

Supporting Information

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Phytochemical Profile and *in vitro* Assessment of the Cytotoxicity of Green and Roasted Coffee Oils (*Coffea arabica* L.) and their Polar Fractions

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Table of Contents	Page
Experimental details	2-4
S1: Fatty acid composition of green and roasted coffee oils extracted through the cold-press process (% total fatty acids content; mg/g oil)	5
S2: Total phenolic content of green, roasted coffee oils and their polar fractions.	6
S3: Chromatographic profiles of a - green and b - roasted coffee oil polar fractions obtained by ultra-high performance liquid chromatography (UHPLC) at $\lambda = 280$ nm.	7
S4: LC-DAD-MS/MS at $\lambda = 280$ nm of green and roasted coffee oil polar fractions (methanol soluble).	8
S5: Radical scavenging activity of green and roasted coffee oils and their lipophilic (LF) and polar (PF) fractions. (TEAC ^a , mmol/g oil).	9
S6: Viability (%) of BALB/c 3T3, clone a31, fibroblasts exposed to 1 mM H ₂ O ₂ after treatment with a - Green and roasted coffee oil (0.001-1 mg/mL) and b - Green and roasted coffee oil polar fraction (PF) (0.0001-3 μ g gallic acid equivalents/mL).	10
References	11

Experimental Details

Gas Chromatography (GC) Analysis: For fatty acid analysis, lipids were extracted and quantified following the method of Folch *et al.* [1] with modifications by Ways and Hanahan [2]. Methyl esters of fatty acids were obtained according to the method of O'Fallon *et al.* [3]. Chromatographic separation was performed using a Shimadzu GC-2014 gas chromatograph (Kyoto, Japan) equipped with a capillary column (RTX[®] 2330, 90% biscyanopropyl/10% phenylcyanopropyl polysiloxane, 105 m x 0.25 mm ID, 0.20 μm film thickness; Restek[®], Bellefonte, CA). Injector temperature was set to 250 °C; flame ionization detector (FID) at 260 °C. Synthetic air was used as carrier gas with the flow of 1 mL/min, column heating ramp: initially at 130 °C, held for 5 min, followed by an increase from 130 to 180 °C at 5 °C per min, held at 180 °C for 10 min, increased from 180 to 240 °C at 3 °C per min and held at 240 °C for 13 min. For fatty acid identification and quantification, retention times and the methyl ester areas were compared to the external analytical standard FAME 37 and to the internal standard, i.e., tricosanoic acid 23: 0. Peak areas were corrected by theoretical relative FID response factors [4]. Analysis was carried out in triplicate, and data were expressed as mean values \pm standard deviation (SD) as % of fatty acid composition and as mg of fatty acids per g of coffee oil.

Isolation of Polar Fractions (PFs): PFs were isolated according to Abramovič *et al.* [5], with modifications. Samples (5 g) of GCO and RCO were dissolved in 50 mL hexane and transferred to separatory funnels. Then, 20 mL of 80% methanol (v/v) were added, and after 10 min of stirring, the lower methanol–water layer was recovered. The extraction was repeated twice, and the methanol phases were combined, followed by storage at -80 °C under modified atmosphere (N₂). For further use in cell culture, the extracts were dried in an Eppendorf Speed-Vac Concentrator Plus 5305 (Hamburg, Germany), followed by storage at -80 °C until use. On the day of use, the dried extracts were resuspended in 10% ethanol (v/v) and filtered through 0.22 μm syringe filters. Phenolic content was determined as described below.

Determination of the Phenolic Contents: The total phenolic content of coffee oils (GCO, RCO and PFs) was evaluated colorimetrically with Folin-Ciocalteu reagent [6]. The reaction mixture contained 300 μL GCO and RCO (solubilized in petroleum ether), 225 μL Folin-Ciocalteu reagent, and 2.475 mL 2% sodium carbonate solution (w/v). Due to the non-polar characteristics of the oils, the mixture was kept in the dark for 1 h under constant stirring to complete the Folin-Ciocalteu reduction reaction, followed by recording the absorbance at $\lambda = 750 \text{ nm}$, using a UV-VIS spectrophotometer (BEL LGS 53, Monza, Italy). PFs assays were performed in the same way, but using 80% methanol as solvent. Analysis was carried out in triplicate and total phenolic contents were quantified using a standard curve of gallic acid (Detection range 7.8 - 125 $\mu\text{g}/\text{mL}$; Methanolic solution $y = 0,0104x$, $r^2 = 1$; Petroleum ether solution $y = 0,0149x$, $r^2 = 1$). Results were expressed as mg gallic acid equivalents per g of oil (mean values \pm SD).

RP-UHPLC-DAD Analysis of the Coffee Oil Polar Fractions: Reversed phase-ultrahigh performance liquid chromatography-diode array detector (RP-UHPLC-DAD) analysis was adapted from Rodrigues and Bragagnolo [7] and was performed in a Thermo ScientificUltiMate 3000 RS Dual System (Thermo Fisher Scientific, San Jose, CA). Chromatographic separation of coffee oil PFs was achieved using a Thermo Scientific C18 reverse phase column (250 \times 4.6 mm, 5 μm particle) and a diode array detector operating at 240 nm, 260 nm, 280 nm, and 320 nm. The gradient elution consisted of water acidified with formic acid (pH 2.3 - Eluent A) and methanol (Eluent B) at a flow rate of 1.0 mL/min. The gradient was as follows: 0 - 5 min, 15% B; 5 - 45 min, 100% B; 45 - 50 min, 15% B. The oven temperature was set at 35 °C. Compounds were identified by comparison of their retention times with those of commercial standards. Caffeine was quantified at $\lambda = 280 \text{ nm}$ based on the integration of the peak areas and calibration curve of that alkaloid (detection range 62 - 1000 $\mu\text{g}/\text{mL}$, $y = 0.414x$, $r^2 = 1$). Analysis was carried out in triplicate and results expressed as mean (mg/g dry extract) \pm SD.

HPLC-DAD-ESI-MS/MS Analysis of the Coffee Oil Polar Fractions: High performance liquid chromatography (HPLC) analysis was performed in a Shimadzu system (Kyoto, Japan) and Liquid

Chromatography Coupled Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-MS/MS) was performed on a MicrOTOF QII Bruker spectrometer (Rheinstetten, Germany), using a hybrid quadrupole/TOF mass analyzer equipped with electrospray ionization (ESI) interface. The liquid chromatography (LC) system includes a quaternary pump (LC-20AD), an on-line degasser (DGU-20A3), an autosampler (SIL-20AHT), a thermostatic column control (CTO-20A), a photodiode array detector (SPD-M20A), and a controller (CBM-20A), all of which are controlled by the LC Solution software. Chromatographic conditions were set as described previously (section 2.3.4). The following ESI parameters were optimized depending on the compounds: nebulizer, 1 bar; dry gas (N₂) flow, 6 L/min, and dry gas temperature of 200° C. The ion trap mass spectrometer operated in negative and positive ion mode with a scanning range from *m/z* 50 to *m/z* 800. In addition, the activation energy for the MS/MS experiment was set between 20 – 70 eV.

Antioxidant Activity: The *in vitro* antioxidant activity of coffee oils, including both lipophilic fractions (LFs) and polar fractions (PFs), was determined using the DPPH assay. Prior to the assay, LF and PF were separated as follows: a total of 250 mg of coffee oil was mixed with 2 mL of 80% methanol (v/v) and kept under stirring during 10 min. The mixture was centrifuged (1.789 g, 4° C, 15 min), and the methanolic phase with the polar compounds (PF) was separated from the oil phase (LF). A stock solution of 7.9 mg DPPH was diluted in 2.5 mL methanol and then further diluted to a final concentration of 1: 100 (v/v) in 80% methanol (v/v). PF was tested using 100 µL of extract added to 2.9 mL of methanolic DPPH. Whole oil and LF assays were performed in the same way, but using DPPH dissolved in ethyl acetate [8]. The decrease in absorption was measured using a UV–VIS spectrophotometer (UV-2000A Instrutherm - São Paulo, Brazil) at λ = 515 nm. The ability to scavenge DPPH radicals was calculated as the percentage of DPPH discoloration based on Equation 1:

$$DPPH\text{inhibition}(\%) = \left(\frac{A_c - A_s}{A_c} \right) * 100$$

In which A_C represents the absorbance of DPPH solution (control – methanolic/ethyl acetate solution), and A_S is the absorbance in the presence of sample. Extractions were conducted with three independent samples performed in triplicate for each one. A Trolox calibration curve (Detection range 0.039 – 1.25 mM; Methanolic solution $y = 140.6x$, $r^2 = 1$; Ethyl acetate solution $y = 71.09x$, $r^2 = 0.99$) was prepared, and data were expressed as mean values ± SD as Trolox equivalent antioxidant capacity (TEAC, mmol/g oil) and DPPH inhibition (%).

Cytotoxicity Assessment: BALB/c 3T3, clone a31, fibroblasts were seeded into 96-well plates (5x10³ and 1x10⁴ cells/well) containing DMEM culture medium, supplemented with 10% FBS and incubated during 24 h (37°C, 95% humidity, and 5% CO₂). After this period, cells were treated with increasing concentrations of coffee oils (0.001 - 1 mg/mL) and coffee oil PFs (0.0001 - 10 µg gallic acid equivalents/mL) during 24 h and 48 h. To allow the emulsification of coffee oils in the culture medium, polysorbate 80 (0.1 mg, w/v) was added to each well. The same concentration of polysorbate 80 in DMEM was used as control. After the treatment period, the culture medium was replaced by 100 µL fresh DMEM, along with 10 µL MTT solution (5 mg/mL in PBS) per well, and incubated in the dark for 3 h. Subsequently, the culture medium was removed, and 100 µL DMSO were added to each well. Absorbance at λ = 540 nm was determined by an ELISA plate reader (Spectramax Paradigm, Molecular Devices, Sunnyvale, CA). Cell viability was expressed as percentage of growth based on the control cells, and results were calculated according to the following equation:

$$Cv = \left[\left(\frac{C_{me} - B_m}{C_{trl_m} - B_m} \right) \right] * 100$$

In which C_V is the cell viability (%), C_{me} is the mean absorbance of cells treated with the tested samples, B_m is the mean absorbance of the blank, and $Ctrl_m$ is the mean absorbance of control cells. Data were presented as mean values \pm SD of three individual experiments performed in sextuplicate for each one.

Hydrogen Peroxide-induced Oxidative Stress and Evaluation of Cell Survival: Hydrogen peroxide was used to induce oxidative stress [9]. BALB/c 3T3, clone a31, fibroblast cells were seeded into 96-well plates (5×10^3 cells/well) containing DMEM culture medium supplemented with 10% FBS and incubated during 24 h (37° C, 95% humidity, and 5% CO₂). After this period, cells were treated with increasing concentrations of coffee oils (0.001 - 1 mg/mL) and coffee oil PFs (0.0001 – 3 µg gallic acid equivalents/mL) for different intervals, as follows:

- (1) Cells treated for 24 h, followed by 1.0 mM of H₂O₂ exposure for 3 h;
- (2) Cells concomitantly exposed to treatments and 1.0 mM of H₂O₂ for 24 h;
- (3) 1.0 mM of H₂O₂ exposure for 3 h, followed by cell treatments for 24 h.

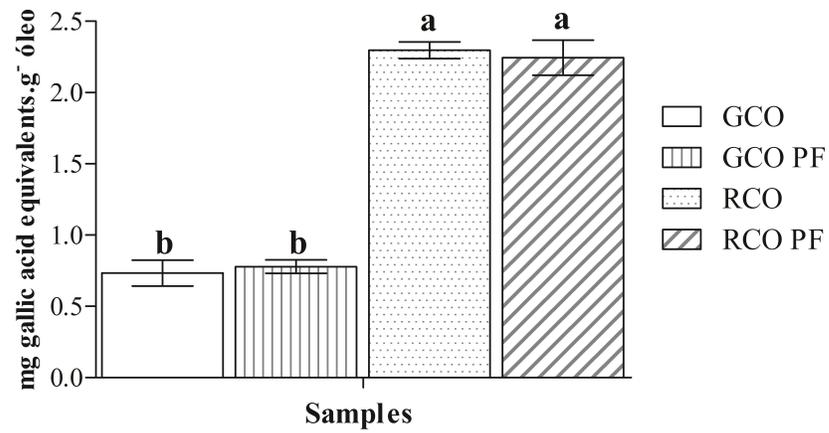
Evaluation of cell survival was performed using MTT assay as described in section 2.5.1.

Statistical Analysis: Data were collected and summarized, followed by statistical analysis using one-way ANOVA and *post-hoc* test as necessary (*t* test, Dunnett's, Tukey). The statistical analyses were performed using GraphPad Prism software for Windows (version 6.01, GraphPad Software, San Diego, CA). P values lower than 0.05 were considered to be statistically significant.

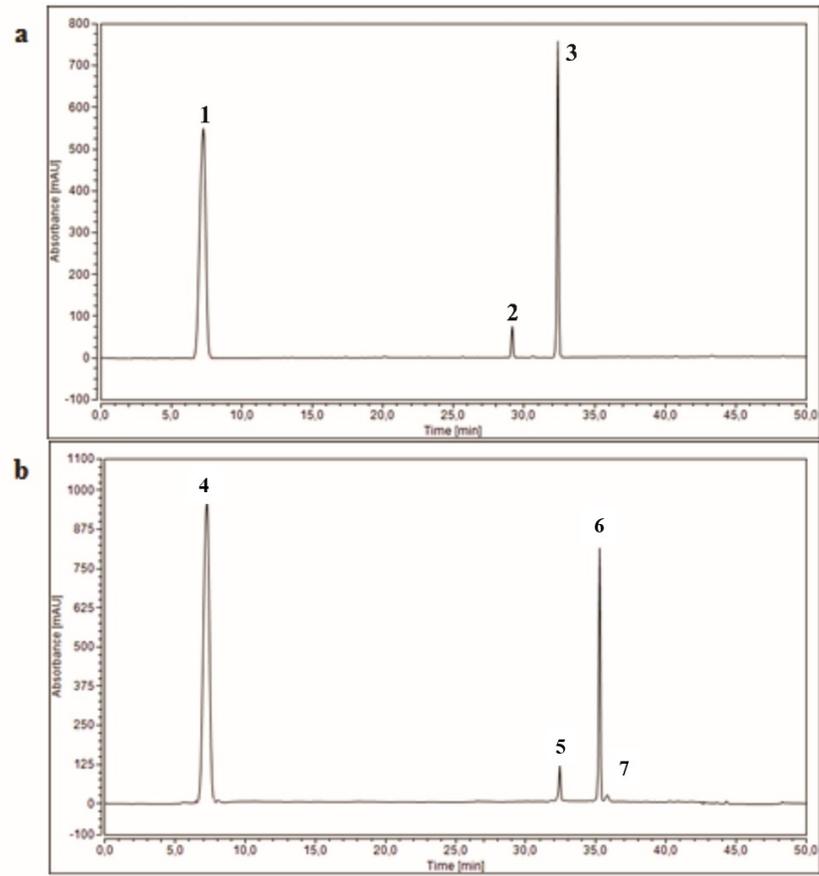
S1. Fatty acid composition of green and roasted coffee oils extracted through the cold-press process (% total fatty acids content; mg/g oil)^a.

Fatty acids (%)	GCO % (mg/g oil)	RCO % (mg/g oil)
Myristic acid C14: 0	0.10±0.001 (0.73±0.01)	0.06±0.003 (0.54±0.02)
Palmitic acid C16: 0	34.78±0.24 (254.30±0.89)	33.81±0.27 (279.52±1.33)
Stearic acid C18: 0	7.45±0.04 (54.49±0.87)	7.42±0.02 (60.92±0.38)
Oleic acid C18: 1 n9	7.92±0.06 (57.91±0.93)	8.79±0.01 (72.32±0.49)
Vaccenic acid C18: 1 n7	0.43±0.006 (3.17±0.06)	0.41±0.009 (3.35±0.12)
Linoleic acid C18: 2 n6	43.28±0.07 (316.45±3.70)	43.59±0.13 (357.70±3.31)
γ-Linolenic acid C18: 3 n6	2.73±0.038 (19.95±0.48)	2.45±0.013 (20.24±0.11)
α-Linolenic acid C18: 3 n3	1.43±0.01 (10.49±0.18)	1.37±0.008 (11.24±0.15)
<i>Cis</i> -11-Eicosenoic acid C20: 1 n9	0.26±0.009 (1.89±0.09)	0.27±0.005 (2.18±0.07)
Others	1.62	1.82

^a Values are means ± standard deviation (SD) of triplicate determinations.



S2. Total phenolic content of green, roasted coffee oils and their polar fractions. Values are means \pm standard deviation (SD), n=3. Different letters indicate statistical differences among samples by ANOVA one-way analyses, followed by Tukey's *post-hoc* test ($p < 0.0001$).



S3. Chromatographic profiles of **a** - green and **b** - roasted coffee oil polar fractions obtained by ultra-high performance liquid chromatography (UHPLC) at $\lambda = 280$ nm.

S4. LC-DAD-MS/MS at $\lambda = 280$ nm of green and roasted coffee oil polar fractions (methanol soluble).

Peak number ^a	RT (min) ^b	[M-H] ⁻ <i>m/z</i>	[M+H] ⁺ <i>m/z</i>	Fragments	Identified compounds
1	7.2	-	195	138, 110	Caffeine
2	29.2	-	313	277, 237, 197, 131	-
3	32.5	-	297	279, 251, 185, 145, 121	-
4	7.2	-	195	138, 110	Caffeine
5	32.5	-	297	185, 121	-
6	35.2	-	297	279, 251, 223, 189, 133, 105, 91	-
7	35.9	-	281	263, 225, 199, 173, 147, 133, 105, 91	-

^a Numbered according to the chromatogram shown in S3.

^b Retention time on C18 column.

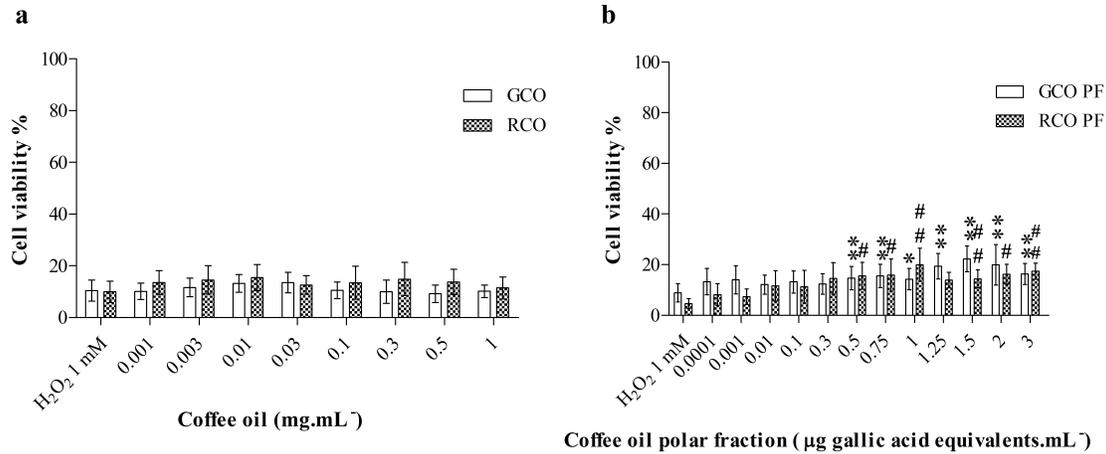
S5. Radical scavenging activity of green and roasted coffee oils and their lipophilic (LF) and polar (PF) fractions. (TEAC^a, mmol/g oil)

Oil type	TEAC ^{b,c} (mmol/g oil)		
	OIL	LF	PF
GCO	8.76 ± 0.20	7.83 ± 0.28	2.02 ± 0.03
RCO	9.96 ± 0.13 *	8.82 ± 0.06 *	5.23 ± 0.02 ***

^a TEAC is the millimolar concentration of a Trolox solution having an antioxidant capacity equivalent to that of the dilution of the samples.

^b Values are means ± standard deviation (SD) of triplicate determinations.

^c Values within a column for each sample having * are significantly different from each other, using *t* test * (p<0.05) ***(p<0.0001).



S6. Viability (%) of BALB/c 3T3, clone a31, fibroblasts exposed to 1 mM H₂O₂ after treatment with **a** - Green and roasted coffee oil (0.001 - 1 mg/mL) and **b** - Green and roasted coffee oil polar fraction (PF) (0.0001 - 3 µg gallic acid equivalents/mL). Data are presented as mean values ± standard deviation (SD), n= 16. Different symbols indicate statistical differences among treatments and control group by ANOVA one-way analyses, followed by Dunnett's *post-hoc* test at p<0.05. * Indicates p<0.05; ** Indicates p<0.01; # Indicates p<0.05 and ## p<0.01.

References

- [1] J. Folch, M. Lees and G.H. Sloane-Stanley (1957). A simple method for the isolation and purification of total lipides from animal tissues, *J. Biol. Chem.* **226**, 497–509.
- [2] P. Ways and D.J. Hanahan (1964). Characterization and quantification of red cell lipids in normal man, *J. Lipid Res.* **5**, 318–328.
- [3] J.V. O’Fallon, J.R. Busboom, M.L. Nelson and C.T. Gaskins (2007). A direct method for fatty acid methyl ester synthesis: Application to wet meat tissues, oils, and feedstuffs, *J. Anim. Sci.* **85**, 1511–1521.
- [4] C.F. Corrêa, R.O. Nobrega, B. Mattioni, J.M. Block and D.M. Fracalossi (2017). Dietary lipid sources affect the performance of Nile tilapia at optimal and cold, suboptimal temperatures. *Aquac. Nutr.* **2017**, 1–11.
- [5] H. Abramovič, B. Butinar and V. Nikolič (2007). Changes occurring in phenolic content, tocopherol composition and oxidative stability of *Camelina sativa* oil during storage, *Food Chem.* **104**, 903–909.
- [6] M. Schiavon, I. Moro, E.A.H. Pilon-Smits, V. Matozzo, M. Malagoli and F. Dalla Vecchia (2012). Accumulation of selenium in *Ulva* sp. and effects on morphology, ultrastructure and antioxidant enzymes and metabolites, *Aquat. Toxicol.* **122–123**, 222–231.
- [7] N.P. Rodrigues and N. Bragagnolo (2013). Identification and quantification of bioactive compounds in coffee brews by HPLC-DAD-MSn, *J. Food Compos. Anal.* **32**, 105–115.
- [8] J.C. Espín, C. Soler-Rivas and H.J. Wichers (2000). Characterization of the total free radical scavenger capacity of vegetable oils and oil fractions using 2,2-diphenyl-1-picrylhydrazyl radical, *J. Agric. Food Chem.* **48**, 648–656.
- [9] H.S. Pitz, A. Pereira, M.B. Blasius, A.P.L. Voytena, R.C.L. Affonso, S. Fanan, A.C.D. Trevisan, R.M. Ribeiro-do-valle and M. Maraschin (2016). *In vitro* evaluation of the antioxidant activity and wound healing properties of jaboticaba (*Plinia peruviana*) fruit peel hydroalcoholic extract, *Oxid. Med. Cell. Longev.* **2016**, 1–6.