Supplementary materials associated with Łukasz Szoka, Jolanta Nazaruk, Marcin Stocki and Valery Isidorov article Cytotoxicity of flavonoids from Betula pubescens and Betula pendula buds in human digestive system cancer cells

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Figure S2. Mass spectra of flavonoids quantified in extracts from birch buds.

Figure S3. Flavonoids from birch buds differentially affect viability of digestive system cancer cells and fibroblasts. Gastric adenocarcinoma AGS cells, colorectal adenocarcinoma DLD-1 cells, hepatocellular carcinoma HepG2 cells and normal human skin CCD25Sk fibroblasts were treated with different doses of flavonoids for 24, 48 and 72 h. Cell viability was determined using the MTT assay. 0.1% DMSO was used as a vehicle control. Data were expressed as mean ± SD (n = 3). The asterisk indicates P < 0.05.

Figure S4. Representative merged photographs of cells stained with annexin V-FITC conjugate (green fluorescence), propidium iodide (red fluorescence), and Hoechst 33342 (blue fluorescence), visualised by fluorescence microscopy. Scale bar = 50 μm.

Table S1. Ions used for quantification of individual flavonoids.

and

Full-length blots
2.5. Colony formation assay

In total, 250 cells were plated in each well of 12-well plates and allowed to adhere for 24 h. Cells were treated with various concentrations of flavonoids (3.125, 6.25, 12.5, 25, 50 and 100 μM) for 48 h. Medium was discarded and fresh medium was added. After seven days, culture medium was removed and cells were washed once with PBS. Then, cells were fixed with 4% formaldehyde and stained with 0.1% crystal violet.

2.6. Apoptosis assay

Cells were seeded at a density of $1 \times 10^5$ cells per well in 6-well plates and allowed to adhere for 24 h. Cells were treated with various concentrations of flavonoids (12.5, 25, 50 and 100 μM) for 48 h. Floating and adherent cells were collected and assayed using a Dead Cell Apoptosis Kit with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI), for flow cytometry (#V13242, Thermo Fisher Scientific, USA), according to the manufacturer’s protocol. Briefly, cells were dispersed in 100 μL annexin-binding buffer containing 5 μL annexin V-FITC conjugate solution, 1 μg/mL PI and 1 μg/mL Hoechst 33342, and incubated for 15 min at room temperature. Then 400 μL of annexin-binding buffer was added, and the cell suspension was transferred to 96-well plates and visualised using fluorescence microscopy on a BD Pathway 855 system (Becton Dickinson, USA). Early apoptotic cells showed green and blue fluorescence, while late apoptotic cells showed green, red and blue fluorescence.

2.7. Western immunoblot

Adherent and floating cells were harvested and sonicated. Obtained homogenates were supplemented with 1% protease and phosphatase inhibitor cocktail (#78440, Thermo Fisher Scientific, USA). A Lowry assay was performed to quantify protein content in the homogenates. Proteins (20–40 μg) were resolved on 7.5%, 10% or 12% SDS-PAGE gels using the Mini-Protean Tetra system (Bio-Rad, USA). Proteins were transferred to nitrocellulose membranes (Bio-Rad, USA) using the Mini Trans-Blot Cell wet blotting system (Bio-Rad, USA). Membranes were blocked with 5% skim milk for 1 hour at room temperature and probed overnight at 4 °C with primary antibodies. The following antibodies were used: anti-caspase-9 antibody (#9508, 1:1000), anti-caspase-8 antibody (#9746, 1:1000), anti-caspase-3 antibody (#9662, 1:1000), anti-caspase-7 (#12827, 1:1000), anti-PUMA (#12450, 1:1000), anti-PARP (#9542, 1:1000) and anti-cleaved PARP antibody (#5625, 1:1000), purchased from Cell Signaling Technology (USA); anti-p53 antibody (#sc-126,
1:500) obtained from Santa Cruz Biotechnology (USA); and anti-actin antibody (#A2066, 1:2000) purchased from Sigma-Aldrich (USA). After extensive washes, a secondary antibody solution in 5% skim milk (anti-mouse IgG-HRP, Sigma-Aldrich, #A9044, 1:5000; anti-rabbit IgG-HRP, Sigma-Aldrich, #A9169, 1:5000) was added for 1 h at room temperature. Membranes were incubated with ECL-HRP substrate (GE Healthcare, USA) and the signal was detected using the BioSpectrum Imaging System (Ultra-Violet Products, Ltd., UK).

2.8. Immunofluorescence microscopy

Cells were plated in 96-well plates at $1 \times 10^4$ cells per well and allowed to adhere for 24 h. Cells were treated with the respective concentration of flavonoids and incubated for 48 h. Medium was removed and cells were fixed at room temperature for 15 minutes with 4% paraformaldehyde, rinsed three times with PBS, and permeabilised at room temperature for 5 minutes with 0.1% Triton X-100. Then, non-specific binding was blocked with 10% heat-inactivated goat serum in PBS for 1 h at room temperature. The cells were incubated with the following antibodies at 4 °C, overnight: anti-p53 antibody (#sc-126, 1:200) obtained from Santa Cruz Biotechnology (USA), and anti-cleaved-caspase-3 antibody (#9664, 1:200) and anti-cleaved-caspase-7 antibody (#8438, 1:200) purchased from Cell Signaling Technology (USA). After three washes with PBS, cells were incubated with Alexa Fluor 594 conjugated antibody (#A11032, #A11037; Invitrogen, USA) for 1 h in the dark. Cell nuclei were stained with Hoechst 33342 (Invitrogen, USA) and F-actin was stained with Phalloidin-Atto 488 (Sigma-Aldrich, USA). Fluorescent signals were examined and captured using a BD Pathway 855 confocal microscope.

Quantitative determination of flavonoids in SFE extracts of buds of two birch species was carried out by GC-MS method. The mass spectrometric detector was calibrated by analyzing flavonoid solutions in the concentration range 0.06–2.0 mg/ml in the mode of selective monitoring of the most intense ions ($\text{M}^+ - \text{CH}_3$) in the spectra of TMS derivatives of the determined components (Table S1).
Figure S1. Calibration plots obtained as a result of GC-MS analysis of artificial solutions of flavonoids in the selective ion monitoring (SIM) mode (m/z = 357, 415, 443, 473).
Figure S2. Mass spectra of flavonoids quantified in extracts from birch buds. 1 - TMS derivative of naringenin-7,4'-O-dimethyl ether, 2 - di-TMS derivative of sakuranetin, 3 - di-TMS derivative of kumatakenin, 4 - di-TMS derivative of cirsimaritin, 5 - di-TMS derivative of ermanin, 6 - di-TMS derivative of santin.
Figure S3. Flavonoids from birch buds differentially affect viability of digestive system cancer cells and fibroblasts. Gastric adenocarcinoma AGS cells, colorectal adenocarcinoma DLD-1 cells, hepatocellular carcinoma HepG2 cells and normal human skin CCD25Sk fibroblasts were treated with different doses of flavonoids for 24, 48 and 72 h. Cell viability was determined using the MTT assay. 0.1% DMSO was used as a vehicle control. Data were expressed as mean ± SD (n = 3). The asterisk indicates $P < 0.05$. 
Figure S4. Representative merged photographs of cells stained with annexin V-FITC conjugate (green fluorescence), propidium iodide (red fluorescence), and Hoechst 33342 (blue fluorescence), visualised by fluorescence microscopy. Scale bar = 50 μm.
Table S1. Ions (m/z = M⁺-15) used for quantification of individual flavonoids.

| Flavonoid                             | m/z = M⁺ - 15 |
|---------------------------------------|---------------|
| Naringenin-7,4′-O-dimethyl ether, TMS | 357           |
| Sakuranetin, di-TMS                   | 415           |
| Kumatakenin, di-TMS                   |               |
| Cirsimaritin, di-TMS                  | 443           |
| Ermanin, di-TMS                       |               |
| Santin, di-TMS                        | 473           |
Full-length blots
AGS cells

PARP
cPARP

(cblot presented in figure)
cPARP
(duplicate)
caspase-8

cleaved caspase-8

p53
HepG2 cells

- caspase-7
- actin
- PARP
- cPARP
cPARP (blot presented in figure)
cPARP (duplicate)
caspase-8
cleaved caspase-8
caspase-9

cleaved caspase-9

caspase-3
DLD-1 cells

Caspase-7

Actin
