

Supplementary data for article:

Krstić-Ristivojević, M.; Stojadinović, M. M.; Smiljanić, K.; Stanić-Vučinić, D.; Ćirković-Veličković, T. The Anti-Cancer Activity of Green Tea, Coffee and Cocoa Extracts on Human Cervical Adenocarcinoma HeLa Cells Depends on Both pro-Oxidant and Anti-Proliferative Activities of Polyphenols. *RSC Advances* **2015**, 5 (5), 3260–3268.

<https://doi.org/10.1039/c4ra13230k>

1 **Electronic Supplementary Information (ESI)**

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

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24 **EXPERIMENTAL**

25

26 **MTT assay**

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

37

38 **DNA staining and cell cycle analysis**

39 HeLa cells were seeded at density of 50,000 cells per well in 24-well plates and left overnight
40 prior to the addition of polyphenol extracts. Cells were incubated for 24 or 48 h with 100 μg
41 GAE mL^{-1} of polyphenols in the final volume of 1 mL per well. Upon incubation, cells were
42 harvested (adherent and detached cells from each well were collected), washed 1x with PBS and
43 fixed in ice-cold 70% ethanol overnight at 4°C. The next day, cells were washed 2x with PBS
44 but centrifuged at 850 g to minimize cell loss. Fixed cells were treated with 200 μg mL^{-1} of
45 RNase A for 30 min at 37°C (to degrade RNA that also stains with PI) and subsequently stained
46 with 50 μg mL^{-1} of PI for the next 30 min at room temperature prior to flow cytometric analysis

47 on FACSCalibur (Becton Dickinson, Franklin Lakes, NY, USA). Data were further analyzed in
48 ModFit LT deconvolution software (Verity, Topsham, ME, USA). Analysis included following
49 steps: (1) cells were gated according to their size and granularity (FSC vs. SSC dot plot, G1), (2)
50 singlet cell population was gated out from debris and aggregates (FL-2 width vs. FL-2 area dot
51 plot, G2), (3) FL-2 area histogram was created and formatted to show only the cells inside the
52 single cell region (G1 and G2). FL-2 area histograms were further modeled by fitting the best
53 Gaussian distribution curve to each peak, G0-G1 and G2-M, and then calculating the resulting S-
54 phase. For each measurement 2,000 events inside single cell region were collected.

55

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

57 HeLa cells were harvested, washed 2x with PBS, and 1.5×10^7 cells were resuspended in 250 μ L
58 of medium without FCS. CFDA-SE stock solution (5 mmol L⁻¹ in DMSO) was diluted with
59 medium without FCS to final concentration of 20 μ mol L⁻¹. To enable uniform labeling, cells and
60 CFDA-SE were separately incubated for 5 min at 37°C prior to mixing in 1:1 v/v ratio. Final cell
61 concentration was 3×10^7 cell mL⁻¹ and the one of CFDA-SE 10 μ mol L⁻¹. Cells were labeled
62 for 15 min at 37°C in the dark, washed 2x with complete medium (FCS will quench the excess of
63 CFDA-SE) and seeded at density of 50,000 cells/well in 24-well plates. The next day,
64 polyphenol extracts were added to corresponding wells to final concentration of 100 μ g GAE
65 mL⁻¹. After 72 h of incubation with polyphenols, both adherent and detached cells from each
66 well were collected. To exclude dead from living cells, prior to FACS analysis, PI was added to
67 final concentration of 50 μ g mL⁻¹. For each measurement 20,000 events were collected.
68 Distribution of cells in different generations and calculation of proliferating cell index (PCI)
69 were performed in the Wizard module of ModFit LT software using standard proliferation

70 models to fit the raw data. The PCI is indicated as the sum of the cells in all generations divided
71 by the computed number of original parent cells present at the start of the experiments, and it is
72 therefore a measure of the increase in cell number in the culture over the course of the
73 experiment.

74

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

77 HeLa cells were treated with 100 $\mu\text{g GAE mL}^{-1}$ of GT, CF and CC for 12 and 24 h, with controls
78 set at the same time. The total RNA was isolated with RNeasy Mini Kit with Qiazol reagent and
79 gDNA eliminator solution following manufacturer's instruction (Qiagen Maryland, USA).
80 Concentration and purity of the total RNA, enriched in mRNA, was determined with Nano Drop
81 spectrophotometer 2000c (ThermoScientific, Wilmington, Delaware, USA). The qPCR primer
82 sets of human CuZnSOD, MnSOD, catalase and glyceraldehyde 3-phosphate dehydrogenase (as
83 endogenous control) were designed by Primer Express 2.0 software (Applied Biosystems,
84 California, USA). The KAPA SYBRR FAST One-Step qRT-PCR Kit was used according to
85 manufacturer's instructions (Kappa Biosystems, Maryland, USA) with 10 ng of total RNA as
86 template. Relative expression levels were determined using Rotor-Gene Q real time PCR system
87 (Qiagen, Germany) and GeneQuest software (version 2.0.2.4). The cycling parameters were
88 42°C for 5 min, 95°C for 5 min followed by 40 cycles of three step cycling protocol (95°C for 3s,
89 58°C for 20s and 72°C for 20s). The melting curve was analyzed at the end to ensure the product
90 specificity (rising by one degree per 5s until 95°C).

91

92 **Determination of catalase and SOD activity**

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

101

102 *Catalase activity assay.* Working substrate solution was prepared by diluting 30% H_2O_2 with 50
103 mmol L^{-1} phosphate buffer pH 7.0 until reaching the absorbance at 240 nm between 0.525 and
104 0.550. To a cuvette with 160 μL of working substrate solution, 6 μL of cell lysate were added,
105 contents were mixed and cuvette was immediately placed in the spectrophotometer to monitor
106 the changes in absorbance during 180s at room temperature. Catalase activity was calculated and
107 expressed as the number of units per mg of total protein, where one unit of activity corresponds
108 to an absorbance change of 0.01 per one s.

109

110 *SOD in-gel activity assay.* Resolution of proteins from cell lysates was carried out on 10% native
111 polyacrylamide gel electrophoresis according to Laemmli.¹ Per each lane, 13 μg of proteins were
112 applied. When finished, the polyacrylamide gel was first immersed in 1.23 mmol L^{-1} solution of
113 NBT for 15 min, and then in the riboflavin solution (28 $\mu\text{mol L}^{-1}$ riboflavin and 28 mmol L^{-1}
114 TEMED in 100 mmol L^{-1} K-phosphate buffer pH 7.0) for the additional 15 minutes in the dark.
115 Hereafter, the gel was exposed to a light source for 15 minutes to initiate the photochemical

116 reaction. NBT and SOD in the gel compete for the superoxide anion radical. In the NBT-
117 negative staining, SOD protein bands became transparent, while the rest of the gel develops dark
118 purple color due to reduced NBT. Gels were further analyzed in Gel-Pro Analyzer 3.0 program
119 (Media cybernetics, Bethesda, USA) and integrated optical density of each band was calculated.

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121 **REFERENCES**

122

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