Antiproliferative and cytotoxic effects of green coffee and yerba mate extracts, their main hydroxycinnamic acids, methylxanthine and metabolites in different human cell lines.

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Abstract

This work aimed at studying the effects of green coffee bean (GCBE) and yerba mate (YME) extracts, their main phenolic components (5-caffeoylquinic acid, 5-CQA; 3,5-dicaffeoylquinic acid, 3,5-DCQA) and metabolites (ferulic acid, FA; caffeic acid, CA; dihydrocaffeic acid, DHCA; and dihydroferulic acid, DHFA) along with caffeine (CAF) on the viability and proliferation of different human cell lines. Extracts (10-1000 µg/mL) and standards (10-1000 µM) were assayed in colon (Caco-2), lung (A549), oesophageal (OE-33), urinary bladder (T24) human carcinoma cells, and a non-cancer cell line (CCD-18Co). YME significantly reduced viability of cancer cells at all assayed concentrations, the higher doses also reducing cell proliferation. GCBE effects on cell viability were more effective at 100 and 1000 µg/mL, showing modest effects on cell proliferation. The highest doses of 5-CQA and 3,5-DCQA reduced cell viability and proliferation in all cell lines, whereas FA, DHCA and DHFA had lower, variable effects. Caffeine had no effect. Dietary-attainable concentrations (0.1, 1 and 10 µg/mL) of YME were tested for cytotoxicity and reactive oxygen species generation, showing no cytotoxic effect. Low concentrations of all tested compounds were non-cytotoxic to CCD-18Co cells. Conclusion: YME and to a lower degree GCBE, their phenolic components and metabolites may decrease cancer cell viability and proliferation.

Keywords: cancer, yerba mate, green coffee beans, hydroxycinnamic acid metabolites.

1. Introduction

The consumption of green coffee and yerba mate as dietary supplements or beverages is increasing due to their reported health benefits, since they have been associated with lower risk of suffering diseases of oxidative aetiology (Nkondjock, 2009; Bracesco et al., 2011; Esquivel and Jimenez, 2012). Numerous studies have evaluated the association between coffee consumption and the risk of cancer in humans (Bohn et al., 2014). Certain results indicate that coffee reduces the risk of colon (Giovannucci, 1998), endometrial (Shimazu et al., 2008), liver, kidney, bladder, and breast cancers (Nkondjock, 2009), whereas other reports suggest that coffee consumption increases bladder (Kurahashi et al., 2009), breast, lung (Tang et al., 2009; Tang et al., 2010), and laryngeal cancers (Chen and Long, 2014). In contrast, some studies did not relate coffee to increased incidence of prostate, breast (Bhoo-Pathy et al., 2015), pancreas or ovary cancers (Nkondjock, 2009; Turati et al., 2012).

The relationship between yerba mate and cancer has been much less studied compared with coffee. Mate, a beverage prepared from the leaves of *Ilex paraguariensis* widely consumed in South America, has shown antimutagenic effects in cell culture and animal models (Nkondjock, 2009; Bracesco et al., 2011). However, epidemiological studies have described a link between yerba mate consumption and oral cavity, pharynx, oesophagus, and oropharyngeal cancers (Goldenberg, 2002; Dasanayake et al., 2010). This has been associated to the high temperature mate is traditionally consumed in South America, adding boiling water to mate leaves in a “calabaza” recipient, drinking the hot brew through a metallic straw-like implement called “bombilla” and repeating the process adding more boiling water into the “calabaza” several times. Consumption of mate tea with “bombilla” has also been associated with bladder cancer in ever-smokers, but not in never-smokers, whereas “mate cocido” (leaves infused in hot water, consumed at lower temperatures) was not associated with this type of cancer (Bates et al., 2007). There is no sound population-based, case-control study on mate consumption that establishes yerba mate as a risk factor for cancer (Loria et al., 2009)

Cell culture studies have shown an antiproliferative effect of polyphenols in mate against oral cancer cells, inhibiting topoisomerases I and II (Gonzalez de Mejia et al., 2005), in addition to showing an increase in apoptosis and necrosis in human lymphocytes (Wnuk et al., 2009) or a protective effect against DNA damage induced by H₂O₂ in liver, kidney and bladder cells isolated from mice (Miranda et al., 2008). This effect was also observed in Caco-2 cells after incubation with chlorogenic acid, an

extract of green coffee or bread supplemented with antioxidants from green coffee (Glei et al., 2006). The cell viability reduction and induction of apoptosis in tumour cells after treatment with hydroxycinnamic acids (Miccadei et al., 2008) was related to their antioxidant activity, increasing the activity and expression of antioxidant enzymes in lung and skin tumour cells, suppressing cell signals involved in survival and proliferation (NF- κ B, AP-1 and MAPK) (Feng et al., 2005), although in another cell model (human hepatoma cells) such effects were not observed (Granado-Serrano et al., 2006).

The main phenolic compounds present in yerba mate and green coffee are hydroxycinnamic acids, which may amount up to 8.1-9.8% of the yerba mate dry weight (Bravo et al., 2007) and to 4.1-11.3% (w/w) of the coffee bean (Baeza et al., 2016b). Major hydroxycinnamic acids described in these products are 3-, 4-, and 5-caffeoylquinic acids (3-, 4-, and 5-CQA), 3,4-, 3,5-, and 4,5-dicaffeoylquinic acids (3,4, 3,5-, and 4,5-DCQA), and 3-, 4-, and 5-feruloylquinic acids (3-, 4-, and 5-FQA), among others (Bravo et al., 2007; Baeza et al., 2016b). Yerba mate and green coffee have also in common a high content of the trimethylxanthine caffeine. The bioavailability and biotransformation of hydroxycinnamic acids and caffeine have been addressed in *in vitro* and *in vivo*, including human studies (Mateos et al., 2006; Stalmach et al., 2010; Martínez-López et al., 2014; Gomez-Juaristi, 2015). These compounds are absorbed, metabolised and modified by the colonic microbiota. Derived CQAs, FQAs and caffeine metabolites have been reported to reach maximum plasma concentrations of 143 nM, 158 nM, and 10.50 μ M, respectively, with urine concentrations of 11.1 μ M, 40.3 μ M, and 4.12 μ M, respectively (Stalmach et al., 2010; Martínez-López et al., 2014; Gomez-Juaristi, 2015). Dihydrocaffeic acid (DHCA) and dihydroferulic acid (DHFA) are major microbial metabolites found in plasma and urine derived from CQAs and FQAs. Maximum concentration (C_{max}) of DHFA and DHCA in plasma of 0.878 μ M and 0.678 μ M, respectively, have been described 8 h after coffee consumption (Lang et al., 2013), with C_{max} of 0.306 μ M and 0.027 μ M found in plasma around 7 h after mate intake, respectively (Gomez-Juaristi, 2015).

Free caffeic (CA) and ferulic (FA) acids can be minor components in coffee and mate, although they are more commonly found esterified to quinic acid, glycosylated, etc. (Baeza et al., 2016b). However, they are also formed after absorption of the corresponding parent molecules due to the action of phase I metabolizing enzymes (i.e. esterases, glycosidases), and further metabolized by phase II enzymes (sulphated, glucuronidated, methylated, etc.). Plasma concentrations of metabolites in the nM

range have been determined after the ingestion of coffee (45 nM) and yerba mate (65 nM) (Gomez-Juaristi, 2015).

The aforementioned discrepancies on the effect of coffee and yerba mate in cancer may be due to the bioactive components originally present in the beverages and/or to the molecules that are formed after being absorbed, metabolised and modified by the colonic microbiota. Therefore, to further understand the association between coffee and yerba mate with cancer, the aim of this study was to assess the effects of phenolic extracts of coffee and yerba mate, their main compounds and resulting metabolites on the viability and proliferation of colon (Caco-2), lung (A549), oesophagus (OE-33) and urinary bladder (T24) cancer cell lines, as well as in a non-cancer cell line (CCD-18Co).

2. Material and methods

2.1 Reagents

Green coffee (*Coffea arabica* L.) from Colombia, and yerba mate (*Ilex paraguariensis* St. Hil.), from Argentina, were purchased in a local supermarket in Madrid (Spain). Gentamicin, penicillin, streptomycin, non-essential amino acids (NEAA), McCoy's 5A medium, and minimum essential culture medium (MEM) were acquired from Sigma-Aldrich (Madrid, Spain). Fetal bovine serum (FBS), Dulbecco's modified eagle's medium (DMEM)-F12 and RPMI 1640 with glutamine were from Biowhitaker Europe (Lonza, Madrid, Spain). Cell proliferation ELISA 5-bromo-2'-deoxyuridine (BrdU) kit and cell viability reagent WST-1 were purchased from Roche Diagnostics (Barcelona, Spain). Protein assay reagent was from Bio-Rad Laboratories (Madrid, Spain). CA, FA, 5-CQA, DHCA, DHFA, caffeine, and dimethylsulphoxide (DMSO) were from Sigma-Aldrich (Madrid, Spain). 3,5-DCQA was from Phytolab (Vestenbergsgreuth, Germany). Human lung carcinoma A549, human colorectal adenocarcinoma Caco-2, human oesophageal carcinoma OE-33, human urinary bladder carcinoma T24, non-cancer fibroblastic cell line CCD-18Co were supplied by the Cell Bank of the Scientific Instrumentation Centre at the University of Granada (CIC-UGR, Granada, Spain).

2.2 Extraction of phenolic compounds from green coffee and yerba mate

Green coffee beans were first milled in a domestic coffee grinder and then in a mill to a final particle size of 0.5 µm. Yerba mate was used without further milling or selection of leaves (the mate contained minor amounts of stems). Phenolic compounds were

extracted in triplicate by organic solvent extraction following the protocol described by Bravo and Saura-Calixto (1998). Briefly, 1 g of sample was mixed with 40 mL of 2N HCl in aqueous methanol (50/50, v/v) using an orbital shaker at room temperature for 60 min, and centrifuged (3000 rpm, room temperature, 10 min). The supernatant was collected and the pellet was re-extracted with 40 mL of 70% (v/v) acetone by orbital shaking at room temperature for 60 min and centrifuged (3000 rpm, room temperature, 10 min). The supernatant was collected and combined with the former.

The extracts were analysed by HPLC-DAD using an Agilent 1200 series equipment (Agilent Technologies, Germany) using a Superspher 100 RP column (4.6 x 250 mm, 4 μ m, Agilent Technologies) with gradient elution using a ternary system consisting of 1% formic acid, methanol and acetonitrile at a constant flow rate of 1 mL/min at 30°C. The total phenolic content of the extracts amounted to 64.1 mg/g (dry matter) in the green coffee beans and 84.9 mg/g in yerba mate. Caffeine was the main methylxanthine in both extracts, amounting to 12.6 mg/g in the green coffee beans and 7.2 mg/g in the yerba mate leaves. The main phenolic compounds were monocaffeoylquinic acids (85% and 64% of the total phenolic content in GCBE and YME, respectively), with lower amounts of dicaffeoylquinic acids (8% and 19% in GCBE and YME, respectively), and feruloylquinic acids (7% and 2%, respectively). Mate also contained up to 9% flavanol-glycosides. Detailed description of the phenolic and methylxanthine composition of the extracts can be found elsewhere (Baeza et al., 2016b; Mateos et al., 2017).

The green coffee bean extract (GCBE) and yerba mate extract (YME) were evaporated in a rotary evaporator and freeze dried. The powdered samples were homogenized with a mortar and pestle, dissolved in 100 mg/mL stock solutions in 1% (v/v) DMSO and stored in aliquots at -20°C for cell experiments. Pure standards (CA, FA, 5-CQA, 3,5-DCQA, DHFA, DHCA and caffeine) were dissolved in 1% (v/v) DMSO in deionized water. Extracts and pure standards were diluted with serum free medium to prepare the different test solutions employed with cell cultures (final concentration of DMSO in the cell culture was 0.1%). All samples were filtered through 0.45 μ m sterile filters.

2.3 Effects of green coffee and yerba mate extracts and phenolic compounds on human cancer cell lines

CCD-18Co cell line was incubated in MEM supplemented with 10% FBS, sodium bicarbonate (1.5 g/L), 1 mM sodium pyruvate, and 0.1 mM NEAA; A549 and Caco-2 cells were incubated in DMEM F-12 medium supplemented with 10% FBS; T24 cell line

was grown in McCoy's 5A medium supplemented with 10% FBS; OE-33 cells were incubated in RPMI 1640 with glutamine medium supplemented with 10% FBS. All culture media were also supplemented with 50 mg/L of antibiotics (gentamicin, penicillin and streptomycin). Cells were grown in 100 mm culture dishes in a humidified atmosphere of 5% CO₂ and 95% air at 37°C.

In the viability and proliferation assays, the tested concentrations ranged from 10 to 1000 µg/mL for GCBE and YME, and from 10 to 1000 µM for the pure standards, in order to study the effect of physiological and supra-physiological doses, simulating a situation of normal vs. overconsumption or pharmacological use, respectively. The exposure to the studied compounds and GCBE and YME during 2 and 24 h was established to mimic acute and chronic consumption, respectively.

Further studies of cytotoxicity (LDH leakage) and reactive oxygen species (ROS) production were also carried out in the non-cancer cells with lower doses of YME (0.1-10 µg/mL), 5-CQA, 3,5-DCQA, DHCA, and CA (0.1-10 µM), and for all the studied cells with YME (0.1-10 µg/mL). Control cells were treated with 0.1% of DMSO in comparison with treated cells. The day before the experiments, cells were changed to FBS-free medium to avoid potential FBS interference with the assays.

2.3.1 Evaluation of cell viability (WST-1 assay)

The colorimetric WST-1 reagent was used to test cell viability following manufacturer's instructions. This method is based on the reduction by viable cells of WST-1 reagent by NAD-dependent dehydrogenase activity to the water-soluble coloured formazan. The amount of dye formed directly correlates to the number of viable cells. Cells were seeded (100 µL, 4 x 10³ cells/well) in 96-well plates. After 24 h of incubation, the medium was removed and cells were treated with 100 µL of the different concentrations of the extracts and pure compounds for 2 or 24 h. Then, 10 µL of WST-1 reagent were added into the wells and the plates were incubated for 1 h. The absorbance at 440 nm was measured using a Bio-Tek microplate reader. Controls were prepared by adding culture medium or sample plus WST-1 reactive in the absence of cells. Results are expressed as percentage of cell viability referred to the absorbance measured in control cells.

2.3.2 Cell viability and cell proliferation tests (BrdU assay)

To study cell proliferation, the BrdU colorimetric assay was used following manufacturer's instructions. This method is based on the incorporation of the pyridine

analogue 5-bromo-2'-deoxyuridine (BrdU) instead of thymidine into the DNA of proliferating cells and its quantification by direct ELISA with anti-BrdU antibodies. Cells were seeded at 10^4 cells per well (100 μ L) in 96-well microplates, incubated for 24 h for attachment, and the different compounds were added for 2 or 24 h prior to assessing cell proliferation. The absorbance was measured at 370 nm. Results are expressed as a percentage of cell proliferation referred to the absorbance determined in untreated control cells.

2.3.3 Cell cytotoxicity (LDH assay)

Cytotoxicity was determined by the lactate dehydrogenase (LDH) assay (Welder and Acosta, 1994). Leakage of intracellular LDH to the culture medium indicates cell damage. Cells (1.5×10^6 cells per plate) were seeded in 60 mm diameter plates and treated with 3 mL of the different concentrations of extracts and compounds for 2 and 24 h as indicated above (section 2.3). After treatment, the culture medium was collected; separately, the cells were scraped in 3 mL of PBS, sonicated to ensure the breakdown of the cell membrane and the release of the total amount of LDH, and centrifuged at 420 g at 4°C for 10 min. To determine LDH levels, 200 μ L of 1.35 M Tris, 0.08 M pyruvate and 2 mg/mL NADH were added to 40 μ L of culture medium or supernatant cell content in a 96 well plate and the absorbance at 340 nm was immediately read in a microplate reader for 5 min. The percentage of LDH leakage was estimated from the ratio between the LDH activity in the culture medium and that of the whole cell content.

2.3.4 Determination of ROS production

Cellular ROS were quantified by the dichlorofluorescein (DCF) assay (Alia et al., 2006), which is based on the measurement of the fluorescence produced by oxidation of the fluorescent probe DCFH-DA due to reactive oxygen species. Briefly, cells were seeded in 24-well plates (2×10^5 cells per well) in medium containing FBS and replaced with the FBS-free medium the following day. Different concentrations of extracts and compounds were treated in the cells as indicated above. After treatment, 10 μ L of DCFH-DA (10 μ M in DMSO) were added to the wells and cells were incubated at 37°C for 30 min in order that DCFH-DA could penetrate into the cells. Then, cells were washed with PBS (0.5 mL) and placed in fresh FBS-free medium with the different concentrations of extracts and compounds. ROS production was monitored for 60 min by measuring the fluorescence in a microplate reader at 485 and 530 nm excitation and emission wavelengths, respectively.

2.4. Statistical analysis

SPSS version 19.0 program for Windows (SPSS Inc., Chicago, Ill., U.S.A.) was used for the statistical analysis of data. Homogeneity of variances was checked by the test of Levene. Multiple comparisons within the different treatments were carried out using One-way ANOVA followed by Bonferroni test when variances were homogeneous or by the Games-Howell test when variances were not homogeneous. Differences were considered as statistically significant when $p < 0.05$.

3. Results

3.1 Effects of extracts, polyphenols and metabolites on cell viability and proliferation

YME reduced cell viability to a higher degree than GCBE (Table 1). Treatment for 2 and 24 h with YME induced a patent negative dose-dependent effect on cell viability in all the studied cell lines. GCBE also decreased cell viability, although contrary to YME, the lowest concentration (10 $\mu\text{g/mL}$) only showed differences with respect to the control after 24 h of treatment in OE-33 and T24 cancer cells. Even 100 $\mu\text{g/mL}$ GCBE showed modest effects and only the highest dose (1000 $\mu\text{g/mL}$) affected cell viability with reductions between 87% (CCD-18Co, 2h) to only 22% (Caco-2, 24 h) with respect to control cells (Table 1).

While cell viability determines the capacity of cells to survive in the presence of the tested extracts or compounds, cell proliferation provides evidences of the capacity of the cells to grow and multiply, which is of especial relevance in cancer cells with enhanced proliferation capacity. Short treatment with GCBE did not modify the proliferation of A549 and OE-33 cancer cells, although it decreased proliferation in Caco-2 and bladder T24 cells (in the latter only the highest concentrations). At longer incubation time (24 h), only the highest levels of GCBE were able to reduce proliferation of all but OE-33 cells, also affecting non-cancerous CCD-18Co cells (Table 1). As for YME, the highest concentration (1000 $\mu\text{g/mL}$) was able to significantly reduce cell proliferation in all cell lines after 2 h, totally blocking proliferation of CCD-18Co and T24 cells. The 100 $\mu\text{g/mL}$ dose also decreased proliferation of OE-33 and T24 cells, while Caco-2 cells, as with GCBE, were affected by all YME concentrations after 2 h. Treatment with YME for 24 h dose-dependently decreased cell proliferation in all cell

lines (except Caco-2 and OE-33, where the 10 µg/mL dose was not effective) (Table 1).

Treatment for 2 and 24 h with 100 and 1000 µM 5-CQA dose-dependently reduced cell viability in both non-cancer and cancer cell lines (Table 2). The 10 µM dose, however, only affected viability of OE-33 cells (and CCD-18Co) at 2 h incubation time, surprisingly increasing the viability of A549 cells. This, however, was not accompanied by an increased proliferation, since only the 1000 µM dose of 5-CQA was able to decrease proliferation of CCD-18Co and T24 cells at short incubation times, also decreasing proliferation of all cell lines after 24 h treatment, except A549, which again showed a different response with higher proliferation after 24 h with 100 µM 5-CQA (Table 2).

As with the monocaffeoylquinic acid, 3,5-DCQA dose-dependently reduced viability of all cell lines after 2 and 24 h incubation, but only with the highest concentrations. This compound showed some effects decreasing cell proliferation of cancer cells at 2 and 24 h treatment with the highest doses. Only Caco-2 and T24 cells showed a slight yet statistically significant reduction of cell proliferation after 24 h treatment with 10 µM 3,5-DCQA (Table 2).

Concerning CA and FA, both compounds were less effective than the previous caffeoylquinic esters, since in general only the highest dose tested reduced cell viability (Table 3). Only OE-33 cells were slightly more susceptible, with 100 µM CA also decreasing cell viability and, interestingly, 10 µM FA at short incubation time. Normal, non-cancer cells were also affected by CA, showing a reduced viability after 2 h incubation with 10 µM CA. However, low doses of CA had no antiproliferative effects in any of the studied cell lines, except in T24 cells after treatment for 24 h. On the contrary, FA showed decreased proliferation of all the cancer cell lines, with the 10 µM concentration having a significant antiproliferative effect in Caco-2 and A549 cells after 2 h, and in OE-33 and T24 at longer incubation time (24 h), pointing to an increased antiproliferative effect of the methylated molecule as compared to unmethylated CA.

The microbial metabolites DHCA and DHFA also showed a limited activity reducing cell viability since, in general, only the highest concentrations decreased the viability of all cell lines (Table 4). Exceptionally, OE-33 cells showed an apparent increased viability with 10-100 µM DHCA after 24 h incubation, the 100 µM dose also increasing cell proliferation. Low concentrations of DHCA (10 µM) decreased proliferation of Caco-2 and A549 cells, also affecting normal CCD-18Co cells. Contrary to FA, the reduced

methyated molecule DHFA only had a significant antiproliferative effect on urinary bladder T24 cells (Table 4).

Both coffee and yerba mate contain caffeine in relevant amounts (7.2 mg/g in mate and 12.6 mg/g coffee; Baeza et al., 2016b; Mateos et al., 2017), which is present in the tested extracts. Therefore, the potential antiproliferative effect of this methylxanthine was also examined. Only the highest concentration showed some effect reducing cell viability in all but Caco-2 cells, also having a modest antiproliferative effect in A549 and T24 cells after incubation for 24 h (Table 5). However, the 10 μ M dose, a concentration found in plasma after consumption of a single cup of coffee (Martinez-Lopez et al., 2014), had no effect on cell viability nor proliferation.

As it can be seen, all the four cancer cell lines showed different response to treatments with GCBE, YME and the major phenolic constituents and metabolites. As a summary, looking only at their response to the physiologically plausible concentration of 10 μ g/mL or 10 μ M, in terms of cell viability OE-33 were more susceptible, with GCBE, YME, 5-CQA, FA, and DHCA decreasing the viability of this oesophageal cancer cell line. However, only FA showed antiproliferative effect in these cells. The other cell lines were less susceptible, with only YME and, at the most GCBE (in T24 cells), reducing cell viability (Table 6). When considering cell proliferation, however, A549 was affected by YME, FA and DHCA, but interestingly proliferation of Caco-2 and T24 cells could be significantly decreased by up to five out of the eight extracts/phenolic compounds tested. Thus, Caco-2 cells were susceptible to 10 μ g/mL GCBE and YME, and 10 μ M 3,5-DCQA, FA, and DHCA, while T24 cells were affected by 10 μ g/mL YME, and 10 μ M 3,5-DCQA, CA, FA, and DHFA (Table 6).

A similar analysis of the results show that YME was the most active compound, the 10 μ g/mL dose reducing viability of all the tested cancer cell lines and decreasing proliferation of all but OE-33 cell lines. Among the pure polyphenols, FA seemed the most active, with antiproliferative effects in the four cancer cell lines. However, while the monocaffeoylquinic acid 5-CQA had no antiproliferative action, 3,5-DCQA showed antiproliferative effect in Caco-2 and T24 cells (Table 6).

It is interesting noting the different activity of CA, FA and their reduced microbial metabolites DHCA and DHFA (Figure 1). As just mentioned, 10 μ M FA reduced proliferation of all cancer cells, also decreasing OE-33 cell viability; actually, it was the only compound inhibiting proliferation of this cell line (Table 6). In contrast, the non-methoxylated molecule, CA, only decreased proliferation of T24 cells. On the contrary,

reduction of the molecule by the microflora led to a DHFA molecule that only affected proliferation of T24 cells, whilst DHCA showed antiproliferative effect in Caco-2 and A549 cells, also decreasing viability of OE-33 cells.

Finally, caffeine only decreased proliferation of A549 cells at short and long incubation times, not affecting cell viability and proliferation of the other cell lines (Table 6). However, some test compounds also affected the non-cancer cell line CCD-18Co. Only YME and DHCA reduced the proliferation of these fibroblast colonic cells, although YME, 5-CQA, CA, and DHCA decreased cell viability at the 10 µg/mL or 10 µM dose (Table 6). Therefore, we studied the potential cytotoxicity of these compounds at lower concentrations in CCD-18Co.

3.2 Cytotoxicity and antioxidant effect in CCD-18Co cells

In order to further understand the potential negative effects observed in CCD-18Co cells with the lowest dose of the tested compounds, cytotoxicity of YME (0.1, 1, and 10 µg/mL) and 5-CQA, CA, and DHCA (0.1, 1 and 10 µM) after 2 and 24 h treatment was evaluated by the LDH method. The percentage of LDH indicates the cytotoxicity of the test compounds, so that the higher the values with respect to the control, the higher the cytotoxicity. None of the compounds induced cytotoxicity in CCD-18Co cells. On the contrary, a cytoprotective effect was observed when cells were incubated with 1 and 10 µg/mL YME, with significant reduction of LDH leakage from damaged cells (about 20% and 10% at 1 µg/mL, and 35% and 10% at 10 µg/mL after 2 and 24 h incubation, respectively) (Figure 2). Accordingly, treatment with 10 µM 5-CQA induced a reduction in LDH leakage of 15% and 7%, at 2 and 24 h, respectively, with a similar reduction after 2 h with 1 µM 5-CQA. CA at the three concentrations studied induced a reduction in %LDH after 2 h but not at longer incubation time (24 h). When CCD-18Co cells were treated with DHCA for 2 h, there was a reduction in cytotoxicity although only the effect of the lowest concentration (0.1 µM DHCA) reached statistical significance.

Treatment of CCD-18Co cells with YME resulted in a decrease in the generation of reactive oxygen species (ROS) with the 1 and 10 µg/mL doses, while 0.1 µg/mL had no effect (Figure 3A). Similarly, 0.1 µM 5-CQA, CA, and DHCA had no effect on ROS levels, while the higher concentrations were able to significantly decrease ROS generation from 39-72% (with respect to basal conditions) after 2 h.

3.3 Effect of YME on cell cytotoxicity and ROS production

Since 10 µg/mL YME was very effective decreasing cell viability and proliferation, the cytotoxic effect of lower concentrations of this extract was also studied. Cytotoxicity experiments using the LDH assay were also carried out with physiological doses (0.1-10 µg/mL) of YME in the four cancer cell lines (Figure 4). After 2 h treatment, YME only induced a slight yet statistically significant cytotoxic effect in OE-33 cells at 1 and 10 µg/mL of YME (Figure 4C). No cytotoxicity was observed in the rest of the studied cancer cell lines, with a cytoprotective action of 1 and 10 µg/mL YME in colonic Caco-2 cells, and 0.1 and 10 µg/mL in T24 bladder cells.

The generation of ROS after 2 h treatment with YME is shown in Figure 5. YME significantly decreased ROS generation in a dose-dependent manner in all cell lines, the 0.1 µg/mL dose being able to decrease ROS production in Caco-2 cells as compared to the respective untreated controls (Figure 5A).

4. Discussion

Cancer is associated with oxidative stress and uncontrolled cell proliferation (Nkondjock, 2009). Identifying dietary strategies to control the proliferation of cancer cells may contribute to a preventive therapy against the onset and progression of the pathology. Dietary polyphenols are potent antioxidants, many of them having shown to exert potential anticarcinogenic activities (Surh, 2003; Nichenametla et al., 2006; Ramos, 2008). Thus, flavonols like quercetin present in apple, onions and many other foodstuffs, have shown to regulate proliferation and induce cell death of human hepatoma cells (Granado-Serrano et al., 2006; Granado-Serrano et al., 2008). Tea flavanols (epicatechin gallate, epigallocatechin gallate, etc.) suppressed the growth of different cancer cell lines (Nichenametla et al., 2006; Granado-Serrano et al., 2007; Ramos et al., 2011). Also, cocoa phenolic compounds (epicatechin, procyanidin B2) and cocoa extracts are potent inhibitors of tumour initiation *in vivo* and present antiproliferative activities against tumour cells *in vitro* (Granado-Serrano et al., 2009a; Rodriguez-Ramiro et al., 2011). These and other highly and regularly consumed foods rich in polyphenols such as coffee in industrialized countries and yerba mate in South America and other parts of the world, are particularly interesting for their potential beneficial effects.

However, the relationship between coffee and mate consumption with certain types of cancer is not totally clear. Coffee intake has been associated with lung cancer, as highlighted by a meta-analysis showing that high coffee consumption may increase the

risk of lung cancer [relative risk (RR) 1.27, 95% confidence interval (CI) 1.04-1.54], although a decreased risk was found in non-smokers (RR 0.78, 95% CI 0.60-1.00) (Tang et al., 2010). A case-control study in Uruguay also showed an increased risk of small cell lung cancer [odds ratio (OR) 2.9, 95% CI 1.3-6.2] with mate consumption, although pulmonary adenocarcinoma was not associated with mate drinking (De Stefani et al., 1996).

Mate has been traditionally associated with cancers of the upper aero-digestive tract (UADT; oral cavity, oropharynx, larynx, oesophagus). A recent case-control study in Latin America, where these types of cancers have special high incidence, found a significant effect of mate consumption only on oesophagus cancer (OR 3.81, 95% CI 1.75-8.30) (Szymanska et al., 2010). This was confirmed in a meta-analysis of studies on the consumption of mate in patients with oesophageal squamous cell carcinoma that found an increased risk in patients with high mate intake (OR 2.76, 95% CI 1.33-5.73) compared to low consumption (OR 1.84, 95% CI 1.12-3.00) (Andrici and Eslick, 2013). The high temperature mate is usually consumed has been related to this increased cancer risk (Sewram et al., 2003), although results in the mentioned case-control study were not conclusive (Szymanska et al., 2010). However, a synergistic effect between mate, alcohol and tobacco is clear and has been reported to represent a 7-fold increase in oesophageal cancer risk (Sewram et al., 2003; Loria et al., 2009). Also, mate was associated with increased risk of UADT cancer in Brazil (RR 2.1, 95% CI 1.6-2.7) but not with coffee and tea drinking (Pintos et al., 1994), which has been recently confirmed in a large epidemiological study in nine European countries in the European Prospective Investigation into Cancer and Nutrition (EPIC) study (Zamora-Ros et al., 2014) where even a decreased risk of oral squamous cell carcinoma among male smokers has been suggested.

Coffee and mate consumption has also been associated with increased risk of bladder cancer in observational studies. Mate (OR 2.2, 95% CI 1.2-3.9) and coffee (OR 1.6, 95% CI 1.2-2.3) drinking were strongly associated with bladder cancer in the Uruguayan population (De Stefani et al., 2007). Again, high temperature and tobacco were factors increasing the risk, since mate intake showed a strong association with bladder cancer when consumed by ever-smokers with “bombilla” (OR 3.77, 95% CI 1.17-12.1) but not when consumed by never-smokers or as “mate cocido” (Bates et al., 2007). On the contrary, a recent meta-analysis showed a higher risk associated to coffee consumption in non-smokers (OR 1.72, 95% CI 1.25-2.35) compared to smokers (OR 1.24, 95% CI 0.91-1.70) (Wu et al., 2015).

Finally, several recent case-control studies and meta-analysis have shown a reduced risk of colorectal cancer with coffee drinking, with OR of 0.74-0.78 in case-control studies and RR of 0.93-0.95 in prospective cohort studies (Gan et al., 2016; Schmit et al., 2016). This beneficial effect of coffee intake has been extended to patients with stage III colon cancer, with a significantly reduced cancer recurrence and mortality in patients consuming 4 cups/d [hazard ratio (HR) 0.58, 95% CI 0.34-0.99] (Guercio et al., 2015). However, studies on the effect of mate intake and colon cancer are limited to a prospective study in Brazil that found that colon cancer mortality was positively associated with mate consumption (Sichieri et al., 1996), although yerba mate tea has shown to reduce colon carcinogenesis in rats (Zapaterini et al., 2010).

All the previous evidences, often contradictory, justify the interest of studying the effect of GCBE and YME in the selected cancer cell lines in this preliminary screening work. The wide range of concentrations of extracts (10-1000 µg/mL) and the major phenolic constituents, metabolites and methylxanthines (10-1000 µM 5-CQA, 3,5-DCQA, CA, FA, DHCA, DHFA, and caffeine) comprised from physiologically relevant concentrations (plasma levels of chlorogenic acids of 10-18.3 µM have been reported after coffee consumption; Monteiro et al., 2007; Farah et al., 2008) to supra-physiological high concentrations to test for potential pharmacologically relevant effects. Although most works in the literature use concentrations of pure phenolic compounds and/or polyphenol-rich extracts up to 200 µM or µg/mL, respectively (Granado-Serrano et al., 2009a; Wang et al., 2012; Venkatesan et al., 2016), it is not uncommon the use of concentrations up to 500-650 µM or µg/mL of pure compounds or extracts, respectively (Gonzalez de Mejia et al., 2010; Mateos et al., 2013; Calo and Marabini, 2014), or even supra-physiological levels as high as mM concentrations of pure polyphenols or 100 mg/mL of extracts (Iwai et al., 2004; Peng et al., 2013; Yang et al., 2015; Hou et al., 2017).

Our results show that the two extracts affected differently the viability and proliferation of the four cancer cell lines, with YME being more effective even at the lowest dose (10 µg/mL). Although GCBE and YME are rich in hydroxycinnamic acids and methylxanthines, there are quantitative differences, with coffee having more caffeine than mate (up to 12.6 mg/g vs. 7.2 mg/g in mate); however, YME is richer in cinnamoylquinic acids (up to 64.1 mg/g in GCBE and 74.1 mg/g in YME) and dicaffeoylquinic acids (up to 16.6 mg/g vs. 5.3 mg/g in coffee) (Bravo et al., 2007; Baeza et al., 2016b; Mateos et al., 2017). Moreover, mate also contains important amounts of flavonols (up to 9% of total polyphenols in the YME), including rutin and other quercetin-glycosides. DCQA in mate have shown to inhibit human colon cancer

cells CRL-2577 and HT-29 proliferation, inducing apoptosis in a time and dose-dependent manner (Puangpraphant et al., 2011). Also, flavonols like quercetin have well-known antiproliferative effects *in vivo* and *in vitro* (Granado-Serrano et al., 2006; Granado-Serrano et al., 2008). Yerba mate also contains saponins, including ursolic acid, which has been reported to induce apoptosis in HT-29 colon cancer cells (Shan et al., 2009). Therefore, differences in the response of GCBE and YME can be due to the different composition of both extracts.

This effect of YME reducing cell viability and proliferation at a physiological relevant dose (10 µg/mL) was not directly due to a pro-oxidant effect of the extract, since ROS values decreased in all cell lines incubated with low (0.1-10 µg/mL) doses of YME (Figure 5). Similarly, such effects could not be explained by a cytotoxic activity of YME, since LDH values showed no effect (A549 cells) or even a cytoprotective activity (Caco-2 and T24 cells) of the extract, which only was slightly cytotoxic to OE-33 cells (Figure 4). Further studies are needed to explore the mechanisms of action of YME decreasing cell viability and proliferation.

Previous studies with yerba mate, either as aqueous (tea-like) or organic extracts, have shown the ability of mate to inhibit oral cancer cell proliferation (Gonzalez de Mejia et al., 2005), having growth inhibitory and cytotoxic effects on human colon adenocarcinoma cells, with inhibitory concentrations (IC₅₀) of 220 and 224 µg/mL in Caco-2 and HT-29 cells, respectively, although with very different GI₅₀ (growth inhibition, 50%) values, 1 µg/mL vs. 105 µg/mL for Caco-2 and HT-29 cells respectively, showing the different susceptibility of cancer cells (Gonzalez de Mejia et al., 2010). Yerba mate tea has also proved to protect against oesophageal and liver carcinogenesis induced by N-diethylnitrosamine (DEN) in Wistar rats (Silva et al., 2009). All these evidences confirm the activity of YME on different types of cancer.

The observed different susceptibility of the four cancer cell lines studied would reflect their different metabolic capacity, gene expression, differentiation, tumorigenicity, etc. Even different human colon cancer cell lines have shown to respond differently to the same phytochemical as mentioned above for Caco-2 and HT-29 cells (Gonzalez de Mejia et al., 2010), also observed by Ramos et al. (2011) for Caco-2 and SW480 cells. Moreover, distinct capacity to uptake and accumulate polyphenols by the different cell lines would likely affect their susceptibility to the tested extracts and phytochemicals. Caco-2 cells, with characteristics of differentiated enterocytes, are known to efficiently uptake and metabolize polyphenols (Vaidyanathan and Walle, 2003; Pereira-Caro et

al., 2010), including mono- and dicaffeoylquinic acids (Gomez-Juaristi, 2015), although the capacity of A549, OE-33, and T24 cells has not been tested.

The dissimilar metabolic capacity, differentiation, proliferation rate, etc. might also account for the important differences observed between the two colon cell lines here assayed, the mildly differentiated Caco-2 cells and the normal, non-transformed CCD-18Co cells. Thus, while viability of Caco-2 was only affected by 10 μ M YME, CCD-18Co cells viability was decreased by 10 μ M YME, 5-CQA, CA and DHCA (Table 6). On the contrary, Caco-2 cancer cells, with a higher proliferation rate than CCD-18Co, were susceptible to the antiproliferative effect of 10 μ M GCBE, YME, 3,5-DCQA, FA and DHCA, whilst only 10 μ M YME and DHCA had some effect on CCD-18Co cell proliferation. Differences could also be observed with the higher doses tested (100 and 1000 μ M), being Caco-2 cells more resistant than CCD-18Co in general terms (Tables 1-4), although both cell lines were similarly susceptible to the strong effect of YME inhibiting cell viability and proliferation (Table 1). Comparable responses were also observed in two different human colon cancer cells incubated with the green tea flavanol epicatechin gallate, which inhibited cell proliferation in moderately differentiated and invasive SW480 cells but not in Caco-2 (Ramos et al., 2011). Likewise, a more pronounced growth inhibition by propolis ethanol extracts was observed in HCT116 and HT29 cells, with a high proliferation rate, in comparison with Caco-2 cells (Ishihara et al., 2009), whereas high doses of 5-CQA (1000 μ M) elicited similar inhibition of cell viability in HCT116 and HT29 cells (Hou et al., 2017). Also, the antiproliferative effect of green tea polyphenols was more pronounced in human oral cavity cancer cells than in their normal counterparts (Babich et al., 2005), in line with the findings here reported for Caco-2 and CCD-18Co.

When looking at the effect of the major phenolic compounds in YME and GCBE and their main metabolites, important differences could be observed. High doses of 3,5-DCQA were more efficient than 5-CQA reducing cell proliferation (Table 2), while at low levels (10 μ M) only 3,5-DCQA was able to decrease proliferation of Caco-2 and T24 cells. However, cell viability was similarly affected by the mono- and dicaffeoylquinic acids. In agreement with our results, DCQAs have shown to decrease proliferation of human colon cells (Puangpraphan et al., 2011). Similarly, Iwai et al. (2004) reported higher antiproliferative capacity of DCQAs as compared to CQAs and FQAs in several cancer cell lines, with IC₅₀ values ranging from 0.10-0.18 mM in KB human oral carcinoma cells to 0.56-0.76 mM in human histiocytic lymphoma U937 cells for different DCQAs as compared with IC₅₀ values of 0.56-4.65 mM for 5-FQA and 0.14 mM (KB

cells) or as high as 8.18 mM (lung fibroblast WI38VA cancer cells) for 5-CQA. It has to be noted the high IC₅₀ values reported by these authors, in the mM range, much higher than the concentrations tested in the present study. The antiproliferative effects of DCQAs are not limited to those present in yerba mate, since DCQAs extracted from sweet potato leaves (3,4-, 3,5-, and 4,5-DCQA) dose-dependently inhibited cancer cell proliferation, inducing apoptosis mediated by caspase-3 activation and expression of c-Jun (Kurata et al., 2007).

The higher activity of DCQA as compared with CQA and FQA suggested that the number of caffeoyl groups might be a key factor regulating the growth inhibitory activity of hydroxycinnamoylquinic esters (Iwai et al., 2004), which has also been suggested to play a role in the antimutagenic capacity of CQA derivatives (Yoshimoto et al., 2002).

Both 5-CQA and 3,5-DCQA were more effective than the non-esterified CA and FA, since in general the highest doses of CA reduced cell viability and proliferation (with the exception of T24 cells), while FA also required high doses to reduce cell viability (Table 3). Other authors reported a reduction of 25% cell viability of T24 cells incubated 24 h with 2000 μ M FA (Peng et al., 2013), similar to the 29% decreased viability of T24 cells treated for 24 h with 1000 μ M FA in the present study. Contrary to CA, FA showed an effective antiproliferative action even at the lowest dose tested (10 μ M), which was able to decrease between 14 and 27% the proliferation of the tested cancer cells (Table 3). This is in contrast with that reported by Iwai et al. (2004) suggesting that methoxylation of the caffeoyl group at the C3 position would result in lower antiproliferative activity against cancer cells, although that association derived from observations in 5-FQA as compared to 3-, 4-, and 5-CQA. Free, non-esterified hydroxycinnamic acids might behave differently as observed in the present study. The results with the reduced microbial metabolites DHCA and DHFA, however, are in line with that suggested by Iwai et al. (2004), since the methoxylated DHFA was in general less effective than DHCA reducing cell viability and proliferation (Table 4).

As for caffeine, the lack of effect of physiologically relevant doses of this methylxanthine on the viability of the studied cell lines, requiring 1000 μ M concentrations to reduce viability of all cell lines (except Caco-2) and showing an antiproliferative effect only in A549 cells (Table 5), agrees with results from a cluster comparative study of 130 chemicals on a panel of 39 human cancer cell lines where caffeine had no cytotoxic effect (Nakatsu et al., 2007).

Among the variability observed in the response of the different cell lines to the different extracts and pure phenols tested, it is interesting noting the unlike effect of some compounds affecting cell viability or proliferation after short (2 h) but not long (24 h) incubation times. Just to mention two examples, 10 μ M 5-CQA, CA and DHCA reduced viability of CCD-18Co cells only after 2 h treatment; also 10 μ M GCBE, YME, FA and DHCA decreased Caco-2 cell proliferation at short incubation time, while the effect on T24 cell proliferation could only be observed after 24 h treatment (Table 6). Regulation of cell survival/proliferation pathways is a complex mechanism that responds differently to phytochemicals. Thus, polyphenols like the flavanols epicatechin and epicatechin gallate have shown to induce cell proliferation/survival through the activation of the NF- κ B cascade and nuclear transcription factor erythroid 2p45-related factor-2 (Nrf2) signalling, stimulating phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT), extracellular regulated kinase (ERK) and protein kinase C- α (PKC α) pathways, and enhancing activator protein-1 (AP-1) binding activity (Granado-Serrano et al., 2009b; Granado-Serrano et al., 2010). On the contrary, the flavonol quercetin has the opposite effect suppressing proliferation/survival pathways by down-regulating PI3K/AKT, ERK and PKC α and up-regulating death signals (c-Jun amino-terminal kinase, JNK), with the activation of caspases-3 and -9 and increasing the expression levels of proapoptotic Bcl-2 family members (Granado-Serrano et al., 2006; Granado-Serrano et al., 2008). Interestingly, studies on the time-course regulation of the up- or down-regulation of such responses show that often these are rapid events that can be triggered at very short incubation times (even after only 5 min), with the early activation/deactivation of signalling cascades having transient or sustained effects, depending on the phenolic compound (Granado-Serrano et al., 2008, 2009a, 2009b, 2010). This might account for the effects observed at short incubation times, reflecting early cell events not sustained at longer times.

Another important aspect to bear in mind when considering the different response of the studied cell lines, also in relation to the time-dependent effect, is the metabolization of the studied compounds. As mentioned above, Caco-2 cells are commonly used as a cell model of enterocytes to study the uptake and transport of polyphenols, being able to metabolize hydroxycinnamic acids mostly into their de-esterified and/or methylated metabolites within 1-2 h incubation *in vitro* (Gómez-Juaristi, 2015). The metabolites formed might be less active than the parent molecules, which would account for the lack of effect on cell viability and proliferation observed at long incubation times. To the best of our knowledge, there is no information on the metabolizing capacity of the other studied cell lines, although it cannot be ruled out that metabolization of phenolic

compounds by the cells, including de-esterification of cinnamoylquinic acids into their parent, less active caffeic and ferulic acids, might contribute to the observed results.

Special mention deserves the effects observed in non-cancer cells, CCD-18Co. As mentioned above, many phytochemicals have shown to be effective reducing viability and/or proliferation of cancer cell lines not affecting normal, non-cancer cells, which is an added value to their potential as natural chemopreventive or even chemotherapeutic agents against cancer. However, the fact that both GCBE and YME decreased viability and proliferation of CCD-18Co cells, in the case of mate even at the 10 µg/mL dose with a 51% lower viability and 12% decreased proliferation after 24 h (Table 1), points to a potential undesired cytotoxic effect in normal cells. Reductions of cell viability and proliferation were also observed with most pure polyphenols and metabolites, only the methylated molecules (FA and DHFA) having lower effect decreasing cell viability after 24 h incubation with the highest dose (1000 µM).

Similar findings were reported by other authors. IC₅₀ values ranging from 500 µM (3, 5-DCQA) to 5870 µM (5-CQA) were reported for normal diploid lung fibroblast WI38 cells (Iwai et al., 2004). In normal CCD-33Co colon cells, high concentrations (500 µg/mL) of mate tea as well as green tea and epigallocatechin gallate (500 µM) reduced growth of the non-cancer cells, although 50 µg/mL concentrations had no effect on cell viability (Gonzalez de Mejía et al., 2010). Puangraphant et al. (2011) reported no cytotoxic effects of 300 µM 3,5-DCQA in CCD-33Co normal cells. In contrast, in our study the 100 µM dose of 3,5-DCQA inhibited 12% and 14% cell viability of CCD-18Co cells after 2 and 24 h treatment, respectively, while the 1000 µM concentration reduced viability to only 34% after 2 h incubation, totally blocking cell viability after 24 h (Table 2), suggesting a strong cytotoxic effect. Such effect was more remarkable for YME, 5-CQA, CA, and DHCA, since the lowest doses (10 µg/mL YME and 10 µM of pure phenolics) showed inhibition of cell viability varying between 13% (DHCA, 2 h) and 63% (YME, 2h). However, specific studies on the cytotoxicity of these samples at lower, physiological concentrations (0.1, 1, and 10 µg/mL or µM) showed that apparently none of the tested concentrations was cytotoxic (Figure 2), even having cytoprotective effects and reducing ROS generation (Figure 3), pointing to an antioxidant effect in CCD-18Co cells. LDH leakage occurs upon plasmatic membrane rupture and thus it will reflect necrotic cell death. Conversely, apoptosis and autophagy are other types of death that maintain cell membrane integrity and therefore will not be accounted for by the method used to measure cytotoxicity, which might partially explain the discrepancy observed in the results concerning loss of viability with no apparent

cytotoxic effect. Indeed, it is well established that polyphenols can regulate autophagy and apoptosis in cancer cells mediated by multiple signalling pathways (reviewed in Hasima and Ozpolat, 2014; Lewandowska et al., 2016). An important mechanism regulating cell death involves mitochondrial membrane permeabilisation by ROS, triggering the intrinsic pathway leading to apoptosis (Tajeddine, 2016). ROS also regulate autophagy by activating signalling pathways; in turn, autophagy can regulate intracellular ROS levels mainly through the elimination of damaged mitochondria, one of the major cellular sources of ROS (Zhao et al., 2016). Since polyphenols are redox-active compounds, they can decrease mitochondrial membrane fluidity and metabolism ultimately modifying ROS production (Dorta et al., 2005; Gorlach et al., 2015), which might explain the observed reduction in ROS levels elicited by 10 µg/mL or µM concentrations of the studied compounds, doses that also induced loss of cell viability.

Different mechanisms of action have been proposed to explain the reduction in cell viability and proliferation by hydroxycinnamic acids: (i) blockage of cell cycle progression by suppressing the activation of the nuclear factor-κB (NF-κB); (ii) cell cycle arrest and induction of apoptosis; or (iii) activation/deactivation of protein kinases (Bohn et al., 2014). Previous studies have reported an inhibition of NF-κB in macrophages treated with DCQAs purified from YME, as well as the induction of apoptosis by activation of caspases-8 and -3 in human colon cancer cells (HT-29 and CRL-2577) in a time and concentration dependent manner (Puangpraphant et al., 2011). Also cell cycle arrest in G2/M phase and accumulation of cells in subG0 phase of cell cycle have been observed in human cervical adenocarcinoma HeLa cells incubated with coffee extract (Krstic et al., 2014), but not in human hepatoma HepG2 cells treated with 5-CQA (Ramos et al., 2011), although high doses of 5-CQA (1000 µM) were able to induce cell cycle arrest in S-phase in human colon cancer cell lines (Hou et al., 2017). DCQAs have shown anti-inflammatory activity by suppressing the COX-2/PGE2 and iNOS/NO pathways in macrophages and colon cancer cells (Puangpraphant et al., 2011). Topoisomerase II, a nuclear enzyme that regulates DNA metabolism and one of the targets in the development of drugs against cancer, is inhibited *in vitro* by yerba mate (Gonzalez de Mejia et al., 2005).

Data on ROS production when cancer cells were incubated with YME and when normal cells were incubated with both YME or its CQA derivatives confirmed our previous results with 5-CQA, 3,5-DCQA (Baeza et al., 2014), DHCA and DHFA (Baeza et al., 2016a) or other herbal extracts which contain these phenolic compounds such as green coffee (Baeza et al., 2014) and *Corema album* extracts (León-González et al.,

2012). The antioxidant activity might therefore contribute to reduce the oxidative stress associated with cancer initiation and progression.

5. Conclusions

In conclusion, consumption of green coffee and especially yerba mate, which are rich in bioactive compounds such as CQA and DCQA derivatives, do not increase proliferation of cancer cells, negatively affecting cell viability and proliferation in a dose- and time-dependent manner. 5-CQA and 3,5-DQA were the most potent anti-proliferative phenols of all the studied compounds, their synergistic effect, together with the potential contribution of other bioactive compounds likely explain the stronger activity of YME. At physiological doses YME do not have a cytotoxic effect in cancer and normal, non-cancer cells, reducing basal ROS production showing antioxidant capacity. Therefore, yerba mate might have interesting anti-proliferative potential in cancer prevention, deserving further studies to confirm this relationship as well as the subjacent mechanisms of action.

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Figure Legends

Figure 1. Chemical structures of caffeic acid (A), ferulic acid (B) and the reduced microbial metabolites dihydrocaffeic (C) and dihydroferulic (D) acids.

Figure 2. Cytotoxicity studies in human healthy colonic CCD-18Co cells after treatment with YME (A), 5-CQA (B), CA (C), and DHCA (D) for 2 and 24 h. Different letters within the same incubation time denote statistically significant differences ($p < 0.05$). Results are the mean $\pm$ SD ($n = 4$).

Figure 3. ROS generation in CCD-18Co cells after 2 h treatment with YME (A), 5-CQA (B), CA (C), and DHCA (D). Different letters denote statistically significant differences ($p < 0.05$). Results are the mean $\pm$ SD ($n = 4$).

Figure 4. Cytotoxicity studies in tumour cell lines treated with YME for 2 h. (A) Caco-2 cells; (B) A549 cells; (C) OE 33 cells; (D) T-24 cells. Different letters denote statistically significant differences ($p < 0.05$). Results are the mean $\pm$ SD ($n = 4$).

Figure 5. ROS production in Caco-2 (A), A549 (B), OE-33 (C), and T-24 (D) cells treated with YME for 2 h. Different letters denote statistically significant differences ($p < 0.05$). Results are the mean $\pm$ SD ($n = 4$).

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Table 1. Effect of green coffee bean and yerba mate extracts on the viability and proliferation of non-cancer (CCD-18Co) and cancer (Caco-2, A549, OE-33, and T24) human cell lines.

Cell lines	Treatment ($\mu\text{g/mL}$)	Green coffee bean extract				Yerba mate extract			
		Viability		Proliferation		Viability		Proliferation	
		2 h	24 h	2 h	24 h	2 h	24 h	2 h	24 h
CCD-18Co	C	100 $\pm$ 7 ^a	100 $\pm$ 3 ^a	100 $\pm$ 12 ^a	100 $\pm$ 11 ^a	100 $\pm$ 7 ^a	100 $\pm$ 3 ^a	100 $\pm$ 12 ^a	100 $\pm$ 11 ^a
	10	96 $\pm$ 5 ^a	95 $\pm$ 5 ^a	108 $\pm$ 11 ^a	95 $\pm$ 9 ^a	37 $\pm$ 2 ^b	49 $\pm$ 4 ^b	99 $\pm$ 9 ^a	88 $\pm$ 12 ^b
	100	64 $\pm$ 2 ^b	82 $\pm$ 5 ^b	98 $\pm$ 9 ^a	78 $\pm$ 10 ^b	6 $\pm$ 1 ^c	14 $\pm$ 1 ^c	96 $\pm$ 10 ^a	53 $\pm$ 4 ^c
	1000	13 $\pm$ 1 ^c	22 $\pm$ 2 ^c	101 $\pm$ 9 ^a	66 $\pm$ 6 ^c	2 $\pm$ 1 ^d	6 $\pm$ 1 ^d	1 $\pm$ 0 ^b	1 $\pm$ 1 ^d
Caco-2	C	100 $\pm$ 10 ^a	100 $\pm$ 4 ^{ab}	100 $\pm$ 5 ^a	100 $\pm$ 6 ^a	100 $\pm$ 10 ^a	100 $\pm$ 4 ^a	100 $\pm$ 5 ^a	100 $\pm$ 6 ^a
	10	102 $\pm$ 8 ^a	105 $\pm$ 5 ^a	92 $\pm$ 4 ^b	103 $\pm$ 10 ^a	77 $\pm$ 7 ^b	89 $\pm$ 6 ^b	85 $\pm$ 5 ^b	99 $\pm$ 6 ^a
	100	93 $\pm$ 6 ^a	95 $\pm$ 7 ^b	86 $\pm$ 4 ^{bc}	98 $\pm$ 10 ^a	33 $\pm$ 5 ^c	62 $\pm$ 9 ^c	82 $\pm$ 6 ^b	73 $\pm$ 7 ^b
	1000	52 $\pm$ 3 ^b	78 $\pm$ 7 ^c	80 $\pm$ 5 ^c	79 $\pm$ 8 ^b	4 $\pm$ 1 ^d	0 $\pm$ 0 ^d	25 $\pm$ 2 ^c	22 $\pm$ 2 ^c
A549	C	100 $\pm$ 4 ^{ab}	100 $\pm$ 5 ^a	100 $\pm$ 9 ^{ab}	100 $\pm$ 8 ^a	100 $\pm$ 4 ^a	100 $\pm$ 5 ^a	100 $\pm$ 9 ^a	100 $\pm$ 8 ^a
	10	103 $\pm$ 4 ^a	98 $\pm$ 5 ^a	94 $\pm$ 4 ^a	95 $\pm$ 7 ^a	84 $\pm$ 3 ^b	73 $\pm$ 5 ^b	101 $\pm$ 9 ^a	76 $\pm$ 6 ^b
	100	95 $\pm$ 5 ^b	89 $\pm$ 6 ^b	108 $\pm$ 9 ^b	80 $\pm$ 8 ^b	39 $\pm$ 3 ^c	41 $\pm$ 2 ^c	91 $\pm$ 8 ^a	58 $\pm$ 5 ^c
	1000	59 $\pm$ 6 ^c	55 $\pm$ 4 ^c	104 $\pm$ 5 ^{ab}	70 $\pm$ 7 ^b	29 $\pm$ 4 ^d	2 $\pm$ 0 ^d	20 $\pm$ 1 ^b	17 $\pm$ 2 ^d
OE-33	C	100 $\pm$ 4 ^a	100 $\pm$ 6 ^a	100 $\pm$ 5 ^a	100 $\pm$ 7 ^a	100 $\pm$ 4 ^a	100 $\pm$ 6 ^a	100 $\pm$ 5 ^a	100 $\pm$ 10 ^a
	10	95 $\pm$ 3 ^a	85 $\pm$ 8 ^b	106 $\pm$ 10 ^a	112 $\pm$ 7 ^a	52 $\pm$ 3 ^b	55 $\pm$ 4 ^b	98 $\pm$ 10 ^{ab}	96 $\pm$ 8 ^a
	100	70 $\pm$ 5 ^b	74 $\pm$ 7 ^c	99 $\pm$ 9 ^a	109 $\pm$ 10 ^a	16 $\pm$ 1 ^c	27 $\pm$ 2 ^c	85 $\pm$ 8 ^b	65 $\pm$ 5 ^b
	1000	23 $\pm$ 3 ^c	27 $\pm$ 3 ^d	99 $\pm$ 8 ^a	103 $\pm$ 8 ^a	12 $\pm$ 1 ^d	15 $\pm$ 2 ^d	15 $\pm$ 2 ^c	16 $\pm$ 0 ^c
T24	C	100 $\pm$ 5 ^a	100 $\pm$ 9 ^a	100 $\pm$ 7 ^a	100 $\pm$ 6 ^a	100 $\pm$ 5 ^a	100 $\pm$ 9 ^a	100 $\pm$ 7 ^a	100 $\pm$ 6 ^a
	10	99 $\pm$ 4 ^a	78 $\pm$ 3 ^b	105 $\pm$ 5 ^a	102 $\pm$ 9 ^a	74 $\pm$ 5 ^b	88 $\pm$ 8 ^b	92 $\pm$ 9 ^a	61 $\pm$ 4 ^b
	100	84 $\pm$ 3 ^b	78 $\pm$ 7 ^b	83 $\pm$ 9 ^b	103 $\pm$ 8 ^a	31 $\pm$ 3 ^c	70 $\pm$ 7 ^c	71 $\pm$ 7 ^b	14 $\pm$ 3 ^c
	1000	39 $\pm$ 3 ^c	61 $\pm$ 6 ^c	61 $\pm$ 4 ^c	9 $\pm$ 3 ^b	10 $\pm$ 1 ^d	9 $\pm$ 1 ^d	2 $\pm$ 1 ^c	1 $\pm$ 1 ^d

Values are expressed as the mean $\pm$ SD. Different letters within a column and cell line indicate significant differences ($p < 0.05$).

Table 2. Effect of 5-caffeoylquinic and 3,5-dicaffeoylquinic acids on the viability and proliferation of non-cancer (CCD-18Co) and cancer (Caco-2, A549, OE-33, and T24) human cell lines.

Cell lines	Treatment (μ M)	5-caffeoylquinic acid				3,5-dicaffeoylquinic acid			
		Viability		Proliferation		Viability		Proliferation	
		2 h	24 h	2 h	24 h	2 h	24 h	2 h	24 h
CCD-18Co	C	100 $\pm$ 5 ^a	100 $\pm$ 7 ^a	100 $\pm$ 10 ^a	100 $\pm$ 10 ^a	100 $\pm$ 8 ^a	100 $\pm$ 7 ^a	100 $\pm$ 12 ^a	100 $\pm$ 11 ^a
	10	83 $\pm$ 4 ^b	93 $\pm$ 5 ^{ab}	105 $\pm$ 8 ^a	100 $\pm$ 10 ^a	102 $\pm$ 5 ^a	98 $\pm$ 7 ^a	100 $\pm$ 9 ^a	94 $\pm$ 9 ^a
	100	64 $\pm$ 3 ^c	88 $\pm$ 6 ^b	95 $\pm$ 7 ^a	91 $\pm$ 8 ^a	88 $\pm$ 6 ^b	86 $\pm$ 6 ^b	103 $\pm$ 9 ^a	71 $\pm$ 8 ^b
	1000	39 $\pm$ 2 ^d	48 $\pm$ 4 ^c	80 $\pm$ 6 ^b	75 $\pm$ 9 ^b	34 $\pm$ 2 ^c	1 $\pm$ 2 ^c	95 $\pm$ 8 ^a	14 $\pm$ 2 ^c
Caco2	C	100 $\pm$ 5 ^a	100 $\pm$ 7 ^a	100 $\pm$ 8 ^a	100 $\pm$ 8 ^a	100 $\pm$ 4 ^{ab}	100 $\pm$ 9 ^a	100 $\pm$ 8 ^a	100 $\pm$ 6 ^a
	10	102 $\pm$ 7 ^a	105 $\pm$ 7 ^a	96 $\pm$ 9 ^a	110 $\pm$ 10 ^a	108 $\pm$ 6 ^a	102 $\pm$ 7 ^a	97 $\pm$ 10 ^a	89 $\pm$ 4 ^b
	100	86 $\pm$ 6 ^b	81 $\pm$ 4 ^b	100 $\pm$ 10 ^a	107 $\pm$ 6 ^a	97 $\pm$ 6 ^b	97 $\pm$ 8 ^a	86 $\pm$ 6 ^a	64 $\pm$ 5 ^c
	1000	43 $\pm$ 4 ^c	30 $\pm$ 2 ^c	91 $\pm$ 8 ^a	77 $\pm$ 6 ^b	73 $\pm$ 5 ^c	78 $\pm$ 5 ^b	75 $\pm$ 6 ^b	68 $\pm$ 5 ^c
A549	C	100 $\pm$ 6 ^a	100 $\pm$ 7 ^a	100 $\pm$ 8 ^a	100 $\pm$ 8 ^a	100 $\pm$ 2 ^a	100 $\pm$ 7 ^a	100 $\pm$ 10 ^a	100 $\pm$ 8 ^a
	10	116 $\pm$ 8 ^b	97 $\pm$ 5 ^a	89 $\pm$ 6 ^a	107 $\pm$ 11 ^{ab}	101 $\pm$ 8 ^a	92 $\pm$ 6 ^{ab}	89 $\pm$ 8 ^a	97 $\pm$ 10 ^{ab}
	100	94 $\pm$ 7 ^a	79 $\pm$ 6 ^b	92 $\pm$ 7 ^a	120 $\pm$ 10 ^b	84 $\pm$ 6 ^b	82 $\pm$ 7 ^b	69 $\pm$ 5 ^b	86 $\pm$ 6 ^{bc}
	1000	63 $\pm$ 4 ^c	51 $\pm$ 4 ^c	90 $\pm$ 7 ^a	107 $\pm$ 10 ^{ab}	47 $\pm$ 4 ^c	38 $\pm$ 3 ^c	69 $\pm$ 7 ^b	74 $\pm$ 6 ^c
OE-33	C	100 $\pm$ 6 ^a	100 $\pm$ 8 ^a	100 $\pm$ 7 ^a	100 $\pm$ 10 ^a	100 $\pm$ 3 ^a	100 $\pm$ 3 ^a	100 $\pm$ 5 ^a	100 $\pm$ 10 ^a
	10	87 $\pm$ 3 ^b	80 $\pm$ 7 ^b	106 $\pm$ 10 ^a	90 $\pm$ 3 ^{ab}	102 $\pm$ 3 ^a	96 $\pm$ 5 ^a	100 $\pm$ 7 ^a	96 $\pm$ 8 ^a
	100	72 $\pm$ 3 ^c	68 $\pm$ 4 ^c	98 $\pm$ 10 ^a	84 $\pm$ 6 ^b	75 $\pm$ 4 ^b	73 $\pm$ 3 ^b	96 $\pm$ 9 ^{ab}	98 $\pm$ 8 ^a
	1000	30 $\pm$ 3 ^d	15 $\pm$ 1 ^d	91 $\pm$ 9 ^a	61 $\pm$ 9 ^c	21 $\pm$ 2 ^c	5 $\pm$ 0 ^c	87 $\pm$ 8 ^b	18 $\pm$ 2 ^b
T24	C	100 $\pm$ 4 ^a	100 $\pm$ 5 ^a	100 $\pm$ 5 ^a	100 $\pm$ 10 ^a	100 $\pm$ 7 ^a	100 $\pm$ 5 ^a	100 $\pm$ 7 ^a	100 $\pm$ 6 ^a
	10	94 $\pm$ 4 ^a	99 $\pm$ 4 ^a	100 $\pm$ 10 ^a	103 $\pm$ 8 ^a	102 $\pm$ 10 ^a	95 $\pm$ 6 ^a	92 $\pm$ 6 ^a	90 $\pm$ 6 ^b
	100	88 $\pm$ 4 ^b	96 $\pm$ 5 ^a	105 $\pm$ 11 ^a	110 $\pm$ 9 ^a	82 $\pm$ 7 ^b	84 $\pm$ 7 ^b	92 $\pm$ 7 ^a	48 $\pm$ 6 ^c
	1000	21 $\pm$ 2 ^c	29 $\pm$ 4 ^b	75 $\pm$ 8 ^b	64 $\pm$ 4 ^b	45 $\pm$ 4 ^c	0 $\pm$ 0 ^c	20 $\pm$ 2 ^b	3 $\pm$ 2 ^d

Values are expressed as the mean $\pm$ SD. Different letters within a column and cell line indicate significant differences ($p < 0.05$).

Table 3. Effect of caffeic and ferulic acids on the cell viability and proliferation of non-cancer (CCD-18Co) and cancer (Caco-2, A549, OE-33, and T24) human cells.

Cell lines	Treatment (μ M)	Caffeic acid				Ferulic acid			
		Viability		Proliferation		Viability		Proliferation	
		2 h	24 h	2 h	24 h	2 h	24 h	2 h	24 h
CCD-18Co	C	100 $\pm$ 6 ^a	100 $\pm$ 7 ^a	100 $\pm$ 8 ^a	100 $\pm$ 8 ^a	100 $\pm$ 6 ^a	100 $\pm$ 7 ^a	100 $\pm$ 8 ^a	100 $\pm$ 8 ^a
	10	79 $\pm$ 5 ^b	96 $\pm$ 6 ^a	96 $\pm$ 10 ^a	99 $\pm$ 6 ^a	97 $\pm$ 8 ^a	99 $\pm$ 8 ^a	109 $\pm$ 9 ^a	103 $\pm$ 5 ^a
	100	64 $\pm$ 3 ^c	96 $\pm$ 6 ^a	101 $\pm$ 9 ^a	61 $\pm$ 5 ^b	97 $\pm$ 5 ^a	94 $\pm$ 6 ^a	101 $\pm$ 5 ^a	104 $\pm$ 3 ^a
	1000	32 $\pm$ 2 ^d	31 $\pm$ 3 ^b	107 $\pm$ 6 ^a	35 $\pm$ 2 ^c	99 $\pm$ 8 ^a	79 $\pm$ 6 ^b	110 $\pm$ 7 ^a	87 $\pm$ 5 ^b
Caco-2	C	100 $\pm$ 9 ^a	100 $\pm$ 9 ^a	100 $\pm$ 8 ^a	100 $\pm$ 10 ^a	100 $\pm$ 7 ^a	100 $\pm$ 8 ^a	100 $\pm$ 8 ^a	100 $\pm$ 10 ^a
	10	100 $\pm$ 6 ^a	101 $\pm$ 6 ^a	98 $\pm$ 7 ^a	95 $\pm$ 8 ^a	109 $\pm$ 7 ^b	109 $\pm$ 10 ^a	72 $\pm$ 5 ^b	94 $\pm$ 8 ^a
	100	98 $\pm$ 7 ^a	97 $\pm$ 11 ^a	89 $\pm$ 4 ^b	99 $\pm$ 9 ^a	100 $\pm$ 7 ^a	107 $\pm$ 8 ^a	68 $\pm$ 4 ^c	81 $\pm$ 7 ^b
	1000	43 $\pm$ 3 ^b	35 $\pm$ 3 ^b	79 $\pm$ 5 ^c	99 $\pm$ 6 ^a	66 $\pm$ 4 ^c	73 $\pm$ 6 ^b	69 $\pm$ 5 ^c	56 $\pm$ 5 ^c
A549	C	100 $\pm$ 7 ^a	100 $\pm$ 9 ^a	100 $\pm$ 3 ^a	100 $\pm$ 7 ^a	100 $\pm$ 7 ^a	100 $\pm$ 8 ^a	100 $\pm$ 3 ^a	100 $\pm$ 7 ^a
	10	99 $\pm$ 7 ^a	106 $\pm$ 5 ^a	92 $\pm$ 7 ^a	109 $\pm$ 8 ^a	94 $\pm$ 4 ^a	102 $\pm$ 10 ^a	82 $\pm$ 5 ^b	101 $\pm$ 7 ^a
	100	94 $\pm$ 5 ^a	99 $\pm$ 5 ^a	95 $\pm$ 6 ^a	97 $\pm$ 7 ^a	94 $\pm$ 5 ^a	107 $\pm$ 6 ^a	90 $\pm$ 5 ^c	99 $\pm$ 6 ^a
	1000	65 $\pm$ 3 ^b	63 $\pm$ 3 ^b	89 $\pm$ 5 ^b	74 $\pm$ 5 ^b	76 $\pm$ 3 ^b	67 $\pm$ 4 ^b	82 $\pm$ 7 ^b	77 $\pm$ 5 ^b
OE-33	C	100 $\pm$ 6 ^a	100 $\pm$ 3 ^a	100 $\pm$ 10 ^a	100 $\pm$ 10 ^a	100 $\pm$ 6 ^a	100 $\pm$ 3 ^a	100 $\pm$ 10 ^a	100 $\pm$ 10 ^a
	10	98 $\pm$ 8 ^a	96 $\pm$ 4 ^a	109 $\pm$ 7 ^a	92 $\pm$ 10 ^a	89 $\pm$ 3 ^b	97 $\pm$ 3 ^a	100 $\pm$ 7 ^a	86 $\pm$ 8 ^b
	100	86 $\pm$ 7 ^b	83 $\pm$ 2 ^b	105 $\pm$ 4 ^a	90 $\pm$ 5 ^a	101 $\pm$ 8 ^a	97 $\pm$ 4 ^a	99 $\pm$ 6 ^a	81 $\pm$ 8 ^b
	1000	41 $\pm$ 3 ^c	5 $\pm$ 1 ^c	85 $\pm$ 5 ^b	15 $\pm$ 2 ^b	76 $\pm$ 6 ^c	66 $\pm$ 2 ^b	94 $\pm$ 7 ^a	66 $\pm$ 3 ^c
T24	C	100 $\pm$ 6 ^a	100 $\pm$ 5 ^a	100 $\pm$ 11 ^a	100 $\pm$ 8 ^a	100 $\pm$ 6 ^a	100 $\pm$ 5 ^a	100 $\pm$ 11 ^a	100 $\pm$ 8 ^a
	10	97 $\pm$ 8 ^a	103 $\pm$ 4 ^a	95 $\pm$ 17 ^a	77 $\pm$ 7 ^b	94 $\pm$ 8 ^a	104 $\pm$ 6 ^a	100 $\pm$ 10 ^a	72 $\pm$ 7 ^b
	100	87 $\pm$ 7 ^b	102 $\pm$ 4 ^a	99 $\pm$ 16 ^a	52 $\pm$ 5 ^c	100 $\pm$ 8 ^a	103 $\pm$ 7 ^a	108 $\pm$ 9 ^a	75 $\pm$ 5 ^b
	1000	20 $\pm$ 2 ^c	2 $\pm$ 0 ^b	56 $\pm$ 5 ^b	0 $\pm$ 3 ^d	93 $\pm$ 8 ^a	71 $\pm$ 5 ^b	113 $\pm$ 10 ^a	49 $\pm$ 5 ^c

Values are expressed as the mean $\pm$ SD. Different letters within a column and cell line indicate significant differences ($p < 0.05$).

Table 4. Effect of dihydrocaffeic and dihydroferulic acids on the viability and proliferation of non-cancer (CCD-18Co) and cancer (Caco-2, A549, OE-33, and T24) human cell lines.

Cell lines	Treatment (μ M)	Dihydrocaffeic acid				Dihydroferulic acid			
		Viability		Proliferation		Viability		Proliferation	
		2 h	24 h	2 h	24 h	2 h	24 h	2 h	24 h
CCD-18Co	C	100 $\pm$ 5 ^a	100 $\pm$ 7 ^a	100 $\pm$ 10 ^a	100 $\pm$ 10 ^a	100 $\pm$ 8 ^a	100 $\pm$ 7 ^a	100 $\pm$ 8 ^a	100 $\pm$ 8 ^a
	10	87 $\pm$ 5 ^b	97 $\pm$ 7 ^a	79 $\pm$ 7 ^b	94 $\pm$ 10 ^{ab}	99 $\pm$ 6 ^a	102 $\pm$ 8 ^a	94 $\pm$ 10 ^a	104 $\pm$ 9 ^a
	100	75 $\pm$ 3 ^c	106 $\pm$ 5 ^a	74 $\pm$ 8 ^b	85 $\pm$ 9 ^b	99 $\pm$ 6 ^a	100 $\pm$ 4 ^a	98 $\pm$ 10 ^a	101 $\pm$ 10 ^a
	1000	79 $\pm$ 5 ^c	13 $\pm$ 1 ^b	65 $\pm$ 5 ^c	28 $\pm$ 3 ^c	89 $\pm$ 6 ^b	72 $\pm$ 5 ^b	102 $\pm$ 9 ^a	100 $\pm$ 6 ^a
Caco-2	C	100 $\pm$ 10 ^a	100 $\pm$ 10 ^a	100 $\pm$ 8 ^a	100 $\pm$ 8 ^a	100 $\pm$ 10 ^a	100 $\pm$ 4 ^a	100 $\pm$ 8 ^a	100 $\pm$ 8 ^a
	10	92 $\pm$ 7 ^a	102 $\pm$ 8 ^a	75 $\pm$ 5 ^b	106 $\pm$ 8 ^a	91 $\pm$ 7 ^{ab}	93 $\pm$ 8 ^a	101 $\pm$ 10 ^a	106 $\pm$ 8 ^a
	100	91 $\pm$ 5 ^a	99 $\pm$ 9 ^a	77 $\pm$ 8 ^b	98 $\pm$ 9 ^a	90 $\pm$ 5 ^b	83 $\pm$ 8 ^b	107 $\pm$ 9 ^a	98 $\pm$ 9 ^a
	1000	75 $\pm$ 3 ^b	97 $\pm$ 10 ^a	69 $\pm$ 5 ^b	98 $\pm$ 9 ^a	75 $\pm$ 3 ^c	73 $\pm$ 5 ^c	99 $\pm$ 7 ^a	98 $\pm$ 9 ^a
A549	C	100 $\pm$ 7 ^a	100 $\pm$ 8 ^a	100 $\pm$ 3 ^a	100 $\pm$ 3 ^a	100 $\pm$ 2 ^a	100 $\pm$ 7 ^a	100 $\pm$ 9 ^{ab}	100 $\pm$ 8 ^a
	10	97 $\pm$ 6 ^a	96 $\pm$ 7 ^a	86 $\pm$ 7 ^b	82 $\pm$ 5 ^b	100 $\pm$ 4 ^a	96 $\pm$ 9 ^a	106 $\pm$ 12 ^{ab}	100 $\pm$ 10 ^a
	100	102 $\pm$ 9 ^a	100 $\pm$ 6 ^a	92 $\pm$ 6 ^{ab}	90 $\pm$ 5 ^a	98 $\pm$ 5 ^a	92 $\pm$ 7 ^a	93 $\pm$ 9 ^a	99 $\pm$ 6 ^a
	1000	78 $\pm$ 3 ^b	80 $\pm$ 5 ^b	86 $\pm$ 8 ^b	82 $\pm$ 7 ^b	79 $\pm$ 5 ^b	79 $\pm$ 4 ^b	113 $\pm$ 11 ^b	76 $\pm$ 8 ^b
OE-33	C	100 $\pm$ 6 ^a	100 $\pm$ 3 ^a	100 $\pm$ 4 ^a	100 $\pm$ 10 ^a	100 $\pm$ 3 ^a	100 $\pm$ 3 ^a	100 $\pm$ 6 ^a	100 $\pm$ 10 ^a
	10	89 $\pm$ 12 ^a	107 $\pm$ 4 ^b	93 $\pm$ 8 ^a	98 $\pm$ 9 ^a	101 $\pm$ 4 ^a	96 $\pm$ 5 ^{ab}	103 $\pm$ 4 ^a	96 $\pm$ 10 ^a
	100	91 $\pm$ 8 ^a	125 $\pm$ 4 ^c	83 $\pm$ 8 ^a	117 $\pm$ 12 ^b	99 $\pm$ 4 ^a	92 $\pm$ 5 ^b	98 $\pm$ 8 ^a	104 $\pm$ 8 ^a
	1000	100 $\pm$ 8 ^a	10 $\pm$ 0 ^d	73 $\pm$ 5 ^b	30 $\pm$ 4 ^c	86 $\pm$ 4 ^b	61 $\pm$ 4 ^c	88 $\pm$ 5 ^b	76 $\pm$ 6 ^b
T24	C	100 $\pm$ 6 ^a	100 $\pm$ 5 ^a	100 $\pm$ 5 ^a	100 $\pm$ 10 ^a	100 $\pm$ 7 ^a	100 $\pm$ 5 ^a	100 $\pm$ 11 ^a	100 $\pm$ 8 ^a
	10	96 $\pm$ 6 ^a	105 $\pm$ 5 ^{ab}	96 $\pm$ 9 ^a	108 $\pm$ 6 ^a	92 $\pm$ 8 ^{ab}	100 $\pm$ 7 ^a	110 $\pm$ 11 ^a	80 $\pm$ 6 ^b
	100	80 $\pm$ 6 ^b	110 $\pm$ 3 ^b	122 $\pm$ 7 ^b	116 $\pm$ 10 ^a	91 $\pm$ 7 ^{ab}	93 $\pm$ 8 ^a	97 $\pm$ 13 ^a	79 $\pm$ 5 ^b
	1000	15 $\pm$ 1 ^c	7 $\pm$ 1 ^c	13 $\pm$ 1 ^c	2 $\pm$ 1 ^b	86 $\pm$ 8 ^b	74 $\pm$ 7 ^b	91 $\pm$ 8 ^a	52 $\pm$ 5 ^c

Values are expressed as the mean $\pm$ SD. Different letters within a column and cell line indicate significant differences ($p < 0.05$).

Table 5. Effect of caffeine at the indicated concentrations, for 2 and 24 h, on the viability and proliferation of non-cancer (CCD-18Co) and cancer (Caco-2, A549, OE-33, and T24) human cell lines.

Cell lines	Treatment (μ M)	Caffeine			
		Viability		Proliferation	
		2 h	24 h	2 h	24 h
CCD-18Co	C	100 $\pm$ 6 ^a	100 $\pm$ 7 ^a	100 $\pm$ 10 ^a	100 $\pm$ 10 ^a
	10	101 $\pm$ 6 ^a	99 $\pm$ 8 ^a	106 $\pm$ 6 ^a	98 $\pm$ 8 ^a
	100	100 $\pm$ 8 ^a	98 $\pm$ 7 ^a	103 $\pm$ 8 ^a	106 $\pm$ 8 ^a
	1000	94 $\pm$ 6 ^a	86 $\pm$ 6 ^b	110 $\pm$ 10 ^a	107 $\pm$ 9 ^a
Caco-2	C	100 $\pm$ 5 ^a	100 $\pm$ 7 ^a	100 $\pm$ 8 ^a	100 $\pm$ 8 ^a
	10	101 $\pm$ 8 ^a	110 $\pm$ 8 ^a	101 $\pm$ 8 ^a	99 $\pm$ 8 ^a
	100	98 $\pm$ 4 ^{ab}	101 $\pm$ 5 ^a	116 $\pm$ 5 ^b	108 $\pm$ 11 ^a
	1000	91 $\pm$ 6 ^b	109 $\pm$ 6 ^a	98 $\pm$ 8 ^a	106 $\pm$ 9 ^a
A549	C	100 $\pm$ 6 ^a	100 $\pm$ 7 ^a	100 $\pm$ 8 ^a	100 $\pm$ 8 ^a
	10	104 $\pm$ 9 ^a	94 $\pm$ 4 ^{ab}	84 $\pm$ 6 ^b	117 $\pm$ ^b
	100	110 $\pm$ 9 ^a	90 $\pm$ 5 ^b	79 $\pm$ 6 ^{bc}	106 $\pm$ 7 ^a
	1000	109 $\pm$ 5 ^a	91 $\pm$ 3 ^b	73 $\pm$ 4 ^c	82 $\pm$ 7 ^b
OE-33	C	100 $\pm$ 6 ^a	100 $\pm$ 8 ^a	100 $\pm$ 7 ^{ab}	100 $\pm$ 10 ^a
	10	100 $\pm$ 5 ^a	91 $\pm$ 5 ^{ab}	116 $\pm$ 5 ^a	103 $\pm$ 10 ^a
	100	108 $\pm$ 6 ^a	95 $\pm$ 7 ^a	98 $\pm$ 10 ^b	90 $\pm$ 7 ^a
	1000	99 $\pm$ 5 ^a	82 $\pm$ 5 ^b	99 $\pm$ 11 ^{ab}	92 $\pm$ 10 ^a
T24	C	100 $\pm$ 4 ^a	100 $\pm$ 5 ^a	100 $\pm$ 5 ^a	100 $\pm$ 10 ^a
	10	96 $\pm$ 3 ^a	103 $\pm$ 4 ^a	120 $\pm$ 10 ^b	115 $\pm$ 8 ^a
	100	101 $\pm$ 7 ^a	104 $\pm$ 3 ^a	101 $\pm$ 13 ^a	111 $\pm$ 9 ^a
	1000	99 $\pm$ 6 ^a	84 $\pm$ 6 ^b	114 $\pm$ 7 ^{ab}	67 $\pm$ 6 ^b

Values are expressed as the mean $\pm$ SD. Different letters within a column and cell line indicate significant differences ($p < 0.05$).

Table 6. Effect of 10 µg/mL GCBE and YME or 10 µM 5-CQA, 3,5-DCQA, CA, FA, DHCA, DHFA, and caffeine decreasing viability and proliferation of the studied cell lines after short (S, 2 h) or long (L, 24 h) incubation times. (-, no effect).

	Caco-2		A549		OE-33		T24		CCD-18Co	
	Viability	Proliferation	Viability	Proliferation	Viability	Proliferation	Viability	Proliferation	Viability	Proliferation
GCBE	-	S	-	-	L	-	L	-	-	-
YME	S,L	S	S,L	L	S,L	-	S,L	L	S,L	L
5-CQA	-	-	-	-	S,L	-	-	-	S	-
3,5-DCQA	-	L	-	-	-	-	-	L	-	-
CA	-	-	-	-	-	-	-	L	S	-
FA	-	S	-	S	S	L	-	L	-	-
DHCA	-	S	-	S,L	-	-	-	-	S	S
DHFA	-	-	-	-	-	-	-	L	-	-
CAF	-	-	-	S,L	-	-	-	-	-	-