In Vivo and In Vitro Genotoxic and Epigenetic Effects of Two Types of Cola Beverages and Caffeine: A Multiassay Approach

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The aim of this work was to assess the biological and food safety of two different beverages: Classic Coca Cola™ (CCC) and Caffeine-Free Coca Cola (CFCC). To this end, we determined the genotoxicological and biological effects of different doses of lyophilised CCC and CFCC and Caffeine (CAF), the main distinctive constituent. Their toxic/antitoxic, genotoxic/antigenotoxic, and chronic toxicity (lifespan assay) effects were determined in vivo using the Drosophila model. Their cytotoxic activities were determined using the HL-60 in vitro cancer model. In addition, clastogenic DNA toxicity was measured using internucleosomal fragmentation and SCGE assays. Their epigenetic effects were assessed on the HL-60 methylation status using some repetitive elements. The experimental results showed a slight chemopreventive effect of the two cola beverages against HL-60 leukaemia cells, probably mediated by nonapoptotic mechanisms. Finally, CCC and CAF induced a global genome hypomethylation evaluated in LINE-1 and Alu MI repetitive elements. Overall, we demonstrated for the first time the safety of this famous beverage in in vivo and in vitro models.

1. Introduction

Diet may modify cancer risk and tumor behavior since nongenotoxicological modulation as epigenetic regulatory processes may be susceptible to changes caused by environmental factors. Therefore, constituents in food and dietary supplements could be involved in changes in the gene expression, increasing the risk of developing some type of cancer all over the life inducing epigenetic changes [1, 2]. Genotoxicological screening tests have been extensively used over time for assessing the health properties of compounds prior to being considered as safe substances. Nowadays, the list of foods with documented health-benefit activities is endless, and scientific evidence supporting the concept of health-promoting food ingredients is steadily growing [3].

Originally developed as medical supplements, cola-based drinks and several beverages such as beer and wine were proposed as medicinal substances [4, 5]. However, a relationship between the consumption of these beverages and an increase in the prevalence of several diseases such as child obesity, diabetes, hypertension, and dental diseases was also demonstrated [6–8]. In spite of the worldwide importance and spread of cola beverages, studies assessing their effects on health and wellbeing are quite scarce [9]. On the contrary, caffeine (CAF), which is a key ingredient in cola beverages as well as in coffee, tea, and some medicines, is one of the most investigated substances, probably due to the lack of consistent results over time [10–12]. In D. melanogaster, CAF has been related to a positive lifespan increase [13], but the results were contradictory when apoptotic and DNA-programmed fragmentation effects were studied [14, 15].

Drosophila is being used more frequently as a model for many human diseases, including cancer [16–18]. Reiter et al.
2. Materials and Methods

2.1. Samples. Two coke beverages, CCC and CFCC, and one of their principal compounds, CAF (1,3,7-trimethylpurine-2,6-dione), were assayed. Drinks were bought at a local market (Córdoba, Spain), lyophilised (SCAI, University of Córdoba), and stored at room temperature in a dark and dry atmosphere until use. CAF was obtained from ACROS (108.160100).

The analysis of CAF content was performed by HPLC/DAD (Perkin Elmer) in reverse phase (column C-18, 150 × 2.1 mm), with a gradient of water/phosphoric buffer and methanol as mobile phase at 1 mL/min flow rate. The injection volume was 10 μL and the column temperature at 45 °C. The CAF identification was performed by retention time and spectrum adjustment obtained by DAD (SCAI, University of Córdoba).

2.2. In Vivo Fly Stocks. Two Drosophila melanogaster strains with genetic markers that affect the wing-hair phenotype were used: (i) mwh/mwh, carrying the recessive mutation mwh (multiple wing hairs) [31] and (ii) flr3/In (3LR) TM3, rip3 sep bx34ce Ba5, where flr3 (flare) [32] marker is a homozygous recessive lethal mutation which is viable in homozygous somatic cells once larva start developing and produce deformed trichomonas.

2.3. In Vitro Cell Culture Conditions. Promyelocytic human leukaemia (HL-60) cells were grown in RPMI-1640 medium (Sigma, R5886) supplemented with heat-inactivated foetal bovine serum (Linus, S01805), L-glutamine 200 mM (Sigma, G7513), and 1x antibiotic-antimycotic solution (Sigma, A5955). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO2. Cultures were plated at 2.5 × 104 cells/mL density in 10 mL culture bottles and passed every 2 days.

2.4. In Vivo Assays

2.4.1. Toxicity and Antitoxicity Assays. Toxicity was assayed according to our standard protocols. Both lyophilised beverages (CCC and CFCC) were tested at five concentrations: 0.7, 3, 6, 25, and 100 mg/mL. The same number of CAF concentrations (0.04 mM, 0.016 mM, 0.032 mM, 0.128 mM, and 0.51 mM) was also tested according to quantity declared in the present study (75.544 mg/L). Negative (H2O) and positive (0.15 M H2O2) toxicant concurrent controls were also assayed. Test groups consisted of larvae fed with Drosophila Instant Medium (Formula 4–24, Carolina Biological Supply, Burlington, NC) supplemented with the beverage concentrations tested. Emerging adults of all groups were counted and toxicity was determined as the percentage of hatched individuals in each treatment compared with the negative control. Antitoxicity was assessed using the same procedure and experimental concentrations as in toxicity assays, but in combined treatments with 0.15 M H2O2 and comparing the percentage of emerging adults with the positive toxicant control [34]. Chi-square test was used to determine if the tested compounds significantly inhibited the survival of flies. Negative control values were considered as those expected in Chi-square formula used in toxicity assay and positive control values in antitoxicity assays [35]. The same concentrations of toxicity and antitoxicity assays within the same substance were also compared.

2.4.2. Genotoxicity and Antigenotoxicity Assays. Genotoxicity assays were carried out following the wing spot test standard procedure [20]. Briefly, transheterozygous larvae for mwh and flr3 genes were obtained by crossing four-day-old virgin flr3 females with mwh males in a 2:1 ratio. Four days after fertilization, females were allowed to lay eggs in fresh yeast medium (25 g yeast and 4 mL sterile distilled water) for 8 h in order to obtain synchronised larvae. After 72 h, larvae were collected, washed with distilled water, and clustered in groups of 100 individuals. Each group was fed with a mixture containing 0.85 g Drosophila Instant Medium (Formula 4–24, Carolina Biological Supply, Burlington, NC) and 4 mL water supplemented with the tested compounds at fixed concentrations (the highest and second lowest from the toxicity assays) and negative (H2O) and positive (0.15 M H2O2) controls until pupae hatching (10–12 days). Adult flies were collected.
and stored in 70% ethanol until the wings were removed and mounted on slides using Faure’s solution. Mutant spots were assessed in both dorsal and ventral surfaces of the wings in a bright light microscope at 400x magnification. The frequencies of each type of mutant clone per wing (single, large, or twin spot) were compared to the concurrent negative control and analysed applying the binomial Kastenbaum and Bowman Test [36]. Antigenotoxicity tests were performed following the method described by Anter et al. [37]. The same compounds and concentrations were assayed in combined treatment with hydrogen peroxide (0.15 M) acting as concurrent genotoxicant. Single and twin spots per wing were also recorded and compared with the concurrent positive control as described before. The recombination percentage was calculated following Valadares et al. [38] procedure and the control as described before. The recombination percentage was recorded and compared with the concurrent positive control. Single and twin spots per wing were also recorded and compared with the concurrent positive control as described before. The recombination percentage was calculated following Valadares et al. [38] procedure and the inhibition percentages (IP) for the combined treatments were calculated from the control-corrected frequencies of clone formation per 10⁵ cells, according to Abraham [39]: IP = [(genotoxin alone – combined treatment)/genotoxin alone] × 100.

2.5.1. Cytotoxicity Assay. The effect of the assayed compounds on cell viability was determined by the trypan blue exclusion test according to our standard procedures [37]. HL-60 cells were placed in 96-well plates (2 × 10⁴ cells/mL) and cultured for 72 h and supplemented with the same concentrations of CCC, CFCC, and CAF from our toxicity/antitoxicity assays. The wide range of tested concentrations was intended to estimate the cytotoxic inhibitory concentration 50 (IC₅₀). After culture, cells were stained with a 1:1 volume ratio of trypan blue dye (Sigma, T8154) and counted in a Neubauer-chamber at 100x magnification. The survival percentage of each treatment compared with the control was recorded in three independent replicates.

2.5.2. DNA Fragmentation Status. The ability of our compounds to induce DNA fragmentation was determined as described by Anter et al. [40]. Briefly, 10⁶ HL-60 cells were cocultured with 5 different concentrations of CCC, CFCC, and CAF (as selected in the toxicity/antitoxicity assays) for 5 h. After treatment, genomic DNA was extracted using a commercial kit (Blood Genomic DNA Extraction Mini Spin Kit, Canvax Biotech, Cordoba, Spain). Subsequently, DNA was incubated overnight with RNase at 37°C and quantified in a spectrophotometer (Nanodrop® ND-1000). Finally, 1200 ng DNA was electrophoresed in a 2% agarose gel for 120 min at 50 V, stained with ethidium bromide, and visualised under UV light. The apoptosis process is recognised by the appearance of internucleosomal DNA fragments that are multiple of 200 base pairs.

2.5.3. Clastogenicity: SCGE (Comet Assay). DNA integrity was assayed by SCGE as described by Olive and Banáth [41] with minor modifications. HL-60 cells (5 × 10⁵) in exponential growing phase were incubated in 1.5 mL of culture medium supplemented with different CCC, CFCC, and CAF (0.004, 0.032, and 0.51 mM) concentrations for 5 h. After treatment, cells were washed twice and adjusted to 6.25 × 10⁶ cells/mL in PBS. Electrophoresis gels were prepared pouring a 1:4 dilution (cells in liquid low-melting-point agarose at 40°C, A4018, Sigma) into slides. Gels were covered with a coverslip and allowed to solidify at RT for 30 min. Once the slides solidified, the coverslips were carefully removed and slides were bathed in freshly prepared lysing solution (2.5 M NaCl, 100 mM Na-EDTA, 10 mM Tris, 250 mM NaOH, 10% DMSO, and 1% Triton X-100; pH 13) for 1 h at 4°C. Thereafter, slides were equilibrated in alkaline electrophoresis buffer (300 mM NaOH and 1 mM Na-EDTA, pH 13) for 20–30 min at 4°C. Once equilibrated, the slides underwent electrophoresis (20 V, 400 mA for 15 min) in the dark and were immediately neutralised in cold neutral solution (0.4 M Tris-HCl buffer, pH 7.5) for 10 min. Finally, slides were dried overnight at RT in the dark. Gels were stained with 7 μL propidium iodide and photographed in a Leica DM2500 microscope at 400X magnification. At least 100 single cells from each treatment were analysed using the Open Comet™ software [42]. The Tail Moment (TM) data were analysed applying a one-way ANOVA and post hoc Tukey’s test with SPSS Statistics for Windows, Version 19.0 (IBM 2010), to determine the effect of the tested compounds on HL-60 cell DNA integrity.

2.5.4. Methylation Status of HL-60 Cells. HL-60 cells were treated with different concentrations of CCC (3 mg/mL and 100 mg/mL), CFCC (3 mg/mL and 100 mg/mL), and CAF (0.016 mM and 0.51 mM) for 5 hour. Then, DNA was extracted similarly to previously described DNA fragmentation assay. After that, the DNA was converted with bisulphite (EZ DNA Methylation-Gold™ Kit). Bisulphite-modified DNA was used for fluorescence-based real-time quantitative Methylation-Specific PCR (qMSP) using 5 μM of each forward and reverse primer (Isogen Life Science BV), 2 μL of iTaq™ Universal SYBR® Green Supermix (Bio-Rad, it contains antibody-mediated hot-start iTaq DNA polymerase, dNTPs, MgCl₂, SYBR Green I Dye, enhancers, stabilizers,
Table 1: Primers information [43].

| Primers | Forward primer sequence 5’ to 3’ (N) | Reverse primer sequence 5’ to 3’ (N) |
|---------|-------------------------------------|--------------------------------------|
| ALU-C4  | GGTTAGGTATAGGTTTATATTGTAATTTAGTA   | ATTAACCTAACTAAATCTCTTAAACCTCAATTTCA  |
| ALU-M1  | ATTATGTTAGTTAGGTATTTTCGATTTT       | CAATCGACCGAAGCGGA (-17)              |
| LINE-1-M1 | GGACGTATTTGAAAATCCGGA (-21)       | AATCTCAGGATACCGGT (-19)              |
| SAT-α-MI | TTAGGGATTTTAAATATCAGTTTTGAT        | AATTCTAAAAATATTCCCTTCTCAATTACGTAAA   |

Table 2: Toxicity and antigenotoxicity levels of CCC, CFCC, and CAF in D. melanogaster.

| CCC (mg/mL) | Survival (%) | CFCC (mg/mL) | Survival (%) | CAF (mM) | Survival (%) |
|-------------|--------------|--------------|--------------|---------|--------------|
|             | Simple treatment | Combined treatment | Simple treatment | Combined treatment | Simple treatment | Combined treatment |
| 0           | 100          | 100          | 0            | 100      | 100          | 100           |
| H₂O₂        |              |              | H₂O₂         |              |              |               |
| 0.7         | 100          | 100* (3)     | 0.7          | 87.66    | 83.33*       | 0.004         |
| 3           | 100          | 92*          | 3            | 88.66    | 100*         | 0.016         |
| 6           | 100          | 85.66*       | 6            | 96.66    | 84.66*       | 0.032         |
| 25          | 100          | 74.66*       | 25           | 87.33    | 75*          | 0.127         |
| 100         | 92           | 65*          | 100          | 77* (4)  | 45.66*       | 0.51          |

(1) Data are expressed as percentage of survival adults with respect to 300 untreated 72-hour-old larvae from three independent experiments. (2) Combined treatments using standard medium and 0.15 M hydrogen peroxide. (3) Asterisks (*) indicate significant differences (one tail) with respect to the hydrogen peroxide control group and (4) untreated control group. * Chi-square value higher than 5.02 [35]. Delta letter (Δ) means significant differences between the same concentrations used in toxicity and antigenotoxicity assays comparing within the same treated substance.

and a blend of a passive reference dyes including ROX and fluorescein) and 25 ng of bisulphite converted genomic DNA.

PCR conditions included initial denaturalisation at 95 °C for 3 minutes and amplification which consisted of 45 cycles at 95 °C for 10 seconds, 60 °C for 15 seconds, and 72 °C for 15 seconds, taking picture at the end of each elongation cycle. After that, melting curve was determined increasing 0.5 °C each 0.05 seconds from 60 °C to 95 °C and taking pictures.

QMS was carried out in 48-well plates in MiniOpticon Real-Time PCR System (MJ Mini Personal Thermal Cycler, Bio-Rad) and were analysed by Bio-Rad CFX Manager 3.1 software. The housekeeping Alu-C4 was used as a reference to correct for total DNA input. Alu-C4 and the target repetitive elements Alu-M1, LINE-1, and Sat-α were obtained from Isogen Life Science and their sequences are shown in Table 1. Each sample was analysed in triplicate.

The results of each Ct were obtained from each qMS. Data were normalised with the housekeeping Alu-C4 using the Nikolaidis et al. [45] and Liloglou et al. [46] comparative Ct method (ΔΔCt). One-way ANOVA and post hoc Tukey’s test are used to evaluate the differences between the tested compounds, repetitive elements, and concentrations.

### 3. Results

#### 3.1. In Vivo Assays

3.1.1. Toxicity/Antitoxicity. Toxicity assays showed that CCC, CFCC, and CAF are not toxic to D. melanogaster larvae (Table 2, simple treatment).

CFCC was significantly toxic only at the highest concentration. All the studies and results on CAF must be viewed with caution, since CAF shows a dose-dependent effect and it is known to be toxic at high concentrations [47].

Antitoxicity results showed that CCC and CFCC exerted an overall significant protective effect against H₂O₂-induced toxicity in Drosophila larvae, at most of the tested concentrations, with a negative dose-dependent effect (Table 2, combined treatment). Although CCC and CFCC were able to revert in some extent the damage caused by hydrogen peroxide, the survival obtained in antitoxicity assay was lower than toxicity assay in flies treated with 6, 25, and 100 mg/mL of these beverages. On the other hand, the 2 lowest concentrations were able to totally revert the oxidative damage caused by the used genotoxin. On the contrary, none of the assayed CAF concentrations produced any significant protective effect.

3.1.2. Genotoxicity/Antigenotoxicity. Table 3 shows the results obtained in the genotoxicity assays (SMART). After applying binomial Kastenbaum-Bowman Test, all tested substances were nongenotoxic with negative results.

Hydrogen peroxide is a potent inducer of oxidative damage and mediator of ageing [48]. It has been used as a genotoxic in many assays using Drosophila as an experimental animal [23, 40] as well as in other models. The mutation rates obtained in our study for this genotoxin (0.438 clones/wing) fall into the usual range described by different laboratories, validating the accuracy of the geno/antigenotoxicity assays.

One of the important characteristics of the SMART is that it allows quantification of the different types of DNA...
Table 3: Genotoxicity and antigenotoxicity of CCC, CFCC, and CAF in the *Drosophila* wing spot test.

| Compound                  | Number of wings | Small single spots (1-2 cells) | Large simple spots (>2 cells) | Twin spots \(m = 5\) | Total spots \(m = 2\) | Observed | Control corrected | Recombination (%) \(^{(3)}\) | IP (%) \(^{(4)}\) |
|--------------------------|----------------|-------------------------------|-------------------------------|----------------------|----------------------|----------|-------------------|---------------------------|----------------|
| H\(_2\)O \(mwh/\text{flr}^3\) | 80             | 0.25 (20)                     | 0.013 (1)                     | 0                    | 0.263 (21)          | 1.078    |                   |                           |                |
| H\(_2\)O \(mwh/TM3\) \(^{(5)}\) | 80             | 0.04 (3)                      | 0                             | 0.04 (3)             | 0.17                |          |                   |                           |                |
| \(mwh/\text{flr}^3\)     | 80             | 0.313 (25)                    | 0.088 (7)                     | 0.038 (3)            | 0.438 (35)          | 1.795    | 0.717             | 54.37                  |                |
| \(mwh/TM3\)              | 80             | 0.188 (15)                    | 0.013 (1)                     | 0.20 (16)            | 0.819               | 0.286    |                   |                           |                |

**Simple treatment \((mwh/\text{flr}^3)\)**

| CCC (mg/mL) \([3.125]\) | 80             | 0.275 (22)                    | 0.025 (2)                    | 0                    | 0.3 (24)−          | 1.23     | 0.152             |                           |                |
| \([100]\)                  | 78             | 0.19 (15)                     | 0.038 (3)                    | 0.026 (2)            | 0.256 (20)−        | 1.05     | −0.028            |                           |                |
| CFCC (mg/mL) \([3.125]\)  | 80             | 0.175 (14)                    | 0.075 (6)                    | 0                    | 0.25 (20)−         | 1.025    | −0.053            |                           |                |
| \([100]\)                  | 80             | 0.225 (18)                    | 0.075 (6)                    | 0                    | 0.3 (24)−          | 1.23     | 0.152             |                           |                |
| Caffeine (mM) \([0.016]\) | 80             | 0.26 (21)                     | 0.03 (3)                     | 0                    | 0.3 (24)−          | 1.23     | 0.152             |                           |                |
| \([0.51]\)                 | 86             | 0.21 (18)                     | 0.058 (5)                    | 0.012 (1)            | 0.28 (24)−         | 1.148    | 0.07              |                           |                |

**Combined treatment \((mwh/\text{flr}^3)\)**

| CCC (mg/mL) \([3.125]\) | 82             | 0.11 (9)                      | 0.037 (3)                    | 0                    | 0.146 (12)\(^*\)  | 0.6      | −0.478            | 74.6                    | 166.67          |
| \([100]\)                  | 83             | 0.217 (18)                    | 0.048 (4)                    | 0                    | 0.265 (22)\(^*\)  | 1.086    | 0.008             | 69.8                    | 98.88           |
| CFCC (mg/mL) \([3.125]\)  | 82             | 0.195 (16)                    | 0.073 (6)                    | 0                    | 0.268 (22)\(^*\)  | 1.1      | 0.022             | 55.5                    | 96.93           |
| \([100]\)                  | 80             | 0.175 (14)                    | 0.05 (4)                     | 0                    | 0.225 (18)\(^*\)  | 0.922    | −0.156            | 64.4                    | 121.76          |
| Caffeine (mM) \([0.016]\) | 80             | 0.16 (13)                     | 0.025 (2)                    | 0                    | 0.188 (15)\(^*\)  | 0.77     | −0.308            | 89.6                    | 142.96          |
| \([0.51]\)                 | 80             | 0.325 (26)                    | 0.125 (10)                   | 0                    | 0.45 (36)\(^\Delta\) | 1.844    | 0.766             |                           |                |

**Combined treatment \((mwh/TM3)\)**

| CCC (mg/mL) \([3.125]\) | 79             | 0.038 (3)                     | 0                              | 0.038 (3)\(^*\)      | 0.158    | −0.35             |                           |                |
| \([100]\)                  | 80             | 0.08 (6)                      | 0                              | 0.08 (6)\(^*\)       | 0.328    | −0.21             |                           |                |
| CFCC (mg/mL) \([3.125]\)  | 82             | 0.12 (10)                     | 0                              | 0.12 (10)\(^\beta\) | 0.49     | 0.32              |                           |                |
| \([100]\)                  | 80             | 0.08 (6)                      | 0                              | 0.08 (6)\(^*\)       | 0.328    | 0.158             |                           |                |
| Caffeine (mM) \([0.016]\) | 82             | 0.02 (2)                      | 0                              | 0.02 (2)\(^*\)       | 0.08     | −0.09             |                           |                |
| \([0.51]\)                 |                |                               |                                |                      |          |                   |                           |                |

\(^{(1)}\) Statistical diagnosis according to Frei and Würfler [44]; + (positive) and − (negative) versus negative control; • (positive), Δ (negative), and \(\beta\) (inconclusive) versus respective positive control; \(m\): multiplication factor. Kastenbaum-Bowman Test without Bonferroni correction; probability level: \(\alpha = \beta = 0.05\). Number of spots in parentheses.

\(^{(2)}\) Frequency of clone formation: clones/wings/24,400 cells.

\(^{(3)}\) Recombination percentage is calculated according to Valadares et al. [38].

\(^{(4)}\) Inhibition percentage values were included when appropriate.

\(^{(5)}\) Balancers-heterozygous wings.

Damages induced by genotoxic compounds (recombination versus mutation). In the balancer-heterozygous genotype \((mwh/TM3, \text{Bd}^3)\) \(mwh\) spots are produced predominantly by somatic point mutation and chromosome aberrations. By scoring \(mwh/TM3\) balancers-heterozygous wings it is possible to quantify the recombinogenic potency of the positive control. The frequency of \(mwh\) clones on the marker transheterozygous wings (\(mwh\) single spots plus twin spots) was compared with the frequency of \(mwh\) spots on the balancer transheterozygous wings. The difference in \(mwh\)
indicating lifespan decrease. Data are expressed as mean value ± SE.

### 3.1.3. Chronic Treatment

Antimutagenic activity rather than antirecombinogenic activity. Therefore, our compounds induced a remarkable antimutagenic effect on promyelocytic cells (data not shown). The highest tested concentration (20.4 mM), which was higher than 19%.

Clone frequency is a direct measure of the proportion of recombinogenicity. A total mutation rate of 0.2 in the mwh/TM3 wings has been obtained and when it is compared to the mutation rate of the marker wings (0.438) thus 54% [1 – (0.819/1.795)×100] of the genotoxic events induced by H2O2 are due to recombinogenicity.

Antigenotoxicity results indicated that CCC, CFCC, and CAF could desmutagenise the genotoxic effect of H2O2, except for the highest tested concentration of CAF. CCC was the most antigenotoxic tested compound (IP: 166.67% and 98.88% for 3.125 and 100 mg/mL, resp.). CFCC IP was 96.93% and 121.76% for similar CCC concentrations and the 0.016 mM CAF IP was 142.96%. All the clone frequencies in combined treatment were compared to the positive control H2O2.

Recombinogenicity values for combined treatments ranged between 55 and 89%, where these figures are higher than their respective recombinogenicity induced by the positive control (54%). Therefore, our compounds induced antimutagenic activity rather than antirecombinogenic activity.

#### 3.1.3. Chronic Treatment

Kaplan-Meier curves and averages of flies’ lifespan are shown in Figure 1 and Table 4, respectively. The longevity of flies was increased by the CCC tested concentrations 3.125 and 25 mg/mL (p ≤ 0.05). CAF also increased the survival rates of *Drosophila* at intermediate concentrations (0.032 and 0.127 mM). CFCC significantly decreased the lifespan of *Drosophila* only at 100 mg/mL (p ≤ 0.001). On average whereas CCC and CAF increased *Drosophila* lifespan more than 15%, CFCC decreased it less than 19%.

Healthspan results (portion ≥ 80% of lifespan curves) are shown in Table 4. CCC increased the average healthspan of flies; such increase was significant only at 100 mg/mL (p ≤ 0.05) since this concentration raised the mean value by 22.4% to the control. Conversely, CFCC only significantly increased the mean healthspan value at 6.25 mg/mL (12%; p ≤ 0.05). CAF increased healthspan at the lowest (0.004 mM for 55.73%; p ≤ 0.01), the intermediate (0.032 mM for 40.32%; p ≤ 0.05), and the highest (0.51 mM for 26.41%; p ≤ 0.05) concentration.

#### 3.2. In Vitro Assays

3.2.1. Cytotoxicity. Both beverages were cytotoxic to the HL-60 line, inhibiting leukaemia cell growth with a positive dose effect (Figure 2). Furthermore, IC50 was similar for both beverages (19 and 20 mg/mL for CCC and CFCC, resp.). CAF concentrations were experimentally increased to reach IC50 since the original tested concentrations did not induce any remarkable cytotoxic effect on promyelocytic cells (data not shown). The highest tested concentration (20.4 mM), which was 40 times higher than the corresponding content in CCC and CFCC, could only inhibit cell growth in about 40%, without reaching IC50.

| Caffeine (mg/mL) | Mean lifespan (days) | Mean lifespan difference (%) | Healthspan (80th percentile) (days) | Healthspan difference (%) |
|------------------|----------------------|------------------------------|-------------------------------------|--------------------------|
| Control          | 59.68 ± 2.92         | 0.00 ± 0.00                  | 32.63 ± 1.49                       | 0.00 ± 0.00               |
| 0.78             | 59.72 ± 2.6          | 0.04 ± 0.00                  | 29.67 ± 2.28                      | −9.08 ± 0.00              |
| 3.125            | 69.78 ± 2.82         | 16.93 ± 0.00                 | 37.33 ± 2.58                      | 15.63 ± 0.00              |
| 6.25             | 59.81 ± 2.58         | 0.23 ± 0.00                  | 37.30 ± 2.26                      | 14.32 ± 0.00              |
| 25               | 69.16 ± 3.39         | 15.90 ± 0.00                 | 34.48 ± 2.17                      | 5.57 ± 0.00               |
| 100              | 64.34 ± 3.77         | 7.82 ± 0.00                  | 39.95 ± 0.96                      | 22.44 ± 0.00              |

**Table 4:** Effects of CCC, CFCC, and CAF treatments on the *Drosophila melanogaster* mean lifespan and healthspan.

- The difference was calculated by comparing treated flies with the concurrent water control. Positive numbers indicate lifespan increase and negative numbers indicate lifespan decrease. Data are expressed as mean value ± SE. *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001 significances obtained with the log-rank (Mantel-Cox) test.
3.2.2 DNA Stability Evaluation. The typical ladder pattern of cells with fragmented internucleosomal DNA was weakly induced only by CCC and CFCC at 25 mg/mL supplementation (Figure 3) and it was not observed with any CAF treatment.

The ability of the compounds to induce strand breaks in the DNA structure was determined by the alkaline comet assay. Based on the results obtained with the previous in vitro assays (cytotoxicity and DNA internucleosomal fragmentation), only three concentrations of each compound were tested. After 5 h exposure, all compounds induced a significant \( p \leq 0.001 \) increase in the TM parameter with respect to the control, except for CFCC at a 25 mg/mL concentration and CAF at 0.51 mM (Figure 4). Despite such significant increase, all TM values were lower than 4.4, suggesting that these compounds mainly affect HL-60 cells through a necrotic pathway.

The relative normalised methylation status (RMS) of the three repetitive sequences (LINE-1, Alu, and Sat-\( \alpha \)) in HL-60 cell line treated with the tested compounds is shown in Figure 5. RMS decreased when cells were treated with CCC in both Alu MI and LINE-1 sequences in a negative dose-dependent manner. However, we obtained hypomethylation in Sat-\( \alpha \) sequences treated with 3 mg/mL and hypermethylation at the highest concentration (100 mg/mL) of CCC. CFCC induced hypermethylation in LINE-1 at 3 mg/mL concentration and hypomethylation at 100 mg/mL. A decrease of methylation status was found in Alu MI sequences when cells were treated with 100 mg/mL CFCC. On the contrary, both assayed concentrations of CFCC were able to hypermethylate Sat-\( \alpha \) sequences. Regarding CAF, a decrease of methylation status in Alu MI and LINE-1 repetitive elements treated with 0.016 mM CAF and 0.016 and 0.51 mM, respectively, was observed. In contrast, an increase of the methylation status was found in Sat-\( \alpha \) sequences when cells were treated with 0.016 mM CAF. The same demethylation pattern was observed at the three repetitive elements when looking at the same concentration as Tukey’s test demonstrated when cells are treated with CCC and CAF, except for the lowest concentration of CAF when Sat-\( \alpha \) is analysed. Nevertheless,
4. Discussion

4.1. Effect of Cola Beverages and Caffeine on D. melanogaster In Vivo Model. Soft drinks have been related to several harmful effects on health, such as child obesity and appetite increase, diabetes, hypertension, and dental diseases [6–8]. They were even related to school intoxication outbreaks, although in the end these events were associated with a mass sociogenic illness [49]. Nevertheless, studies assessing systematically the toxicological effects of cola beverages are scarce [50, 51] or showed contradictory results, as in the case of CAF. Drosophila is considered an accurate in vivo model to study human disease and further substantial contributions in this sense are expected [52].

To our knowledge, this is the first attempt to characterise the genotoxic effect of these beverages using in vivo (Drosophila melanogaster) and in vitro (HL-60) models, as well as CAF, using experimental doses mimicking the concentration used in cokes.

The lack of toxicity observed in our results is reasonable since these beverages are consumed worldwide and strictly regulated by governments and agencies. Furthermore, the use of "physiological" CAF doses could explain the harmlessness of the compound, since its effect was widely demonstrated as highly dependent of the dose consumed [53]. On the other hand, differences in sugar content between beverages (11.1% versus 10.6% W/V in CFCC and CCC, resp.) could explain the different toxicity levels found in the Drosophila assays. Several toxic and side effects were reported due to the high carbohydrate concentrations of beverages, particularly referred to as glucose and fructose. In our flies, it was also demonstrated that those carbohydrates could be converted into glyoxal which reduces the number of adults emerged and the pupation time [54].

In our study, only CCC and CFCC exerted a significant antitoxic activity against \( \text{H}_2\text{O}_2 \)-induced oxidative damage in Drosophila. On the contrary, CAF showed neither toxic nor antitoxic effects. Since the effect of CAF has been widely described as dose-dependent, the lack of toxicity observed in our experiments was probably due to the low concentrations (equal to those found in the cola beverages) tested. In this sense, it was demonstrated that CAF can exert an antioxidant effect when consumed at moderate doses; it can even be neurotoxic at higher doses by increasing dopamine release [55, 56] or even inhibit autophagy in a dose-dependent manner [57]. Our results are more in agreement with Zhao et al. [58] who very recently found that CAF antioxidant properties are very weak and probably overestimated. On the other hand, it is well known that there are several extra compounds in Coca Cola, such as carbohydrate syrups, phosphoric acid (E-338), and class IV caramel colorants, but none of them has been reported as antioxidant [54, 59]. Therefore, we hypothesise that the antioxidant effects of CCC and CFCC could be explained by other undeclared components of these beverages, considering that part of its formula is an industrial secret.

Research using Drosophila has provided seminal insights into gene function which are relevant to human health [60]. The genomic stability (lack of genotoxicity) observed in Drosophila with all the compounds assayed confirmed their safety. Previous reports determined that cola drinks could be mutagenic by inducing chromosomal abnormalities and liver adducts in mice [61, 62]. However, those results are at least controversial, since the mutagenic effects were observed after 1 day of treatment with cola intakes equal to 600 mL in humans. On the contrary, our study agrees with Tóthová et al. [63] which demonstrated in a 6-month experimental design with rats drinking cola beverages ad libitum neither harmful effects nor changes in the gene expression pattern.

CAF is one of the most investigated genotoxic substances, probably because results obtained over time are not consistent (reviewed by Nehlig and Debry [64]). The absence of genotoxicity was reported a long time ago using different models: in Drosophila germ cells [65], in the Salmonella Ara test [66], or in the micronucleus assay [11]. On the contrary, mutagenic results have been reported after Sex-Linked Recessive Lethal
An interesting finding was the antigenotoxic differences among both cola beverages and CAF. Our hypothesis is that the beverages effects could be mediated in part by the differential CAF content. Although in vitro studies indicated that CAF was able to scavenge hydroxyl radicals [69], this ability was not clearly observed in the highest concentration of our in vivo antigenotoxicity assays. In this sense, 0.51 mM CAF was not able to induce antigenotoxic activity although, contrarily, the lowest CAF concentration (0.016 mM) did induce it, being the most antimutagenic compound according to the recombination percentage data. In contrast, CAF has been demonstrated to be nonantimutagenic in Ames test at 0.19 mM [70] although it depends on the environmental factors [64]. Both cola beverages also revealed an inhibitory effect against the frequency of mutant spots induced by hydrogen peroxide due to an antimutagenic activity [71]. The different IP values of 166.67% and 96.93% for CCC and CFCC, respectively, at the lowest tested concentration could be due to the CAF content in CCC (0.016 mM CAF) since CFCC does not consist of CAF. This is in agreement with several reports showing CAF antigenotoxic capacity against X-rays [72, 73] and ethyl methanesulfonate (SMART assay [74] and yeast (15 mM) [75]). The IP value of CCC at 100 mg/mL decreased up to 98.88% and this fact could be due to the absence of antigenotoxicity observed in the highest CAF concentration. CAF did not present antigenotoxic activity in the micronucleus test of mice [11], although these authors assayed higher concentrations than those tested herein. However, CCC and CFCC antigenotoxic ability could also be due to another undeclared compound in the beverage formula or to the presence of fructose, reported as being demutagenic against heterocyclic amines (Trp-p-1) [76].

Drosophila melanogaster is an excellent model for the study of aging because adults show many similarities with the cellular senescence observed in mammals [77]. This is the

**Figure 3**: Internucleosomal DNA fragmentation after 5h of treatment with CCC ((a)-mg/mL), CFCC ((b)-mg/mL), and CAF ((c)-mM). Letters M and C mean weight size marker and negative control, respectively.

**Figure 4**: Alkaline comet assay (pH <13) of HL-60 cells after 5h treatment with different concentrations of CCC (a), CFCC (b), and CAF (c). DNA migration is reported as mean TM. The plot shows mean TM values and standard errors. Different letters mean different values after one-way ANOVA and post hoc Tukey’s test.
4.2. Effect of Cola Beverages and Caffeine on In Vitro Cancer Model Cells. The in vitro evaluation of the anticancer properties of nutraceutical compounds or foods is the first step of a large pathway to obtain suitable conclusions to be extrapolated to humans. Here, we determined the potential chemopreventive effect of CCC, CFCC, and CAF on a human cancer cell model (HL-60 cell line). CCC and CFCC similarly decreased the survival rate of HL-60 leukaemia cells in a positive dose-dependent manner. Kapicioğlu et al. [81] reported the ability of cola drinks to inhibit proliferation of gastric mucosal cells although they were not cancerous. Conversely, Nowacki et al. [82] reported that CCC was able to induce an increase in fibroblast proliferation probably due to the sugar content, which could trigger a carcinogenic process. However, the rate of increase of this proliferation depended on where the CCC was bought. Our results showed that CAF induced weak cytotoxicity in HL-60 since none of the tested concentrations reached IC_{50}. Therefore, we demonstrated that CCC and CFCC cytotoxicity cannot uniquely be due to CAF content. Previous reports showed that CAF inhibited HL-60 growth at 5 mM [83]. More recently, Rosendahl et al. [84] demonstrated an inhibitory effect of CAF against human breast cancer cells, IC_{50} being roughly at 5 mM. Similarly, Pitaksalee et al. [57] showed inhibition of autophagy with CAF supplementations of 10 mM in a neuroblastoma cell line. These recent reports support our findings, suggesting that CAF could be cytotoxic only at higher concentrations and in a positive dose-dependent manner.

The degradation of genomic DNA into internucleosomal fragments was proposed as a major mechanism affecting cancer cell apoptosis. We determined that CCC and CFCC
only induced a weak proapoptotic DNA internucleosomal fragmentation at higher concentrations. Conversely, this activity was not observed in the concurrent CAF concentration tested. In this sense, previous reports by different authors are contradictory. It has been demonstrated that CAF protects HL-60 [14] and endothelial [85] cells against certain types of induced apoptosis in a dose-dependent manner and only at higher concentrations. The existence of a dose-dependent response pattern [55, 56] has recently been demonstrated by Wang et al. [86] showing that 2 mM CAF enhanced the proapoptotic effect of cisplatin lung cancer cells; these results could also explain the differences in CAF studies since they suggest that low CAF concentrations do not induce apoptosis by themselves, but by enhancing a different apoptotic pathway.

For these reasons, we performed alkaline SCGE in order to detect DNA damage [87], which are widely used to determine whether cells are undergoing apoptotic and/or necrotic pathways [41]. The use of such a test in transformed cells for the screening of substances with clastogenic DNA-strand break activity could be considered as a very early stage screening in the search of molecules for the treatment of acute promyelocytic leukaemia [88]. It is assumed that apoptosis occurs when treatments induce a TM > 30 (hedgehog pattern) whereas control cells remain lower than 2 (no tails). On the contrary, necrosis shows a short comet-tail pattern since the majority of the damaged DNA remains in the comet head [89]. Our results showed that the damage induced by CCC, CFCC, and CAF in HL-60 cells was characterised by necrosis (short tails, TM < 5, Figure 4). These results agree with our cytotoxicity and DNA fragmentation assays, demonstrating that CCC and CFCC induced cell death in HL-60, probably mediated by a necrotic pathway. Both beverages and CAF had the same DNA damage pattern (class I; TM between 1 and 5 according to Fabiani et al. [90]) whereas class 0 was detected in their concurrent controls (TM lower than 1, no visible comet). In the same way, our results agree with those of Rayburn et al. [91] who reported that CAF supplementation (0–2 mM) did not produce DNA-strand breaks in CHO cells. Consistent with our results, several authors demonstrated that CAF induced apoptotic cell death in glioma and lung cancer cells at higher doses (10–20 mM), suggesting again that CAF induced apoptotic cell death in glioma and lung cancer cells at higher doses. The existence of a dose-dependent response pattern [55, 56] has recently been demonstrated by Wang et al. [86] showing that 2 mM CAF enhanced the proapoptotic effect of cisplatin lung cancer cells; these results could also explain the differences in CAF studies since they suggest that low CAF concentrations do not induce apoptosis by themselves, but by enhancing a different apoptotic pathway.

Regarding epigenetics, it is currently known that environmental factors are involved in gene expression. In cancer cells, the genome is globally hypomethylated inducing transposable element activity and thus triggering genome instability [94]. As a proof of that, the silencing of tumor suppressor genes is closely associated with hypermethylation [95]. Repetitive elements are highly methylated in somatic normal cells contributing to a global genomic hypermethylation [43, 94] suppressing the transposable activity of repetitive elements. Nevertheless, a lot of information is still unknown specially in order to ascertain the mechanisms which modulate the epigenetic changes in cancer cells. Biomedical research is focused on hypomethylation agents since this therapy is highly related to gene silencing; thus this fact could activate tumor suppressor genes and be a positive highlight although its benefit on human therapies is not clear because much more investigations should be performed [96].

We studied three different repetitive elements: LINE-1, Alu M4, and SAT-α. Long interspersed nuclear elements (LINE) are abundant retrotransposons and represent about 17% of the human genome. Although LINE1 has a non-random distribution, they are accumulated in primarily G-positive bands, which are AT-rich regions of chromosomes [97]. LINE-1 elements are also accumulated in regions of low recombination rate mainly in X-chromosome [98]. Alu elements belong to the SINE (short interspersed nuclear elements) family, being the most abundant (accounting about 10% of the whole human genome [43]) and predominantly present in noncoding and GC-rich regions [97, 99]. Sat-α (satellite alpha DNA) repeats are composed of tandem repeats of 170 bp DNA sequences, are AT-rich regions, and represent the main DNA component of every human centromere, constituting about 5% of total human DNA [97, 100]. Therefore, examination of the methylation status of LINE-1 and Alu regions has served as an approach for measuring global methylation levels since 32% of the human genome has been evaluated [101].

Our results of methylation status showed that CCC may generally hypomethylate the global genome although 100 mg/mL CCC hypermethylate Sat-α repetitive element. We also observed a significant negative dose-dependent effect in every target repetitive element with 50% hypomethylation average rate. Nevertheless, the overall hypermethylation rate induced in CFCC treatments is 328%, and only a decrease of methylation status is observed at Alu M1 and LINE-1 sequences when treated with CFCC 100 mg/mL. This hypermethylation could be considered as a benefit since LINE-1 is associated with C-met oncogene that would be silenced [102].

Xu et al. [103] demonstrated that caffeine (0.3 mM) enhanced the methylation ratio of multiple single CpG sites, as well as the total methylation ratio at nt –358 to –77 of the hippocampal 11β-HSD-2 promoter of primary fetal hippocampal neurons in rats. However, 4 and 40 μM CAF were able to induce hypomethylation of single CpG site inhibiting the DNMT3 enzyme but not decrease the global status of the proximal promoter of the human STAR gene [104]. The present results of CAF are in agreement with Ting et al. [105] since 16 μM was able to induce hypomethylation of Line-1 and Alu M1 sequences as well as 0.5 mM CAF in LINE-1. However, Sat-α (AT-rich elements) was methylated when cells were treated with 16 μM CAF. It has been demonstrated that the expression of satellite sequences is associated with a hypomethylation triggering cancer cells; thus methylation
process in satellite sequences is a potential mechanism for silencing its satellite expression in transformed cells [105]. These results could suggest that CAF may be one of the compounds responsible for the global hypomethylation status induced by CCC.

Statistical analysis showed that the methylation status induced by CCC and CAF in each repetitive element was not significantly different. Conversely, CFCC resulted in inducing different methylation status. Therefore, the effects of CCC on methylation status of HL-60 cells could be explained by those induced by CAF.

It is clear that much more information is needed for ascertaining on the role of food and beverages on epigenomes since hypomethylation mechanisms are not clear in every type of tumor. In addition, the hypomethylation and hypermethylation status of repetitive elements depend on both their concurrent control [102] and the target repetitive elements selected to evaluate the global methylation status. To our knowledge, it is the first attempt assessing DNA methylation changes induced by CCC, CFCC, and CAF on human leukaemia cells.

An apparent scarce data on the lack of dose-dependent effect is observed at almost all parameters analysed at the individual, cell, and molecular levels. Based on the obtained results, we only found a clear-cut dose-dependent effect when CCC is tested in the anticotoxicity, cytotoxicity, and methylation bioassays. A threshold level of concentration may be needed to obtain some biological effects [106]. We found this threshold in the rest of the assays and compounds for toxicity, anticotoxicity, longevity, healthspan, DNA fragmentation, and SCGE.

5. Conclusions

In conclusion, our experimental results show a slight chemopreventive effect of the two cola beverages against HL-60 leukaemia cells, probably mediated by nonapoptotic mechanisms. CCC and CAF induce a global genome hypomethylation evaluated in LINE-1 and Alu M1.

Competing Interests

The authors declare that they have no competing interests.

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