Electronic Supplementary Information (ESI)

The anti-cancer activity of green tea, coffee and cocoa extracts in human cervical adenocarcinoma HeLa cells depends on both pro-oxidant and anti-proliferative activities of polyphenols

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EXPERIMENTAL

MTT assay

Cells were seeded in 96-well plates at density of 10,000 cells per well and left overnight to allow cell attachment. Hereafter, cells were incubated with each polyphenol extract (0-200 µg GAE mL⁻¹ in 200 µL) for the next 24 and 72 h. Only medium was added to the cells in the control wells. Cell culture medium with or without polyphenol extracts was added to blank wells. After the indicated period of time, 20 µL of MTT solution (5 mg mL⁻¹ in phosphate-buffered saline, PBS) were added to each well and incubated for the next 4 h at 37°C. Medium was carefully removed and 200 µL of DMSO were added to dissolve the formed formazan complexes; absorbance was read at 492 nm. In addition, prior to MTT addition, cells were visualized under the light inverted microscope to observe potential changes in cell morphology. Data are expressed as percentage of viability with untreated cells taken as 100%.

DNA staining and cell cycle analysis

HeLa cells were seeded at density of 50,000 cells per well in 24-well plates and left overnight prior to the addition of polyphenol extracts. Cells were incubated for 24 or 48 h with 100 µg GAE mL⁻¹ of polyphenols in the final volume of 1 mL per well. Upon incubation, cells were harvested (adherent and detached cells from each well were collected), washed 1x with PBS and fixed in ice-cold 70% ethanol overnight at 4°C. The next day, cells were washed 2x with PBS but centrifuged at 850 g to minimize cell loss. Fixed cells were treated with 200 µg mL⁻¹ of RNase A for 30 min at 37°C (to degrade RNA that also stains with PI) and subsequently stained with 50 µg mL⁻¹ of PI for the next 30 min at room temperature prior to flow cytometric analysis.
on FACSCalibur (Becton Dickinson, Franklin Lakes, NY, USA). Data were further analyzed in ModFit LT deconvolution software (Verity, Topsham, ME, USA). Analysis included following steps: (1) cells were gated according to their size and granularity (FSC vs. SSC dot plot, G1), (2) singlet cell population was gated out from debris and aggregates (FL-2 width vs. FL-2 area dot plot, G2), (3) FL-2 area histogram was created and formatted to show only the cells inside the single cell region (G1 and G2). FL-2 area histograms were further modeled by fitting the best Gaussian distribution curve to each peak, G0-G1 and G2-M, and then calculating the resulting S-phase. For each measurement 2,000 events inside single cell region were collected.

56  **CFSE staining and analysis of cell proliferation**

HeLa cells were harvested, washed 2x with PBS, and 1.5 x 10^7 cells were resuspended in 250 µL of medium without FCS. CFDA-SE stock solution (5 mmol L^{-1} in DMSO) was diluted with medium without FCS to final concentration of 20 µmol L^{-1}. To enable uniform labeling, cells and CFDA-SE were separately incubated for 5 min at 37ºC prior to mixing in 1:1 v/v ratio. Final cell concentration was 3 x 10^7 cell mL^{-1} and the one of CFDA-SE 10 µmol L^{-1}. Cells were labeled for 15 min at 37ºC in the dark, washed 2x with complete medium (FCS will quench the excess of CFDA-SE) and seeded at density of 50,000 cells/well in 24-well plates. The next day, polyphenol extracts were added to corresponding wells to final concentration of 100 µg GAE mL^{-1}. After 72 h of incubation with polyphenols, both adherent and detached cells from each well were collected. To exclude dead from living cells, prior to FACS analysis, PI was added to final concentration of 50 µg mL^{-1}. For each measurement 20,000 events were collected. Distribution of cells in different generations and calculation of proliferating cell index (PCI) were performed in the Wizard module of ModFit LT software using standard proliferation
models to fit the raw data. The PCI is indicated as the sum of the cells in all generations divided by the computed number of original parent cells present at the start of the experiments, and it is therefore a measure of the increase in cell number in the culture over the course of the experiment.

Relative gene expression of catalase and SOD as measured by real time quantitative reverse transcription PCR (real-time RT-qPCR)

HeLa cells were treated with 100 µg GAE mL$^{-1}$ of GT, CF and CC for 12 and 24 h, with controls set at the same time. The total RNA was isolated with RNeasy Mini Kit with Qiazol reagent and gDNA eliminator solution following manufacturer's instruction (Qiagen Maryland, USA). Concentration and purity of the total RNA, enriched in mRNA, was determined with Nano Drop spectrophotometer 2000c (ThermoScientific, Wilmigton, Delaware, USA). The qPCR primer sets of human CuZnSOD, MnSOD, catalase and glyceraldehyde 3-phosphate dehydrogenase (as endogenous control) were designed by Primer Express 2.0 software (Applied Biosystems, California, USA). The KAPA SYBRR FAST One-Step qRT-PCR Kit was used according to manufacturer’s instructions (Kappa Biosystems, Maryland, USA) with 10 ng of total RNA as template. Relative expression levels were determined using Rotor-Gene Q real time PCR system (Qiagen, Germany) and GeneQuest software (version 2.0.2.4). The cycling parameters were 42°C for 5 min, 95°C for 5 min followed by 40 cycles of three step cycling protocol (95°C for 3s, 58°C for 20s and 72°C for 20s). The melting curve was analyzed at the end to ensure the product specificity (rising by one degree per 5s until 95°C).

Determination of catalase and SOD activity
Cell suspension at concentration of 600,000 cells per mL was incubated with 100 µg GAE mL\(^{-1}\) of GT, CF and CC for 24 h at 37°C. After incubation, cells were washed twice with ice-cold PBS and resuspended in 180 µL of lysis buffer (1% Triton X-100, 1% phenylmethanesulfonylfluoride, 1% protease inhibitor cocktail, 50 mmol L\(^{-1}\) Tris pH 7.5, 150 mmol L\(^{-1}\) NaCl, 0.25% Na-deoxycholate, 50 mmol L\(^{-1}\) NaF, 10 mmol L\(^{-1}\)pyrophosphate) and left in the ice-bath for the next 30 minutes until samples were resolved. Cell lysates were then centrifuged for 15 minutes at 13,400 rpm and the obtained supernatants were stored at -80°C until further analysis.

**Catalase activity assay.** Working substrate solution was prepared by diluting 30% H\(_2\)O\(_2\) with 50 mmol L\(^{-1}\) phosphate buffer pH 7.0 until reaching the absorbance at 240 nm between 0.525 and 0.550. To a cuvette with 160 µL of working substrate solution, 6 µL of cell lysate were added, contents were mixed and cuvette was immediately placed in the spectrophotometer to monitor the changes in absorbance during 180s at room temperature. Catalase activity was calculated and expressed as the number of units per mg of total protein, where one unit of activity corresponds to an absorbance change of 0.01 per one s.

**SOD in-gel activity assay.** Resolution of proteins from cell lysates was carried out on 10% native polyacrylamide gel electrophoresis according to Laemmli. Per each lane, 13 µg of proteins were applied. When finished, the polyacrylamide gel was first immersed in 1.23 mmol L\(^{-1}\) solution of NBT for 15 min, and then in the riboflavin solution (28 µmol L\(^{-1}\) riboflavin and 28 mmol L\(^{-1}\) TEMED in 100 mmol L\(^{-1}\) K-phosphate buffer pH 7.0) for the additional 15 minutes in the dark. Hereafter, the gel was exposed to a light source for 15 minutes to initiate the photochemical
reaction. NBT and SOD in the gel compete for the superoxide anion radical. In the NBT-
negative staining, SOD protein bands became transparent, while the rest of the gel develops dark
purple color due to reduced NBT. Gels were further analyzed in Gel-Pro Analyzer 3.0 program
(Media cybernetics, Bethesda, USA) and integrated optical density of each band was calculated.

REFERENCES