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1 **The anti-cancer activity of green tea, coffee and cocoa extracts in human cervical**
2 **adenocarcinoma HeLa cells depends on both pro-oxidant and anti-proliferative**
3 **activities of polyphenols**
4
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† Electronic Supplementary Information (ESI) available: more experimental details on MTT assay, DNA staining and cell cycle analysis, CFSE staining and analysis of cell proliferation, relative gene expression and activity of catalase and SOD.

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23 **Abbreviations:** C, catechin; CC, cocoa polyphenol extract; CF, coffee polyphenol extract;
24 CFDA-SE, carboxyfluorescein diacetate succinimidyl ester; CMF-DA, 5-
25 chloromethylfluorescein diacetate; CTRL, control, cells not treated with polyphenols; DHE,
26 dihydroethidium; $DiOC_6$, 3,3′-dihexyloxacarbocyanine iodide; ECG, epicatechin-3-gallate;
27 EGC, epigallocatechin; EGCG, epigallocatechin-3-gallate; FCS, fetal calf serum; GAE, gallic
28 acid equivalents; GSH, reduced glutathione; GT, green tea polyphenol extract; H_2DCF-DA,
29 2, 7-dichlorodihydrofluorescin diacetate; MFI, mean fluorescence intensity; mTMP,
30 mitochondrial transmembrane potential; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-
31 diphenyltetrazolium bromide; NBT, nitro blue tetrazolium; PCF, proliferating cell fraction;
32 PCI, proliferating cell index; PI, propidium iodide; ROS, reactive oxygen species; SOD,
33 superoxide dismutase; TEMED, tetramethylethylenediamine.

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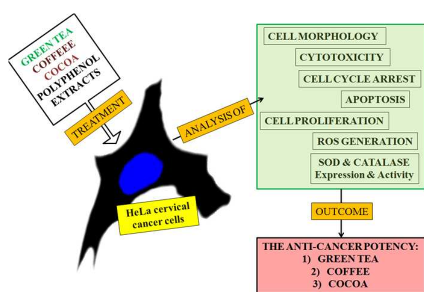
34 **TABLE OF CONTENTS ENTRY**



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36 The *in vitro* anti-cervical cancer potency of tested polyphenol extracts is exhibited in the
37 following order: green tea>coffee>cocoa, with only green tea showing both pro-oxidative and
38 anti-proliferative action.

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ABSTRACT

It has been shown before that dietary polyphenols possess cancer chemopreventive effects. As cervical cancer is the second leading genital malignancy in women after breast cancer, the anti-cervical cancer effects of polyphenol extracts of commonly used beverages (green tea, coffee and cocoa) were tested and compared in HeLa cells. All extracts induced apoptosis of HeLa cells, but green tea was the most potent. However, as opposed to green tea which induced strong anti-proliferative response in HeLa cells, coffee and cocoa extracts promoted the proliferation of surviving cells. After short-term exposure, green tea and coffee extracts, but not cocoa, induced formation of intracellular reactive oxygen species. Only green tea extract increased production of superoxide anion radical and decreased reduced glutathione levels. Gene expression of Cu/Zn and Mn-superoxide dismutase or catalase was unaltered in cells treated with extracts, but green tea partially inhibited catalase activity. Cytotoxic activity of green tea and coffee extracts was partially inhibited by vitamin C. The *in vitro* anti-cervical cancer potency of tested polyphenol extracts is related to their pro-oxidant and anti-proliferative activities and exhibited in the following order: green tea>coffee>cocoa, with only green tea showing both pro-oxidative and anti-proliferative action.

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56 **INTRODUCTION**

57

58 Polyphenols are widely distributed in human diet and are mainly known for their antioxidant
59 activity.[1] As antioxidants, polyphenols may protect cell constituents against oxidative
60 damage. However, it has become clear that in complex biological systems, polyphenols also
61 exhibit pro-oxidant activity and several additional properties that are independent of
62 immediate antioxidant/pro-oxidant activities. Through these mechanisms of action, dietary
63 polyphenols could interfere with cell signaling and modulate expression of transcription
64 factors which control cell survival, transformation and apoptosis.[2]

65

66 Cervical cancer is the second leading cause of cancer deaths in women worldwide with
67 500,000 new diagnoses annually, most occurring in the developing world.[3] A large body of
68 evidence from epidemiological and experimental studies indicates that dietary polyphenols,
69 especially green tea catechins such as epigallocatechin-3-gallate (EGCG), act as effective
70 chemopreventive agents towards different organ specific cancers.[4-9] Epidemiological data of
71 green tea consumption and its chemoprevention against cervical cancer are still lacking or are
72 under the way.[10] *Ex vivo* data demonstrated that EGCG induced apoptosis in cancer cells in
73 more than 50% of women with cervical cancer.[11] In almost 70% of green tea extract-treated
74 patients with different stages of cervical cancer significant anti-cancer effects were present,
75 when compared to the untreated group.[12] In addition, there are several *in vitro* studies
76 addressing the anti-proliferative and apoptotic mechanism of green tea polyphenol extract or
77 EGCG in HeLa cell lines, a type of immortalized cervical carcinoma cell line.[13-18] Except for
78 one study investigating the effects of spent coffee beans extract,[19] there are no studies
79 examining the potential anti-cancer effects of coffee and cocoa polyphenol extracts on HeLa
80 cells or any other kind of cervical cancer cells.

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81

82 In the current study, we have investigated the effects of green tea, coffee and cacao
83 polyphenol extracts on viability, cell cycle, and proliferation of HeLa cells. All extracts were
84 previously characterized and precise data on their polyphenol composition were reported by
85 Tantoush et al.[20] and Stojadinovic et al.[21] In addition, we tried to explore the mechanism of
86 the anti-cancer action of polyphenol extracts by studying apoptosis and onset of early
87 oxidative stress. Our experimental data suggest that there is a causal link between the
88 polyphenol pro-oxidant and cytotoxic action in HeLa cells.

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EXPERIMENTAL

Reagents

RPMI-1640 medium was purchased from PAA The Cell Culture Company (Linz, Austria). Gallic acid, Folin-Ciocalteu reagent, fetal calf serum (FCS), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), penicillin, streptomycin, glutamine, 2,7-dichlorodihydrofluorescin diacetate (H_2DCF-DA), dihidroethidium (DHE), 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$), propidium iodide (PI), ribonuclease A (RNAse A), Triton X-100, riboflavin, protease inhibitor cocktail for general use, vitamin C and carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Green 5-chloromethylfluorescein diacetate (CMF-DA) was from Invitrogen (Carlsbad, California, USA). Nitro blue tetrazolium (NBT), Tris and tetramethylethylenediamine (TEMED) were from Serva Electrophoresis GmbH, Heidelberg, Germany. All other chemicals were from Applichem (Darmstadt, Germany). Deionized water used in the experiments was purified in a Barnstead Smart2Pure water purification system (Thermo Scientific Barnstead, USA). Green tea, coffee and cocoa were purchased from a local grocery store.

Preparation of green tea, cocoa and coffee polyphenol extracts

Green tea catechin-enriched polyphenol extract (GT) was prepared from the commercial green tea food supplement (local distributer "Zeleni čaj" Pharmanova, Obrenovac, Serbia and producer Chengdu Wagott Pharmaceuticals Co. Ltd, Chando, China).[20] Cocoa (CC) and coffee polyphenol extracts (CF) were made from the commercial cocoa powder or ground roasted coffee (mixture of Arabica and Robusta).[21] Concentration of total phenolic compounds was measured following Chun et al.[22] spectrophotometric assay optimized for 96-

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114 well plates using Folin–Ciocalteu's reagent and expressed in μg of gallic acid equivalents per
115 ml or μg GAE mL^{-1}.
116
117 **Cell culture and MTT vitality assay**
118 HeLa cells were cultured in RPMI-1640 medium, supplemented with 10% FCS, 2 mmol L^{-1}
119 glutamine, 100 IU mL^{-1} penicillin and 0.1 mg mL^{-1} streptomycin. Cells were incubated at
120 37°C in a humidified atmosphere with 6.0% CO_2. Cytotoxicity of GT, CC and CF in HeLa
121 cells was evaluated by MTT assay after 24 and 72 h of treatment, according to slightly
122 modified procedure of Mosmann[23] (for detailed description see ESI†).
123
124 **Cell cycle analysis**
125 Cells at different phases of cell cycle were distinguished by flow cytometric measurement of
126 cellular DNA content.[24] First, cells were seeded at density of 50,000 cells per well in 24-well
127 plates and left overnight prior to the addition of polyphenol extracts. Cells were incubated for
128 24 or 48 h with 100 μg GAE mL^{-1} of extracts in the final volume of 1 mL per well. Upon
129 incubation, both adherent and detached cells from each well were collected and their DNA
130 was stained with PI. Flow cytometric analysis was performed on FACSCalibur (Becton
131 Dickinson, Franklin Lakes, NY, USA). The obtained FL-2 area histograms were further
132 analyzed in ModFit LT deconvolution software (Verity, Topsham, ME, USA) by fitting the
133 best Gaussian distribution curve to G0-G1 and G2-M peaks, and then calculating the resulting
134 S-phase. For etailed DNA staining and analysis procedure see ESI†.
135
136 **Detection of apoptosis using Annexin V-FITC and PI double staining**
137 HeLa cells were treated with 100 μg GAE mL^{-1} of extracts for 48 h. Upon incubation, both
138 adherent and detached cells from each well were collected and stained with FITC-Annexin V

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139 and PI using the apoptosis detection kit from Becton Dickinson. For each measurement
140 20,000 events were collected. FL-1 vs. FL-3 dot plots were created in Cyflogic software
141 (CyFlo Ltd, Turku, Finland).
142

143 **Estimation of cell proliferation using CFSE dye dilution method**

144 After division, the intensity of CFSE fluorescence in daughter cells is halved, which allows
145 for simple detection of the number of cell divisions by flow cytometry.[25] CFSE-stained cells
146 were treated with 100 µg GAE mL^{-1} for 72 h. Upon incubation, both adherent and detached
147 cells from each well were collected and analyzed for fluorescence by FACSCalibur.
148 Distribution of cells in different generations and calculation of proliferating cell index (PCI)
149 were performed in the Wizard module of the ModFit LT software using the standard
150 proliferation models to fit the raw data. For detailed experimental procedure see ESI†.
151

152 **Monitoring early pro-oxidant action of polyphenol extracts with flow cytometric assays**

153 Generation of multiple ROS, in particular hydrogen peroxide, was measured with H_2DCF as
154 a substrate.[26-27] As H_2DCF is poorly sensitive for superoxide anion radical, DHE was used for
155 its detection.[28] The level of intracellular reduced glutathione (GSH) was determined using
156 CMF-DA.[29] The change in mTMP was monitored by using $DiOC_6$.[30] After trypsinization,
157 HeLa cells (100,000 cells per tube) were treated with 100 µg GAE mL^{-1} of extracts for 15
158 min at 37°C, washed and stained for 30 min at 37°C with 20 µmol L^{-1} DHE or stained for 15
159 min with either 10 µmol L^{-1} CMF-DA or 10 nmol L^{-1} $DiOC_6$ in PBS. To monitor the
160 generation of ROS, cells were treated for 30 min with extracts at 37°C, washed and stained
161 with 10 µmol L^{-1} H_2DCF-DA for 30 min in PBS. For each measurement 10,000 events were
162 collected. Analysis was performed in Cyflogic software, where cells were gated according to

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163 their size and granularity and the gate was applied to corresponding FL-1 or FL-2 histograms
164 of mean fluorescence intensity (MFI) of cells.
165

166 **Monitoring the expression and activity of antioxidant enzymes in HeLa cells after the**
167 **treatment with polyphenol extracts**

168 Relative gene expression of catalase and SOD was measured by real-time RT-qPCR after 12
169 and 24 h of treatment with 100 μg GAE mL^{-1} of extracts, and analyzed with the comparative
170 $2^{-\Delta\Delta Ct}$ method.[31] For determination of SOD and catalase activity cell lysates were prepared
171 after 24 h of treatment with 100 μg GAE mL^{-1} of extracts. Catalase activity was determined
172 by following hydrogen peroxide decomposition at 240 nm, as proposed by Claiborne.[32] The
173 catalase specific activity was expressed as the number of units per mg of total protein, where
174 one unit of activity corresponds to an absorbance change of 0.01 per one s. Semi-quantitative
175 determination of SOD in-gel activity was done as before.[33] Gels were analyzed in Gel-Pro
176 Analyzer 3.0 program (Media cybernetics, Bethesda, USA) and integrated optical density of
177 each band was calculated. For detailed experimental procedure see ESI†.
178

179 **Modulation of the polyphenol growth inhibition capacity by vitamin C**

180 The growth inhibition potency of polyphenols was assayed by the MTT assay as indicated
181 previously. HeLa cells were incubated for 24 h with 100 μg GAE mL^{-1} of polyphenol
182 extracts, 100 μg mL^{-1} of vitamin C or with both simultaneously. Control cells, without the
183 extracts or vitamin C, were set at the same time. The sum of % of growth inhibition obtained
184 for an extract and vitamin C separately, was compared to % of inhibition obtained for the
185 simultaneous stimulation.
186

187 **Statistical interpretation**

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188 Data are presented as mean ± standard deviation and analyzed in Graph Prism 5 program (La
189 Jolla, CA, USA). All incubations were set up in triplicate, except for the SOD and catalase
190 gene expression analysis where each sample was analyzed in duplicate with 2 biological
191 replicates. The differences were analyzed by one-way ANOVA, except for the experiment
192 with vitamin C where student's t-test was used. Differences were considered significant if p
193 <0.05.

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194 **RESULTS**

195

196 **Polyphenol extracts induce HeLa cell death in a dose-dependent manner but only GT**
197 **causes marked morphological changes**

198 All tested extracts showed cytotoxic activity in a dose-dependent manner in HeLa cells, with
199 GT being the most potent (Fig. 1A). At the concentration of 100 µg GAE mL^{-1}, GT reduced
200 cell viability to 66 ± 8% after 24 h or to 31 ± 6% after 72 h of exposure. After 72 h of
201 treatment with 100 µg GAE mL^{-1}, CC and CF cytotoxic activity increased, leaving behind 73
202 ± 3 and 65 ± 7% of living cells, respectively. Concentration of 100 µg GAE mL^{-1} of
203 polyphenol extracts was chosen to be tested in the following experiments for it is the first
204 tested concentration where significant differences were observed between the control and
205 treatment groups after 24 h of treatment. Changes in morphological characteristics of HeLa
206 cells were assessed via light inverted microscopy (Fig. 1B). After the 24 hour-treatment with
207 100 µg GAE mL^{-1} of extracts, GT promoted cell rounding which was followed with cell
208 detachment. In CC and CF treated groups, a majority of cells retained their spindle-shaped
209 appearance and ability to adhere to the flask surface.

210

211 **Polyphenol extracts cause arrest in different phases of HeLa cell cycle**

212 After 24 h of treatment, GT caused significant accumulation of cells in S phase and reduction
213 of cells in G2/M and G0/G1 phase (Fig. 2A, left). Percentage of viable cells in S phase
214 changed dramatically, from 38.11% in the control to 61.75% in GT treated cells, as illustrated
215 from one representative experiment (Fig. 2A, right). The S-phase cell cycle arrest resulted in
216 significant increase in number of GT treated cells in subG0 phase after 48 h, rising from
217 1.92% in the control to 28.95% in GT treated group (Fig 2B). Treatment with CF promoted
218 cell accumulation in G2/M and sub G0 phase after 48 h. Although CC exhibited milder effect

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219 on the cell cycle in the monitored time frame, a trend for cycle arrest in G2/M phase was
220 noticeable after 48 h (Fig. 2B).
221
222 **Polyphenol extracts induce HeLa cell death via apoptosis**
223 By using Annexin-V/PI double staining we were able to distinguish between viable cells
224 (Annexin V-PI-, lower left quadrant), cells in early apoptosis (Annexin V+PI-, lower right
225 quadrant) and dead cells (late apoptotic and necrotic, Annexin V+PI+, upper right quadrant)
226 (Fig. 3A). All tested extracts induced apoptosis of HeLa cells, as demonstrated *via* significant
227 increase in percentage of early apoptotic cells after 48 h of treatment with 100 μg GAE mL^{-1}
228 of extracts. As in previous experiments, GT proved to be the most potent ($p<0.001$, Fig. 3B).
229 In one selected experiment, 32.6% of GT treated cells were in early apoptosis while 20.8%
230 and 18.7% were detected in CF and CC treated cells, compared to 8.5% in the control cells
231 (Fig. 3A).
232
233 **Polyphenol extracts modulate HeLa cell proliferation**
234 HeLa cells were treated with 100 μg GAE mL^{-1} of extracts and cell division was analyzed
235 after 72 h of treatment. When compared to control, statistically significant decrease ($p<0.001$)
236 in PCI was observed only in GT treated cells while, on the contrary, significant increase
237 ($p<0.001$) in PCI was measured in CF and CC treated cells (Fig. 4). The percentage of total
238 cells in the proliferating cell fraction (PCF) was only 13.3 ± 3% in GT treated cells, which
239 was significantly lower than 78 ± 4% in the control group. From the selected histograms in
240 Fig 4, it can be noticed that generation 4 was the mostly populated generation in the control
241 group with 59.41% of cells. The same trend was observed in CF and CC treated cells, with
242 67.96 and 68.98% of cells in the generation 4, respectively. In addition, CC was more potent
243 than CF in stimulation of cell proliferation yielding 8.98% of cells in generation 5, which was

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244 populated with only 1.69% in untreated cells. As for GT, abatement of proliferation resulted
245 in cells accumulating in generation 3 (55.93%) and 2 (7.79%), at expense of generation 4
246 (34.79%) and 5 (1.5%).
247
248 **Generation of ROS in HeLa cells differs between the tested extracts**
249 When used at the concentration of 100 μg GAE mL^{-1}, GT and CF, but not CC, induced
250 oxidative stress in HeLa cells after a short-term exposure. As shown in Fig. 5, GT
251 polyphenols significantly increased the basal level of intracellular superoxide anion radical
252 (increased ethidium fluorescence after 15 min of exposure) and other ROS such as hydrogen
253 peroxide and hydroxyl radicals (increased DCF fluorescence after 30 min of exposure). As a
254 consequence of increased oxidative stress in GT treated cells, the intracellular pool of GSH
255 got depleted (reduction in CMF-GSH fluorescence after 15 min of exposure) and mTMP
256 potential dropped below the basal level (measured as increased number of cells with low
257 DiOC6 uptake after 15 min of exposure). Although CF induced the formation of ROS that
258 oxidize H_2DCF and a drop in mTMP, no changes were noticed in the other tested parameters.
259 Finally, CC polyphenols induced only a drop in mTMP.
260
261 **Changes in antioxidant enzymes (gene expression levels and activity) after the exposure**
262 **to polyphenol extracts**
263 HeLa cells were treated with 100 μg GAE mL^{-1} of extracts for 12 and 24 h when relative
264 gene expression of CuZnSOD, MnSOD and catalase was assessed with RT-qPCR. As seen in
265 Fig. 6A, there were no significant differences in the relative gene expression levels among the
266 control and polyphenol-treated cells for any of the tested genes. Also, CuZnSOD activity did
267 not change after the 24-hour treatment with 100 μg GAE mL^{-1} of polyphenol extracts (Fig.

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268 6B, left). However, a significant decrease in catalase activity was detected in GT treated cells
269 after 24 h, when compared to the control (Fig. 6B, right).
270
271 **Vitamin C interferes with the cytotoxic activity of poyphenols**
272 After 24 h of separate exposure, 100 µg mL^{-1} of vitamin C and 100 µg GAE mL^{-1} of
273 polyphenol extracts induced HeLa cell death. But, when vitamin C was added to each extract,
274 their cytotoxicities were partially antagonized, resulting in lower percentage of growth
275 inhibition when compared to the calculated sum of their individual activities (Fig. 7).
276 Significant reduction was observed for GT and CF, but not for CC.

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277 **DISCUSSION**

278

279 The aim of this study was to investigate the effects of total polyphenolic compounds
280 extracted from commonly used green tea supplement, roasted ground coffee and cocoa
281 powder on human cervical carcinoma HeLa cells *in vitro*. Our results indicate that out of all
282 three extracts, GT possesses the strongest cytotoxic activity in HeLa cells. In fact, when used
283 at the same concentration of total phenolic compounds (100 μg GAE mL^{-1}), after 72 h of
284 treatment, GT cytotoxicity was roughly twice the rate of CF and CC. Next, we have shown
285 that all extracts induce apoptosis of treated cells but cause different alterations in cell cycle
286 progression. After 24 h of treatment with GT, cells were detained in S phase of cell cycle
287 resulting in subsequent accumulation of cells with fragmented DNA (subG0 phase) i.e.
288 apoptotic cells, as later confirmed by Annexin V/PI double staining. Cells that survived the
289 72-hour stimulation with GT and retained cell integrity, only 13% of total cells, proliferated
290 much slower than the untreated cells. Although CF and CC exhibited similar cytotoxicity in
291 MTT, their effects on cell cycle were different. After 48 h of exposure, CF caused cycle
292 arrest in G2/M phase and accumulation of cells in subG0 phase, while only slight differences
293 were observed in CC treated cells, suggesting different mechanism of action. Although CF
294 and CC induced cell apoptosis, HeLa daughter cells originating from the parent cells that
295 survived the CF and CC treatment seem to be more aggressive, with CC treated cells showing
296 the highest proliferation rate after 72 h, when compared to other groups.

297

298 Independent of the magnitude of their cytotoxicity, for all extracts it was observed that their
299 effect on HeLa cells was strongly dependent on the concentration of polyphenols and the
300 length of incubation, suggesting a possible correlation with their pro-oxidant action. Previous
301 reports have suggested that EGCG, a major green tea constituent, at high concentrations

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302 produces intracellular ROS, especially hydrogen peroxide.[34, 35] Our current findings confirm
303 that GT polyphenols induce the generation of ROS in HeLa cells, but the early over-
304 production of superoxide anion radical may be the reason for its high cytotoxic potency. For
305 instance, the less potent CF polyphenols induced similar levels of H_2DCF oxidizing ROS,
306 mainly hydrogen peroxide, but did not induce the generation of superoxide anion radical. It is
307 known that formation of superoxide anion radical precedes the formation of hydrogen
308 peroxide, and these two products of molecular oxygen can in turn react with each other to
309 generate the highly damaging hydroxyl radical.[36] An unbalanced redox environment and
310 mitochondrial dysfunction are important regulators of cell progression to apoptosis.[37, 38] An
311 early depletion of the cellular pool of GSH was detected only after the GT treatment.
312 However, a drop in mTMP was detected after the exposure to all three extracts, especially CF
313 and GT. The decline in mTMP measured after the CC treatment is ROS-independent and
314 could be a consequence of interactions formed between the rigid polymeric flavonoids
315 present in CC and the mitochondrial inner-membrane, as reported before for quercetin and
316 galangin.[39] It should be emphasized that we measured the pro-oxidant response in HeLa cells
317 15 or 30 min after the initiation of treatment with polyphenols, thus generating more accurate
318 results, since the redox state of polyphenols changes rapidly. As an example, Sang et al.[40]
319 observed by mass-spectrometry in real time that the intensity of EGCG ion in Tris-HCl buffer
320 of pH 7.2 decreases due to oxidation, with an approximate half-life of 2 h.
321
322 Several antioxidant enzymes such as SOD and catalase provide a first line of defense against
323 superoxide anion radical and hydrogen peroxide.[41] However, HeLa cells did not down- or up-
324 regulate the expression of antioxidant enzymes during the 12- or 24-hour treatment with
325 extracts. Therefore the partial inhibition of catalase activity by GT polyphenols, observed
326 after 24 h, is independent of changes in gene expression. Slight decrease in catalase activity

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327 with no changes in catalase protein levels was also observed in OSC-4 cells (squamous cell
328 carcinoma line), after the 24-hour treatment with 50 μmol L^{-1} of EGCG.[42] It is possible that
329 phenolic compounds from GT or their cell-modified derivatives inhibit catalase activity, as
330 reported before for maize catalase and *o*-coumaric acid.[43] The decline in catalase activity
331 indicates that GT treated cells are less capable of adapting to hydrogen peroxide produced by
332 both GT polyphenols and normal cell metabolism.
333
334 To further explain the causal connection between the polyphenol pro-oxidant and cytotoxic
335 activity, we incubated HeLa cells for 24 h with a mixture of vitamin C and individual
336 extracts. Vitamin C interfered with the cytotoxic action of GT and CF. Relaying on previous
337 experiments with EGCG and vitamin C,[44] we assume that GT and CF polyphenols efficiently
338 scavenge the ascorbate radicals formed due to vitamin C pro-oxidant action at high
339 concentrations, having as a consequence reduction of polyphenol pro-oxidant activity. On the
340 other hand, CC presumably scavenges ascorbat radicals but the decrease in cytotoxicity is not
341 that pronounced, mainly because of low CC pro-oxidant activity.
342
343 Although we tested different extracts at the same concentration of total phenolic compounds,
344 their polyphenol composition is very different.[20, 21] From our findings, we can conclude that
345 the most potent polyphenols against HeLa cells were those containing both catechol and
346 galloyl moieties (such as EGCG in GT), followed by phenolic acids containing catechol
347 group with an α, β-unsaturated side chain (such as chlorogenic acids in CF), and at the end,
348 by polyphenols with catechol group only (such as catechin or procyanidin in CC). It was
349 shown before that a 5`(3`)-hydroxyl group in the B-ring and pyrogallol structure in a
350 molecule is a minimum requirement for the induction of apoptosis by catechin compounds[45]

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351 and that only galloyl derivatives of catechins inhibit the proliferation of several cancer cell
352 lines.[46]
353
354 **CONCLUSION**
355
356 Although polyphenol compounds influence many cellular signaling pathways, their pro-
357 oxidant action is important for the overall anti-cancer activity. Based on the *in vitro* results
358 from this study, we conclude that green tea and coffee polyphenols may have potential use
359 for the treatment of cervical cancer. Given that the blood concentration of green tea and
360 coffee polyphenols is extremely low in plasma after oral administration,[47] and that they are
361 metabolized as other xenobiotics,[1] their topical administration in further *in vivo* studies may
362 be more suitable as it will allow reaching a very high concentration of polyphenols in their
363 active pro-oxidant form.
364
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370 **REFERENCES**

371

372 1. C. Manach, A. Scalbert, C. Morand, C. Remesy and L. Jimenez, *Am. J. Clin. Nutr.*, 2004,
373 **79**, 727-747.

374

375 2. R. Masella, R. Di Benedetto, R. Vari, C. Filesi and C. Giovannini, *J. Nutr. Biochem.*, 2005,
376 **16**, 577-586.

377

378 3. A. North and C. South, in *Cancer incidence in five continents*, eds. M. P. Curado, B.
379 Edwards, H.R. Shin, H. J. Ferlay, M. Heanue and P. Boyle, IARC Scientific Publications,
380 Lyon, 2007, No. 160.

381

382 4. I. T. Johnson, *Pro. Nutr. Soc.*, 2007, **66**, 207-215.

383

384 5. C. S. Yang, *Nature*, 1997, **389**, 134-135.

385

386 6. G. Y. Yang, J. Liao, K. Kim, E. J. Yurkow and C. S. Yang, *Carcinogenesis*, 1998, **19**, 611-
387 616.

388

389 7. H. Mukhtar and N. Ahmad, *Toxicol. Sci.*, 1999, **52**, 111-117.

390

391 8. M. G. Hertog, D. Kromhout, C. Aravanis, H. Blackburn, R. Buzina, F. Fidanza, S.
392 Giampaoli, A. Jansen, A. Menotti, S. Nedeljkovic, M. Pekkarinen, B.S. Simic, H. Toshima,
393 E. J. M. Feskens, P. C. H. Hollman and M. B. Katan, *Arch. Intern. Med.*, 1995, **155**, 381-386.

RSC Advances Accepted Manuscript

394

395 9. J. D. Lambert and R. J. Elias, *Arch. Biochem. Biophys.*, 2010, **501**, 65-72.

396

397 10. C. Zou, H. Liu, J. M. Feugang, Z. Hao, H. H. Chow and F. Garcia, *Int. J. Gynecol.*

398 *Cancer.*, 2010, **20**, 617-624.

399

400 11. F. Asif Siddiqui, M. Naim and N. Islam, *Diagn. Cytopathol.*, 2011, **39**, 482-488.

401

402 12. W. S. Ahn, J. Yoo, S. W. Huh, C. K. Kim, J. M. Lee, S. E. Namkoong, S. M. Bae and I.

403 P. Lee, *Eur. J. Cancer Prev.*, 2003, **12**, 383-390.

404

405 13. Y. Qiao, J. Cao, L. Xie and X. Shi, *Arch. Pharm. Res.*, 2009, **32**, 1309-1315.

406

407 14. M. Singh, R. Singh, K. Bhui, S. Tyagi, Z. Mahmood and Y. Shukla, *Oncol. Res.*, 2011,

408 **19**, 245-257.

409

410 15. M. Singh, K. Bhui, R. Singh and Y. Shukla, *Life Sci.*, 2013, **93**, 7-16.

411

412 16. Y. Wang, H. Zhang, A. Holmgren, W. Tian and L. Zhong, *Oncol. Rep.*, 2008, **20**, 1479-

413 1487.

414

415 17. A. P. Sommer, D. Zhu and T. Scharnweber, *Photomed. Laser Surg.*, 2010, **28**, 429-430.

416

417 18. M. Yokoyama, M. Noguchi, Y. Nakao, A. Pater and T. Iwasaka, *Gynecol. Oncol.*, 2004,

418 **92**, 197-204.

RSC Advances Accepted Manuscript

419

420 19. J. Bravo, L. Arbillaga, M. P. de Pena and C. Cid, *Food Chem. Toxicol.*, 2013, **60**, 397-
421 403.
422
423 20. Z. Tantoush, D. Apostolovic, B. Kravic, I. Prodic, L. Mihajlovic, D. Stanic-Vucinic and
424 T. Cirkovic Velickovic, *J. Funct. Foods*, 2012, **4**, 650-660.
425
426 21. M. Stojadinovic, J. Radosavljevic, J. Ognjenovic, J. Vesic, I. Prodic, D. Stanic-Vucinic
427 and T. Cirkovic Velickovic, *Food Chem.*, 2013, **136**, 1263-1271.
428
429 22. O. K. Chun, D. O. Kim and C. Y. Lee, *J. Agric. Food Chem.*, 2003, **51**, 8067-8072.
430
431 23. T. Mosmann, *J. Immunol. Methods*, 1983, **65**, 55-63.
432
433 24. P. Pozarowski and Z. Darzynkiewicz, in *Checkpoint controls and cancer*, ed. A.
434 Schönthal, Humana Press, New York, 2004, pp. 301-311.
435
436 25. C. R. Parish, *Immunol. Cell Biol.*, 1999, **77**, 499-508.
437
438 26. M.-L. Kuo, A. J. Sy, L. Xue, M. Chi, M. T. C. Lee, T. Yen, M.-I. Chiang, L. Chang, P.
439 Chu and Y. Yen, *Sci. Rep.*, 2012, **2**, 822.
440
441 27. W. O. Carter, P. K. Narayanan and J. P. Robinson, *J. Leukoc. Biol.*, 1994, **55**, 253-258.
442

RSC Advances Accepted Manuscript

443 28. R. Franco, M. I. Panayiotidis and J. A. Cidlowski, *J. Biol. Chem.*, 2007, **282**, 30452-
444 30465.
445
446 29. T.-J. Hsieh, T.-Z. Liu, Y.-C. Chia, C.-L. Chern, F.-J. Lu, M.-c. Chuang, S.-Y. Mau, S.-H.
447 Chen, Y.-H. Syu and C.-H. Chen, *Food Chem. Toxicol*., 2004, **42**, 843-850.
448
449 30. P. X. Petit, H. Lecoeur, E. Zorn, C. Dauguet, B. Mignotte and M. L. Gougeon, *J. Cell*
450 *Biol.*, 1995, **130**, 157-167.
451
452 31. K. J. Livak and T. D. Schmittgen, *Methods*, 2001, **25**, 402-408.
453
454 32. A. Clairborne, in *Handbook of methods for oxygen radical research*, ed. R. A.
455 Greenwald, CRC Press, Boca Raton, 1985, pp. 283-284.
456
457 33. C. J. Weydert and J. J. Cullen, *Nat. Protoc.*, 2010, **5**, 51-66.
458
459 34. G.-Y. Yang, J. Liao, C. Li, J. Chung, E. J. Yurkow, C.-T. Ho and C. S. Yang,
460 *Carcinogenesis*, 2000, **21**, 2035-2039.
461
462 35. W. Li, S. Nie, Q. Yu and M. Xie, *J. Agric. Food. Chem.*, 2009, **57**, 6685-6691.
463
464 36. B. Halliwell and J. M. C. Gutteridge (eds.), *Free radicals in biology and medicine*,
465 Clarendon Press, Oxford, 3rd edn., 1999.
466
467 37. S. Pervaiz and M.-V. Clément, *Biochem. Biophys. Res. Commun.*, 2002, **290**, 1145-1150.

RSC Advances Accepted Manuscript

468

469 38. S. Desagher and J. C. Martinou, *Trends. Cell Biol.*, 2000, **10**, 369-377.

470

471 39. D. J. Dorta, A. A. Pigoso, F. E. Mingatto, T. Rodrigues, I. M. R. Prado, A. F. C. Helena,
472 S. A. Uyemura, A. C. Santos and C. Curti, *Chem. Biol. Interact*., 2005, **152**, 67-78.

473

474 40. S. Sang, I. Yang, B. Buckley, C. T. Ho and C. S. Yang, *Free Radic. Biol. Med.*, 2007, **43**,
475 362-371.

476

477 41. J. M. Mates, C. Perez-Gomez and I. Nunez de Castro, *Clin. Biochem.*, 1999, **32**, 595-603.

478

479 42. T. Yamamoto, J. Lewis, J. Wataha, D. Dickinson, B. Singh, W. B. Bollag, E. Ueta, T.
480 Osaki, M. Athar, G. Schuster and S. Hsu, *J. Pharmacol. Exp. Ther.*, 2004, **308**, 317-323.

481

482 43. E. Horváth, T. Janda, G. Szalai and E. Páldi, *Plant Science*, 2002, **163**, 1129-1135.

483

484 44. H. Sakagami, H. Arakawa, M. Maeda, K. Satoh, T. Kadofuku, K. Fukuchi and K. Gomi,
485 *Anticancer Res*, 2001, **21**, 2633-2641.

486

487 45. K. Saeki, S. Hayakawa, M. Isemura and T. Miyase, *Phytochemistry*, 2000, **53**, 391-394.

488

489 46. N. P. Seeram, Y. Zhang and M. G. Nair, *Nutr. Cancer*, 2003, **46**, 101-106.

490

491 47. G. Williamson, F. Dionisi and M. Renouf, *Mol. Nutr. Food Res.*, 2011, **55**, 864-873.

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492 **FIGURE CAPTIONS**

493

494 **Figure 1.** Vitality and cell morphology in the presence of polyphenols. (A) Decrease of HeLa
495 cells viability after 24 and 72 h of incubation with GT, CF and CC at concentrations ranging
496 from 0 of 200 µg GAE mL^{-1}. Viability was examined by MTT assay; the viability of
497 untreated cells was taken as 100%. (B) HeLa cell morphology after 24 h of incubation with
498 100 µg GAE mL^{-1} of extracts, as assessed by light inverted microscopy. One representative
499 experiment out of three is shown.

500

501 **Figure 2.** Distribution of cell cycle phases and DNA content in HeLa cells after the treatment
502 with 100 µg GAE mL^{-1} of polyphenol extracts for 24 h (A) and 48 h (B). The differences
503 between the treated and control groups were considered statistically significant if $p<0.05$ (*),
504 $p<0.005$ (**) or $p<0.001$ (***). In addition, representative histograms for control and GT
505 treated groups are shown.

506

507 **Figure 3.** HeLa cell apoptosis after 48 h of incubation with 100 µg GAE mL^{-1} of polyphenol
508 extracts. (A) One selected cell gating profile out of three. B) The histogram showing
509 percentage of viable (Annexin V-PI-), early apoptotic (Annexin V+ PI-) and dead (late
510 apoptotic and necrotic, Annexin V+PI+) cells in the control and treated groups. The
511 differences between the treated and control groups were considered statistically significant if
512 $p<0.05$ (*), $p<0.005$ (**) or $p<0.001$ (***).

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514 **Figure 4.** Proliferation of HeLa cells in the presence of 100 μg GAE mL^{-1} of polyphenol
515 extracts, as measured by CFSE dye dilution method after 72 h of treatment and further
516 analyzed in ModFit LT software. Representative histograms are presented with the calculated
517 proliferating cell index (PCI) and the percentage of proliferating cell fraction (PCF).
518 Gaussian distribution curves were fitted to detect cells in different generations, from parent to
519 daughter generation number 5.
520
521 **Figure 5.** The intracellular level of ROS (including hydrogen peroxide, hydroxyl radical,
522 peroxyl radical and peroxynitrite anion), superoxide anion radical ($O_2^{\bullet-}$), reduced glutathione
523 (GSH) and mitochondrial transmembrane potential (mTMP) after short exposure (15 min for
524 $O_2^{\bullet-}$, GSH and mTMP or 30 min for ROS) to 100 μg GAE mL^{-1} of GT, CF or CC. The
525 differences between the treated and control values were considered statistically significant if
526 $p<0.05$ (*), $p<0.005$ (**) or $p<0.001$ (***).
527
528 **Figure 6.** Changes in antioxidant enzymes after the exposure to polyphenol extracts**.** (A)
529 Relative gene expression of CuZnSOD, MnSOD and catalase after 12 h and 24 h of treatment
530 with GT, CF and CC at concentration of 100 μg GAE mL^{-1}. (B) CuZnSOD in-gel activity and
531 catalase specific activity after 24 h of treatment with 100 μg GAE mL^{-1} of extracts. The
532 differences between the treated and control groups were considered statistically significant if
533 $p<0.05$ (*), $p<0.005$ (**) or $p<0.001$ (***).
534
535 **Figure 7.** To assess the interference of Vitamin C (Vit.C) with the cytotoxic activity of
536 poyphenols, stimulations were carried out for 24 h with 100 μg mL^{-1} of Vit.C and/or 100 μg
537 GAE mL^{-1} of polyphenol extracts. The sum of percentage of growth inhibition obtained for
538 an extract and Vit. C separately (calculated) was compared to the percentage of inhibition

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539 obtained for the extract + Vit. C stimulation (obtained). The differences were considered

540 statistically significant if $p<0.05$ (*), $p<0.005$ (**) or $p<0.001$ (***).

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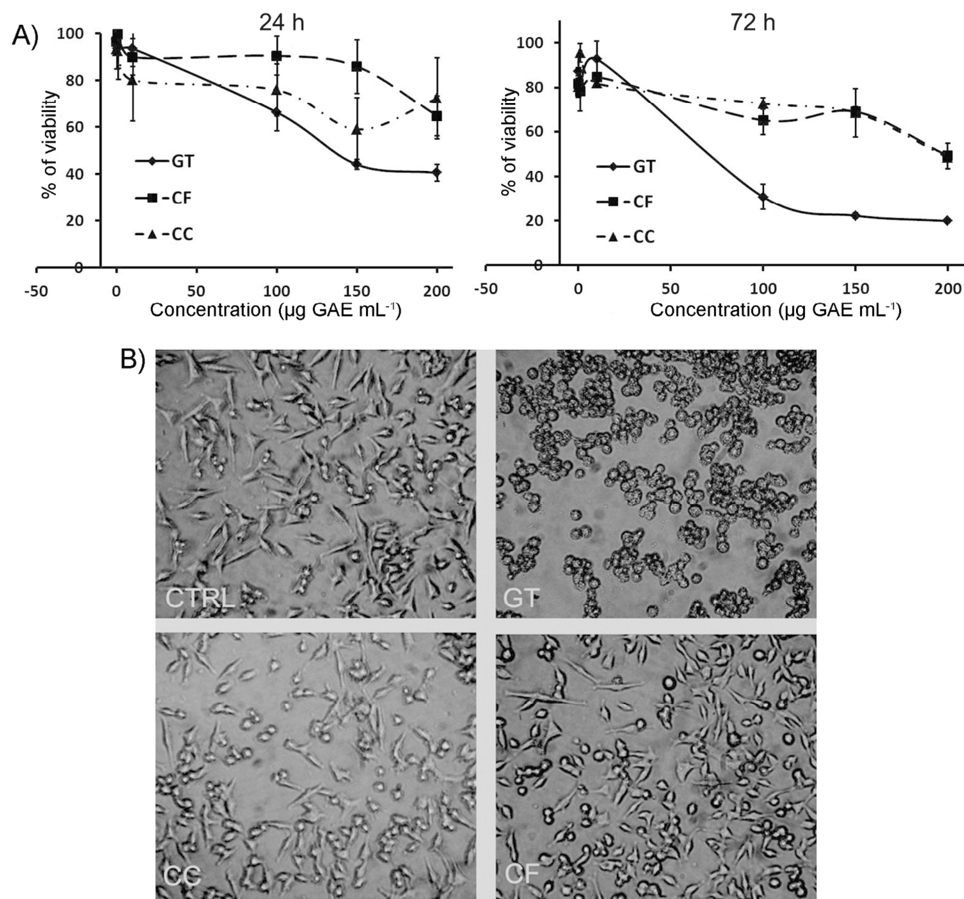

Vitality and cell morphology in the presence of polyphenols. (A) Decrease of HeLa cells viability after 24 and 72 h of incubation with GT, CF and CC at concentrations ranging from 0 of 200 µg GAE mL-1. Viability was examined by MTT assay; the viability of untreated cells was taken as 100%. (B) HeLa cell morphology after 24 h of incubation with 100 µg GAE mL-1 of extracts, as assessed by light inverted microscopy. One representative experiment out of three is shown.
180x162mm (300 x 300 DPI)

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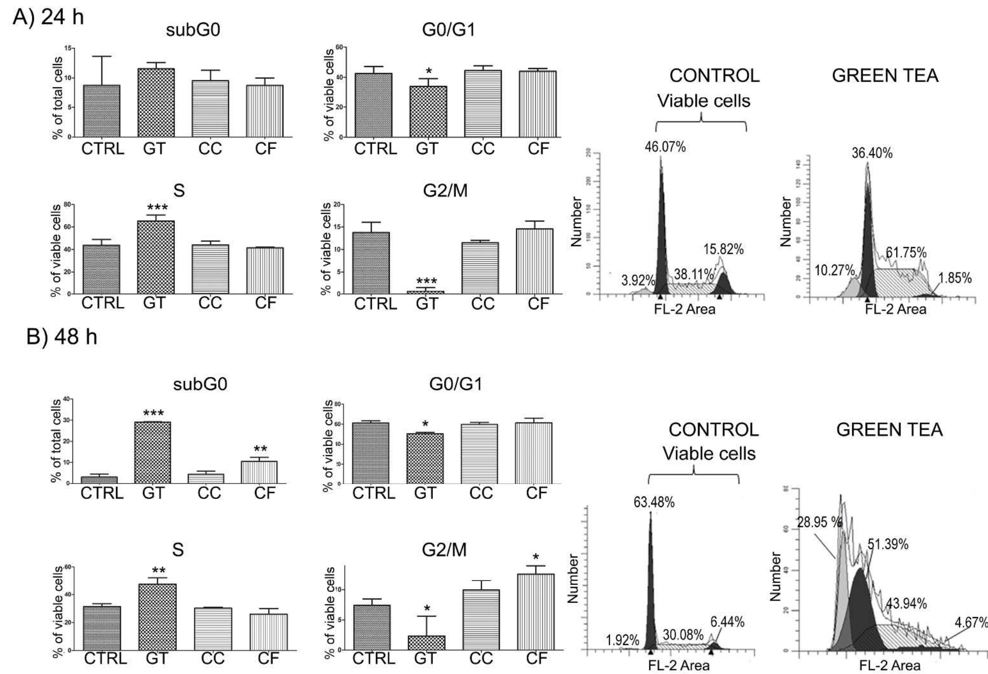

Distribution of cell cycle phases and DNA content in HeLa cells after the treatment with 100 µg GAE mL-1 of polyphenol extracts for 24 h (A) and 48 h (B). The differences between the treated and control groups were considered statistically significant if $p<0.05$ (*), $p<0.005$ (**) or $p<0.001$ (***). In addition, representative histograms for control and GT treated groups are shown.
128x86mm (600 x 600 DPI)

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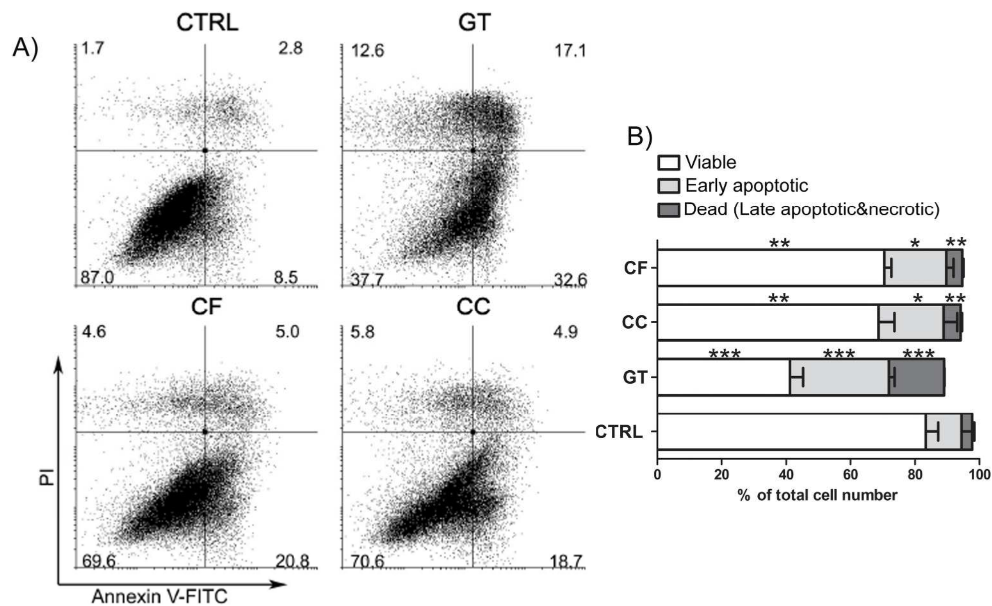

HeLa cell apoptosis after 48 h of incubation with 100 µg GAE mL-1 of polyphenol extracts. (A) One selected cell gating profile out of three. B) The histogram showing percentage of viable (Annexin V-PI-), early apoptotic (Annexin V+ PI-) and dead (late apoptotic and necrotic, Annexin V+PI+) cells in the control and treated groups. The differences between the treated and control groups were considered statistically significant if $p<0.05$ (*), $p<0.005$ (**) or $p<0.001$ (***).
117x72mm (600 x 600 DPI)

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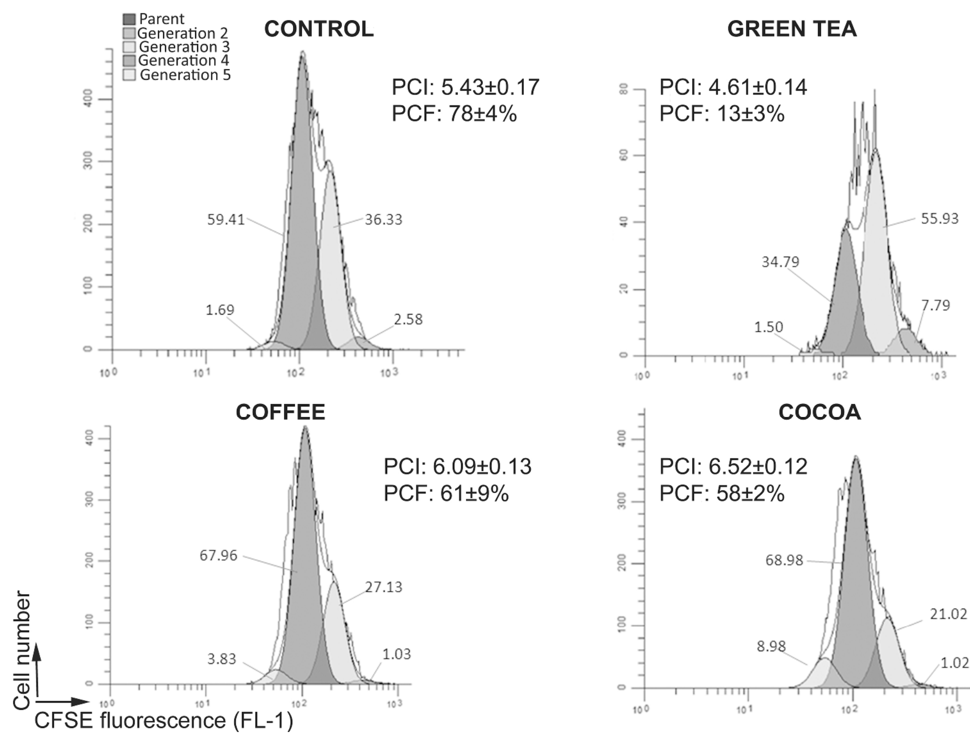

Proliferation of HeLa cells in the presence of 100 µg GAE mL-1 of polyphenol extracts, as measured by CFSE dye dilution method after 72 h of treatment and further analyzed in ModFit LT software. Representative histograms are presented with the calculated proliferating cell index (PCI) and the percentage of proliferating cell fraction (PCF). Gaussian distribution curves were fitted to detect cells in different generations, from parent to daughter generation number 5.
139x102mm (600 x 600 DPI)

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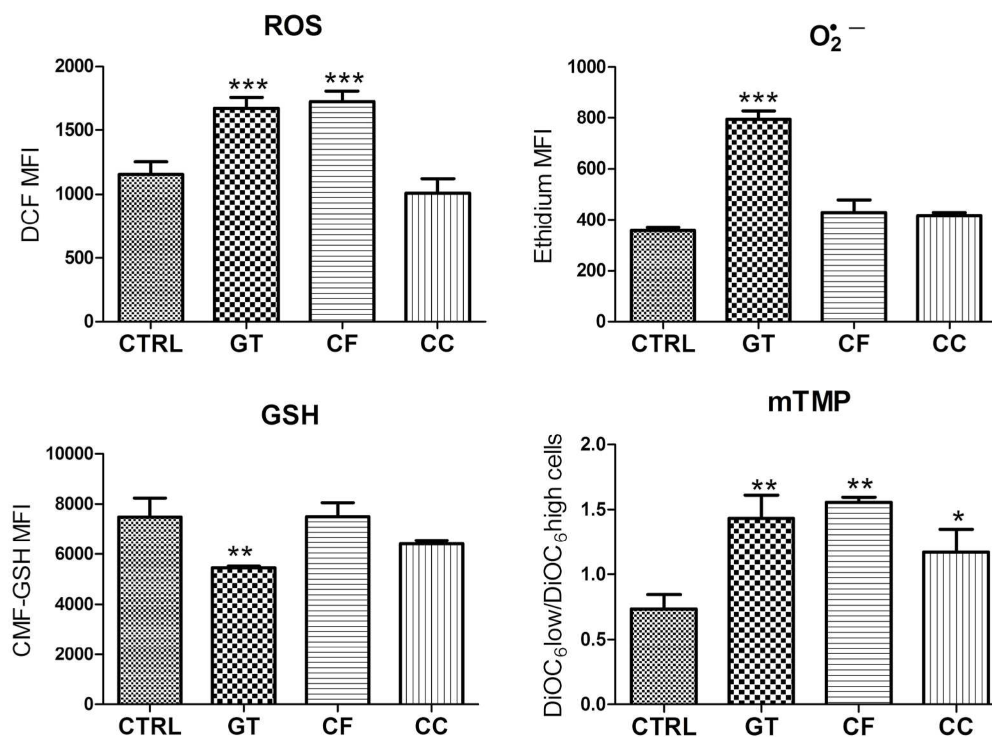

The intracellular level of ROS (including hydrogen peroxide, hydroxyl radical, peroxyl radical and peroxynitrite anion), superoxide anion radical (O2•-), reduced glutathione (GSH) and mitochondrial transmembrane potential (mTMP) after short exposure (15 min for O2•-, GSH and mTMP or 30 min for ROS) to 100 µg GAE mL-1 of GT, CF or CC. The differences between the treated and control values were considered statistically significant if $p<0.05$ (*), $p<0.005$ (**) or $p<0.001$ (***).
104x77mm (600 x 600 DPI)

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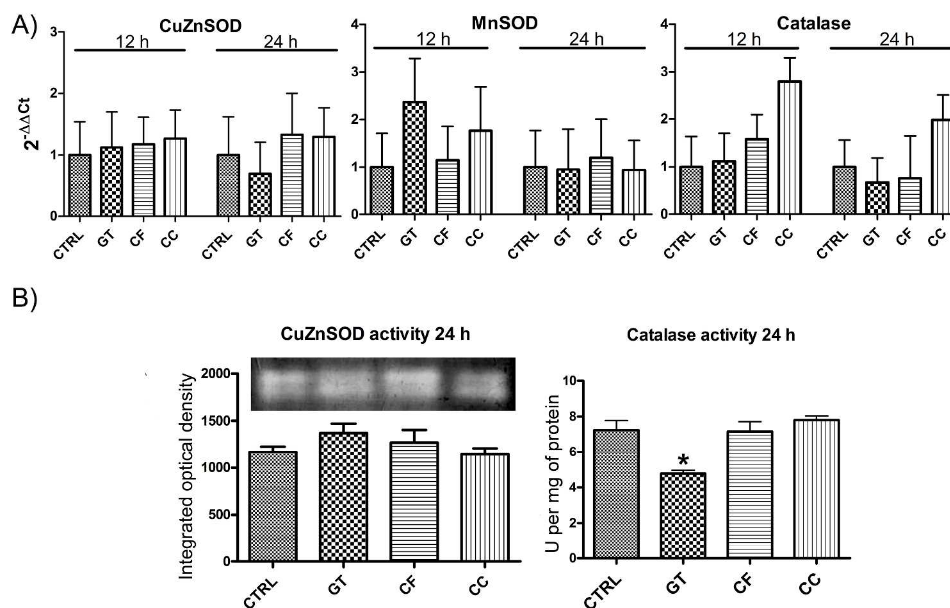

Changes in antioxidant enzymes after the exposure to polyphenol extracts. (A) Relative gene expression of CuZnSOD, MnSOD and catalase after 12 h and 24 h of treatment with GT, CF and CC at concentration of 100 µg GAE mL-1. (B) CuZnSOD in-gel activity and catalase specific activity after 24 h of treatment with 100 µg GAE mL-1 of extracts. The differences between the treated and control groups were considered statistically significant if p<0.05 (*), p<0.005 (**) or p<0.001 (***).
124x81mm (600 x 600 DPI)

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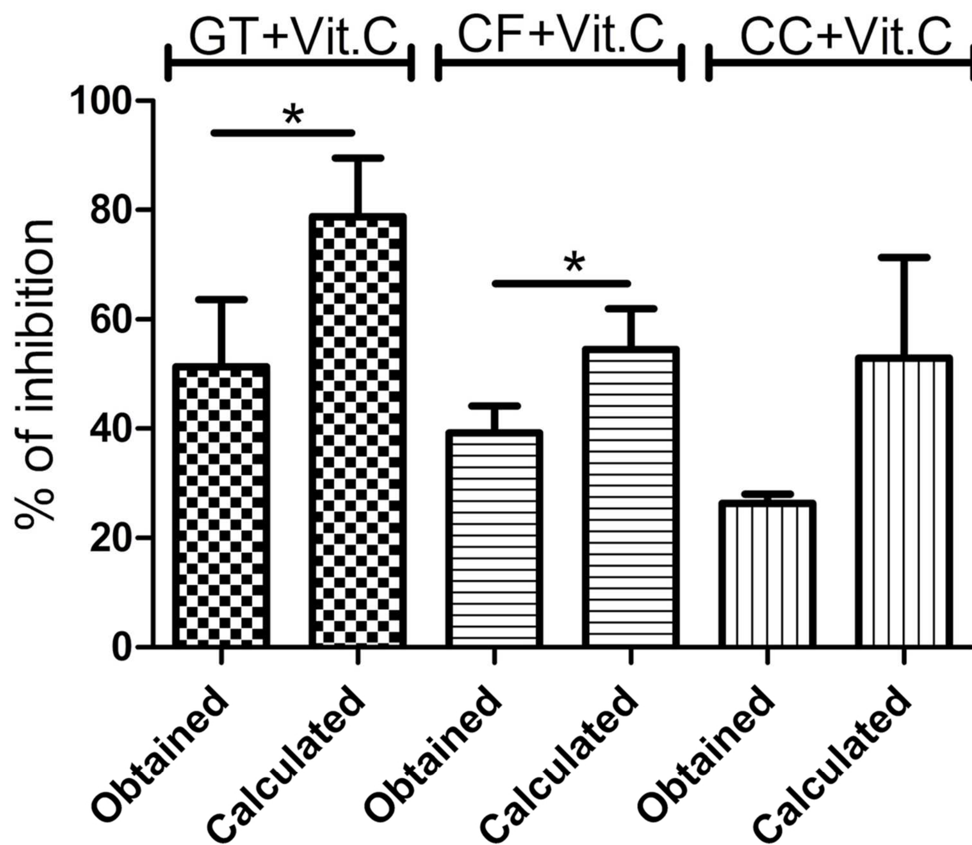

To assess the interference of Vitamin C (Vit.C) with the cytotoxic activity of poyphenols, stimulations were carried out for 24 h with 100 µg mL-1 of Vit.C and/or 100 µg GAE mL-1 of polyphenol extracts. The sum of percentage of growth inhibition obtained for an extract and Vit. C separately (calculated) was compared to the percentage of inhibition obtained for the extract + Vit.C stimulation (obtained). The differences were considered statistically significant if $p<0.05$ (*), $p<0.005$ (**) or $p<0.001$ (***).
52x45mm (600 x 600 DPI)

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