagic activity using the Autophagy LC3 HiBiT Reporter Assay System. **(A)** U2OS HiBiT-HaloTag-LC3 cells were cultivated in Dulbecco's Modified Eagle Medium (high glucose) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin (8×10^3 cells per 80 μ L in each well) in 96-well white clear-bottom plates. The indicated components (50 μ M) in the unfermented or fermented tree bark extract from *A. japonica* and 20 μ M Hir or 20 μ M Ore were then examined for autophagic activity using this screening kit, as described in Methods and Materials. Data are presented as the mean $\pm$ SEM ($n = 3$). Data was analyzed using one-way ANOVA with Tukey's post hoc test (* $p < 0.05$ and ** $p < 0.01$). **(B)** rG2-DC-1C cells stably expressing pMRX-IP-GFP-LC3-RFP-LC3 Δ G were cultured with different inducers and each of the four flavonoids (50 μ M) and unfermented or fermented tree bark extracts, and 20 μ M Hir or 20 μ M Ore for 24 h. Then the GFP/RFP fluorescence ratio of the treated cells was expressed as a percentage relative to that of nontreated cells. Data represent mean $\pm$ SEM ($n = 3$). Bafilomycin A1 (BafA1); Torin1, mTOR inhibitor. Data are presented as the mean $\pm$ SEM ($n = 3$). Data was analyzed using one-way ANOVA with Tukey's post hoc test (* $p < 0.05$ and ** $p < 0.01$).



Figure S7. Full-length western blotting is used in this study.

Uncropped full length Western Blots are shown in these figure panels. The molecular weight markers are also listed.

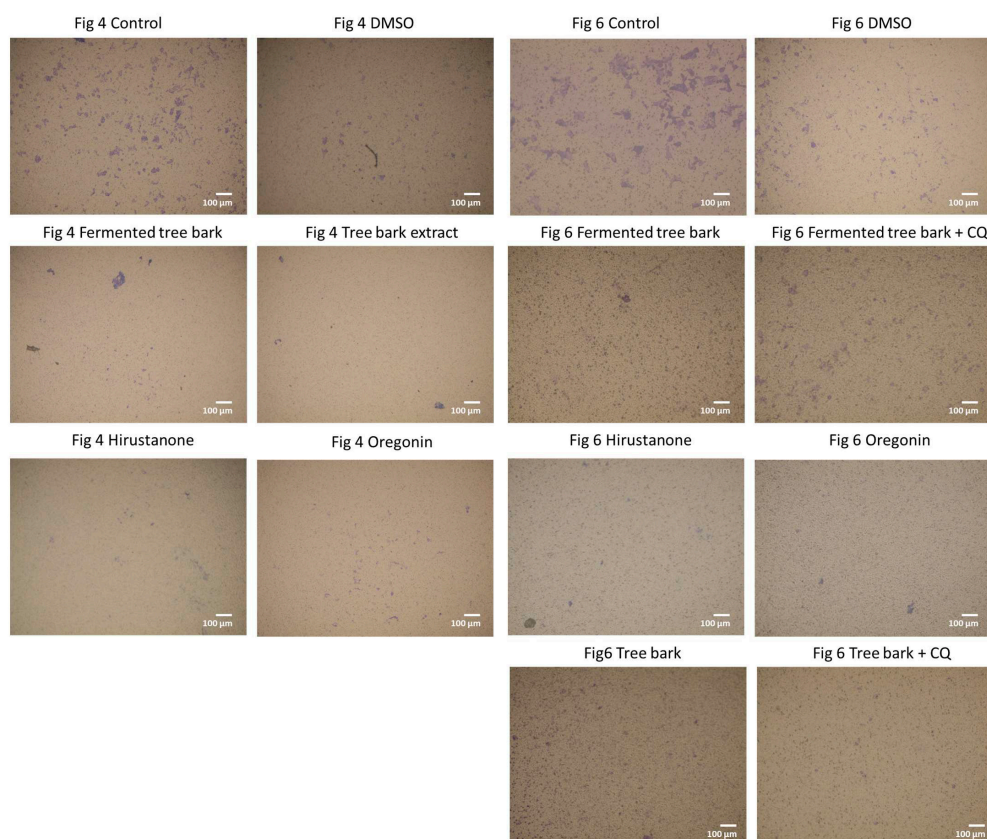


Figure S8. Bright view images taken in this study.

Original images are shown in figure 4 and figure 6 panels, the images were taken with 100x magnification under bright field.

Supplementary Table S1. Chemical constituents of the tree bark extract from *Alnus japonica*. Several candidate compounds derived from the bark were identified and reported by Sati et al. [18]. The major components were listed in this Table. Among these, we focused on four components such as apigenin, luteolin, kaempferol, and quercetin which have been previously implicated in induction of autophagy and apoptosis [45-50]. This Table lists the shared components detected in the non-fermented tree bark extracts and the unique composition of the fermented extract has not yet been fully characterized. Preliminary data suggest that fermentation alters the relative abundance of certain polyphenols, which may contribute to the observed differences in biological activity.

- | | |
|-----|---|
| 1. | Lutein 7,4'-dimethyl ether (Pillion) |
| 2. | Scutellarein-6,7,4'-trimethyl ether (salvigenin) |
| 3. | Kaempferide |
| 4. | Isohamnectin |
| 5. | Rhamnazin |
| 6. | Quercetin-7, 3', 4'-trimethyl ether |
| 7. | 6-hydroxyl-kaempferol-3,6, 4'-trimethyl ether |
| 8. | Acacetin |
| 9. | Apigenin-7,4'-dimethyl ether |
| 10. | Scutellarein-6,4'-dimethyl ether |
| 11. | Methyl (24-E)-3,4-secodammara-4 (28),20,24-trien-26-oic acid 3-oate |
| 12. | Hirsutanonol 5-O-(6-O-galloyl)- β -D-glucopyranoside |
| 13. | 3-deoxo-hisutanonol 5-O- β -D-glucopyranoside |
| 14. | Hirsutanonol-5-O- β -D-glucopyranoside |
| 15. | Hirsutanone |
| 16. | Oregonin |
| 17. | β -amyrin |
| 18. | 3-O-acetyl- β -amyrin |
| 19. | 3-O-acetyltertaxerol |
| 20. | Glutinane |
| 21. | Lupenone |

22.	Quercetin
23.	5-O-methyl hirsutanonol
24.	Glutinel
25.	Taraxrone
26.	Alnus japoninsA
27.	Alnus japonins B
28.	5-O-galloyl-shikimic acid
29.	4,6-di-O-galloyl-D-glucose
30.	1,4.-di-O-galloyl- β -D-glucose
31.	Strictinin
32.	Gemin D
33.	Pedunculagin
34.	Praecoxin A
35.	Flosin A
36.	Stachyurin
37.	Casuarinin
38.	Platyphylloside
39.	Garugamblin-3
40.	Acerogenin L
41.	Oregonoyl A
42.	Oregonoyl B
43.	Platyphyllme
44.	Platyphyllonol 5'-xylopyranpside

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