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Research Article

Effects of Extraction Processes on Recovery, the Phenolic Profile, and Antiglycation Activity from Green Coffee Residues (*Coffea arabica* and *Coffea canephora Pierre*)

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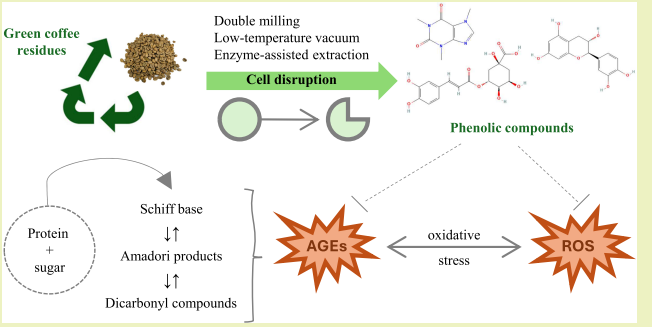
ABSTRACT: Coffee processing generates tons of residues, entailing environmental problems and economical lost. The use of these coffee residues by the food industry could add value to the coffee plant, increasing social and economic prosperity. Green coffees are rich in phenolic compounds that have a strong antioxidant capacity and the ability to prevent the production of advanced glycation agents (AGEs). Traditional extraction methods using solvents have been widely used in recent years; however, there is an increasing need for the use of green technologies, which are nontoxic, eco-friendly, and efficient. Therefore, the objective of this study was to evaluate the effect of different physical (low-temperature vacuum, LTV, and double milling) and enzymatic processes of phenolic extraction and their bioactivity from green coffee residues. The results show that the green coffee is a good source of phenolic compounds, especially chlorogenic acid, and although the hydroalcoholic extract presented higher phenolic content and bioactivity in relation to the LTV extract, the double milling and enzymatic treatment seem to recover this potential in the LTV extracts, making them similar to the hydroalcoholic extract but without the use of organic solvents, showing that it is possible to obtain a phenolic-rich extract with antioxidant and antiglycant activity under scalable extraction conditions, without the use of chemical solvents.



KEYWORDS: *green coffee, phenolic compounds, green extraction, antioxidant, advanced glycation agents*

1. INTRODUCTION

Brazilian coffee farming is the world leader in the ranking of production and export of green, unroasted beans.[1] The main types of coffee marketed are arabica (*Coffea arabica* L.) and conilon or robusta (*Coffea canephora Pierre*), which have higher productivity in tropical and subtropical regions.[2] According to Habtamu and collaborators, 20% of the total coffee produced in Brazil is defective and commercialized locally, producing a lower quality beverage at a reduced price.[3]

Coffee processing generate tons of residues, entailing environmental problems and economical lost.[4] The use of these coffee residues by the food industry could add value to the coffee plant, increasing social and economic prosperity in poorer coffee-growing regions and work against the decreasing coffee price, which is especially worthwhile in the current times of global economic crisis.[2] Green coffee beans can be considered a residue, as they are considered immature; due to incomplete maturation, they have a lower weight when compared to normal beans, and this reduction can be up to 10%, considered a significant defect since the final product will have greater bitterness and astringency. The pre- and postharvest stages, carried out inadequately, are the main source of defective beans—green, overripe (black), and fermented fruits in contact with the soil (overfermented), drying at high temperature (stinker), among others.

Coffee beans are a source of phenolic compounds recognized as natural antioxidants with effects on the prevention of chronic degenerative diseases, correlated with several beneficial effects on health, fighting inflammation, obesity, LDL cholesterol oxidation, diabetes, cancer diseases, and liver diseases, among others.[5,6] Chlorogenic and caffeic acids are present in greater quantities in green coffee beans, among other phenolic compounds such as caffeine, rutin,

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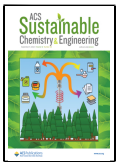

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epigallotanins, theophyllines, etc.[3,6,7] Besides their antioxidant activity, phenolic compounds also have a strong antiglycant capacity. Advanced glycation agents (AGEs) can be formed intracellularly in individuals or in the food itself through the Maillard reaction. The high concentration of AGEs with high oxidative capacity circulating in the bloodstream can harm health, as they interact with cell receptors and/or proteins, resulting in increased expression of changes in the format and functions, inflammatory mediators, and oxidative stress.[8] Although research on the effect of phenolic compounds in preventing the formation of AGEs is recent, it has indicated that this can be a positive approach to diet aimed at maintaining health, in addition to preventing diseases.[9,10]

The extraction methodology used will strongly impact the quantity and quality of the phenolic compounds present in the final extract.[11] Conventional extraction methods, involving the application of solid−liquid extraction, have been used for more than a century for the isolation of polyphenols; however, these techniques present some limitations, such as longer extraction time, requirement of costly and high-purity solvents, and evaporation of the huge amount of solvents.[12] Due to the growing interest in phenolic compounds, there is an increasing need for the use of green and economically feasible technologies without the use of chemical solvents. Modern green extraction techniques, such as ultrasound, low-temperature vacuum (LTV), and enzyme-assisted extraction, are nontoxic, eco-friendly, less time- and energy-consuming, and efficient[13] and represent promising approaches for overcoming current limitations to the extraction of polyphenols as bioactive compounds. LTV is a physical extraction method, characterized by a low time and temperature during cooking and reduced exposure to air. This technique can promote the rupture of the plant and the release of bioactive compounds into the solvent, facilitating polyphenol extraction. Besides, the LTV conditions are beneficial in preserving bioactive compounds such as polyphenols, as the anaerobic condition prevents the oxidation process and the mild temperature conditions prevent possible thermal changes of the compounds.[14] The combination of emerging extraction techniques (i.e., microwave, ultrasound, and supercritical fluid extraction) with enzymes has proven to be versatile to recover bioactive compounds from plant matrices and their byproducts.[15,16]

As far as we know, the use of LTV combined with enzyme-assisted extraction in green coffee beans has never been studied before. Therefore, the objective of this study was to evaluate the effect of different physical (milling and LTV) and enzymatic processes of phenolic extraction from green coffee residues on the phenolic profile, on the extraction productivity, and on the antioxidant and antiglycating capacity of the extracts obtained. A comparison with the traditional methodology of hydroalcoholic extraction was performed.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents. Milli-Q water (EMD Millipore Corporation, Merck, Darmstadt, Germany), gallic acid, potassium hydroxide, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and Trolox were purchased from Sigma−Aldrich (Darmstadt, Germany). The standards catechin, epicatechin, epicatechin gallate, caffeine, chlorogenic acid, and theobromine were also acquired from Sigma-Aldrich (Darmstadt, Germany). Theophylline was acquired from Abbott (São Paulo, Brazil). Phosphate buffered saline (PBS), Dulbecco's modified Eagle medium (DMEM), 0.25% trypsin −10 mM EDTA solution, and a solution containing 10,000 units/mL penicillin and 10,000 μg/mL streptomycin, fetal bovine serum (FBS), and nonessential amino acids were acquired from Gibco (Waltham, MA). Methanol (99.9%, HPLC grade) was obtained from JT Baker (Bridgewater). Folin−Ciocalteu reagent, ethanol (99.5%), sodium citrate, tannic acid, glacial acetic acid, sodium metabisulfite, and anhydrous sodium acetate were acquired from Dinâmica (São Paulo, Brazil).

The enzymes cellulase (Celluclast 1.5 L) and pectinase (Pectinex Ultra SP-L) were donated by Novozymes (LNF, Bento Gonçalves, Brazil). The tannase was produced in the Bioprocesses Laboratory of the Department of Nutrition and Food.

2.2. Samples. The green coffee beans used were a blend of arabica and conilon species donated by the MINASUL Cooperative (Varginha, Minas Gerais, Brazil, coordinates 21°34′54.8″S 45°26′46.0″W). The raw material was classified by the manual harvesting method, following the recommendations found in Brazilian Normative Instruction No. 8, which presents tables detailing the defects found.[17]

The received green coffee residues were stored at −20 °C. A preprocessing was carried out in a knife and hammer granulator mill (Treu, model 740646, Rio de Janeiro, Brazil) where the raw material was milled and its granulometry was subsequently standardized at 1.19 mm (*Bertel*, electromagnetic model, São Paulo, Brazil).

2.3. Obtaining Phenolic Extracts. *2.3.1. Hydroalcoholic Extraction.* Hydroalcoholic extraction was carried out according to Santana and collaborators,[18] with modifications. The hydroalcoholic solution was prepared in centrifuge tubes covered with aluminum foil with a capacity of 50 mL, using a proportion of 50% ethanol and 50% deionized H_20 (w/w). The ratio of ground bean:solvent was 1:25 (1 g of powder and 25 g of 50% hydroalcoholic solution) (w/w), with vortexing of the tubes (Fanem, Modelo 251, São Paulo, Brazil) for 1 min. Subsequently, the samples were centrifuged in an orbital shaker (*Thermo Scientific*, model Max Q 8000, California) at 200 rpm, in the dark, at a temperature of 25 °C for 2 h. Finally, the extract was concentrated in a rotary evaporator at 50 °C for 3 h, where the ethanol present was completely removed. It was frozen at −20 °C for subsequent freeze drying at −45 °C for 48 h (Liotop, model L101, São Carlos, Brazil).

2.3.2. Low-Temperature Vacuum (LTV) Extraction Coupled with Enzyme-Assisted Extraction. To obtain these extracts, two types of milling were performed (single and double). For simple milling, coffee beans were used in 1.19 mm granulometry, as previously described (item 2.2). For the double milling, the green coffee beans went through a second stage of milling in a semi-industrial processor mill (Comitrol Urschel, model 1700, Barueri, Brazil) at 9300 rpm for 2 min. The particle size was standardized using a 1.0 mm strainer (Bertel, electromagnetic model, São Paulo, Brazil). For the LTV extraction, filtered water was added in 1:2 ratio of powder:water (w/w) to the ground beans (both single and double milling). This mixture was cooked with a negative pressure of 500 mmHg at 60 °C for 30 min in a semi-industrial closed evaporator concentrator (Mecamau, model C-055, Esprito Santo do Pinhal, Brazil). The temperature was controlled to ensure that there would be no degradation of the phenolic compounds present.

For enzyme-assisted extraction, the methodology used was the one described by Barbosa and collaborators,[19] with modifications, consisting of the addition of 1 g of cooked bean and 25 mL of sodium acetate buffer (20 mM, pH 5.0), i.e., 1:25, in vials enveloped with aluminum foil so that the reaction occurred in the dark. The sample was subjected to four different enzymatic treatments with the addition of **C** (10 U of cellulase/g dry matter) or **T** (10 U of tannase/g dry matter) or **M** (10 U of commercial mix of pectinase, hemicellulase and β glucanase/g dry matter) and finally **T+M** (5 U of commercial mix of pectinase, hemicellulase and β glucanase/g dry matter combined with 5 U of tannase/g dry matter). At the same time, a treatment was carried out under the same conditions, without the addition of enzymes, to control the reaction, and it was called **Blank**.

The extracts named above were centrifuged in an orbital shaker (*Thermo Scientific*, model Max Q 8000, California) at 50 °C and 130 rpm for 4 h for the extraction of phenolic compounds. The containers

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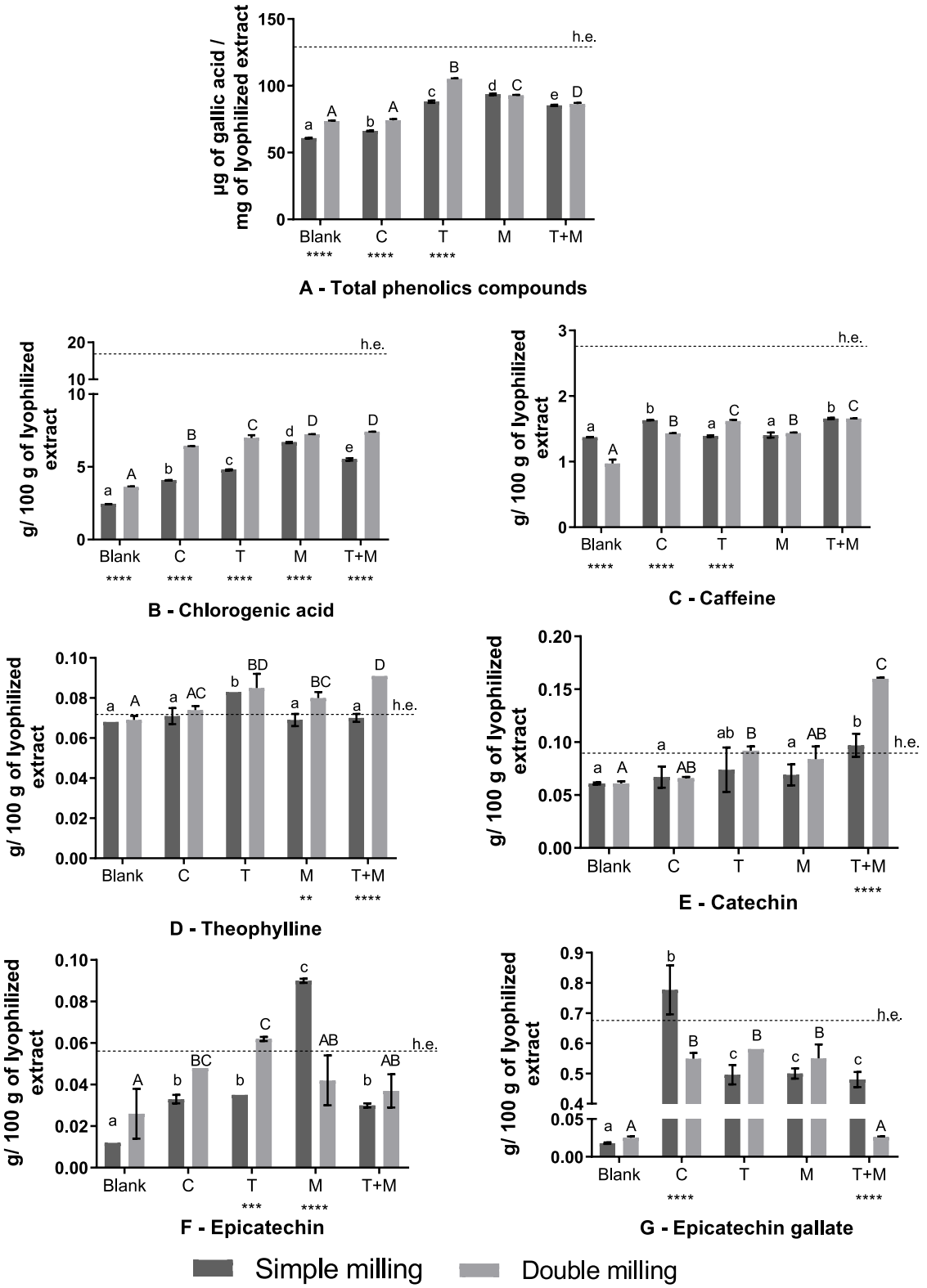

Figure 1. Phenolic profile of hydroalcoholic and LTV extracts assisted or not assisted by enzymes, with single or double milling. Values were expressed as μg of gallic acid/mg of lyophilized extract for total phenolics (A) and as g/100 g of lyophilized extract for chlorogenic acid (B), caffeine (C), theophylline (D), catechin (E), epicatechin (F), and epicatechin gallate (G), as mean ± standard deviation, and were analyzed by two-way ANOVA, followed by Tukey's test $p < 0.05$. Different lowercase letters mean differences within the "single milling" group. Different capital letters signify differences within the "double milling" group. Asterisks mean differences between milling processes (**$p < 0.01$; ***$p < 0.005$; ****$p < 0.0001$) Caption: h.e.: hydroalcoholic extract, LTV: low-temperature vacuum, Blank: aqueous extract without enzymatic treatment, C: aqueous extract with the addition of 10 U of cellulose/g of dry matter, T: aqueous extract with addition of 10 U of tannase/g of dry matter, M: aqueous extract with the addition of 10 U of a commercial mixture of pectinase, hemicellulase, and β glucanase/g of dry matter, and T+M: aqueous extract with addition of 5 U of tannase/g of dry matter + 5 U of commercial mixture of pectinase, hemicellulase, and β glucanase/g of dry matter.

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containing the extracts were cooled in an ice bath for 15 min to paralyze the enzymatic reaction. The filtration was done in a porcelain filter with *Whatman* number 1 paper. Finally, the extracts were frozen (−20 °C) and then taken for freeze drying at −45 °C for 48 h (Liotop, model L101, São Carlos, Brazil).

2.4. Characterization of Phenolic Extracts. *2.4.1. Determination of Total Phenolics.* The determination of total phenolic compounds was performed following the methodology described by Singleton and collaborators.[20] Four different concentrations (0.4, 1.2, 1.6, 2.0 mg/mL) were used in triplicate. The control or standard sample was made of deionized H_2O in place of the sample. Absorbance was analyzed in a 96-well microplate reader at 725 nm (Spectrophotometer DU 640, Beckman CoulterTM, EUA). The calibration curve was elaborated using gallic acid and the results were expressed in μg of gallic acid equivalents per mg of sample.

2.4.2. Identification and Quantification of Major Phenolics by High-Performance Liquid Chromatography Coupled to a Diode Array Detector (HPLC-DAD). Chlorogenic acid, caffeine, theobromine, theophylline, catechin, epicatechin, and epicatechin gallate were identified and quantified in all extracts obtained by high-performance liquid chromatography coupled to a diode array detector (HPLC-DAD). The extraction was performed according to Santana and collaborators.[18] A 10 mg portion of each sample was mixed with 1 mL of 50% (w/v) methanolic solution. The mixture was filtered in a 0.45 μm membrane (HV PVDF, Millipore, Massachusetts) before being injected into the chromatograph. The mobile-phase solutions were formulated as follows: 100% water (A), 100% methanol (B), water/formic acid at 99.9:0.1, v/v (C) and methanol/formic acid at 99.9:0.1, v/v (D). Elution with a flow rate of 0.5 mL/min was performed as follows: 0−15 min of 90−10% C in D; 15−25 min of 90−10% C in D; 25−34 min of 80−20% C in D; 35−40 min of 80−20% C in D; 40−55 min of 90−10% C in D; and 55−60 min of 90−10% C in D. The absorption spectra were obtained at 210, 260, 280, and 330 nm. The chromatograms were processed at 280 nm. The results were expressed as g/100 g of extract (dry basis).

2.5. Determination of the Antioxidant Capacity *In Vitro* of the Extracts Obtained. The determination of the antioxidant capacity of the extracts was performed by reducing the stable radical DPPH (2,2-diphenyl-1-picrylhydrazyl), as described by Peschel and collaborators[21] and adapted by Macedo and collaborators.[22] The linear regression equation of the DPPH determinations of the Trolox standard for the calculation of antioxidant activity and the results are expressed in μmol equivalent in Trolox/mg of extract.

The determination of antioxidant capacity by the ORAC method was carried out in triplicate, following the methodology described by Dávalos and collaborators[23] and adapted by Macedo and collaborators.[22] The ORAC values were established based on the difference between the area under the fluorescence decrease curve of the extracts and the white (net AUC), and the linear regression equations between the net AUC and concentrations were calculated for all the extracts, so the results are expressed in μmol equivalent in Trolox/mg of extract.

2.6. Evaluation of the *In Vitro* Antiglycating Activity of the Extracts Obtained. The extracts obtained were evaluated for their antiglycation capacity related to protein glycation inhibition using the three *in vitro* glycation models, bovine serum albumin (BSA) with fructose (FRU); BSA with methylglyoxal (MGO); and arginine (ARG) with methylglyoxal (MGO), using the method described by Wang and collaborators,[24] considering the amendments proposed by Shen and collaborators.[25] For the analyses in the three models, the extracts were prepared in different concentrations in potassium phosphate buffer (50 mM, pH 7.4 with 0.02% sodium azide to prevent microbiological growth).

The antiglycation assay in the BSA/FRU model was performed by mixing 500 μL of fructose solution (1.5 M) with 500 μL of extracts. This solution was incubated at 37 °C for 2 h. Then, 500 μL of BSA (60 mg/mL) was added to the previous solution and incubated again at 37 °C for 6 days. For the BSA-MGO model, 500 μL of MGO solution (60 mmol/L) and 500 μL of extracts were mixed, and they were kept at 37 °C for 2 h. Then, 500 μL of BSA (60 mg/mL) was added to the solution. In the ARG-MGO model, 500 μL of MGO solution (60 mmol/L) and 500 μL of extracts were added in 10 mL tubes. The tubes were closed and held at 37 °C for 2 h. Then, 500 mL of arginine (60 mg/mL) was added to the solution.

Fluorescent AGEs were monitored (excitation wavelength of 360 nm and emission wavelength of 460 nm) by luminescence (FLUOstar OPTIMA − BMG Labtech, Germany). The percentage of inhibition of AGEs was calculated by the following equation

$$\text{inhibition (\%)} = \left[1 - \left(\frac{\text{Fe}}{\text{Fc}}\right)\right] \times 100 \quad (1)$$

where Fe is the fluorescence emitted by the extract and Fc is the fluorescence emitted by the control.

2.7. Cell Culture. Human colon adenocarcinoma cell lines Caco-2 (ATCC HTB-37) were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM) (4.5 g/L) supplemented with L-glutamine 0.6 g/L, 1% (v/v) nonessential amino acids, 10% (v/v) fetal bovine serum (FBS), and 1% (v/v) antibiotics (100 μg/mL streptomycin and 100 U/mL penicillin), in a humidified 5% CO_2 atmosphere at 37 °C. The cells were subcultured once a week at a split ratio of 1:10 using trypsin−EDTA solution (0.25% trypsin, 0.02% EDTA).

2.8. Generation of Intracellular Reactive Oxygen Species (ROS). The generation of intracellular ROS was measured using CM-H_2DCFDA, which is an oxidation-sensitive fluorescent probe. Briefly, human Caco-2 intestinal epithelium cells (3×10^5 cells/mL) were seeded on black plates with a 96-well transparent bottom in DMEM supplemented with 10% fetal bovine serum. After 24 h, the culture medium was replaced by HBSS containing 10 μM CM-H_2DCFDA and the plates were incubated for 30 min at 37 °C. After being washed with PBS for complete removal of the probe, the cells were treated with the hydroalcoholic extract diluted in HBSS at different concentrations with and without 100 μM AAPH. Fluorescence was monitored every 30 min for 5 h (e.g., 490 nm/in: 520 nm) using the FLUOstar OPTIMA plate reader. The results were expressed as % increase in fluorescence compared to the control.

2.9. Statistical Analysis. To evaluate the effect of processing on the extraction of phenolic compounds, a two-factor analysis of variance (ANOVA) was performed, considering the factors milling (m), addition of enzymes (e), and its interaction (i), followed by Tukey's test, to compare the means of the different extracts ($p < 0.05$). The results were expressed as means ± standard deviation; all measurements were performed in triplicate. A Pearson correlation test was also performed between the antiglycant activity variables × the other analyses (phenolic profile and antioxidant activity *in vitro*).

To evaluate the generation of ROS in Caco-2 cells, a one-factor ANOVA was performed, followed by the Dunnet test, to compare the means ($p < 0.05$). The experiments were carried out in at least triplicates, and the results were confirmed in a second independent experiment. All analyses and graphs were performed using GraphPad Prism 8.0 software.

3. RESULTS AND DISCUSSION

3.1. Effect of Processing on the Extraction and Phenolic Profile of the Extracts Obtained. As can be seen in Figure 1A, the LTV extracts had a lower total phenolic content than the hydroalcoholic extract (mean of all LTV extracts: 82.6 ± 13.7; h.e.: 129.3 ± 0.8 μg of GAE/mg of lyophilized extract). Although the results obtained show a higher efficiency of extraction of phenolic compounds using an organic solvent, the LTV extraction process, associated with equipment and process conditions that allow extraction in higher performance, also proved to be efficient, which can be very interesting from an environmental point of view, since these technologies do not use large quantities of organic solvents, like the conventional extraction methods. Furthermore, LTV extraction is less time-consuming and more cost-effective than the conventional ones. Thus, these technologies

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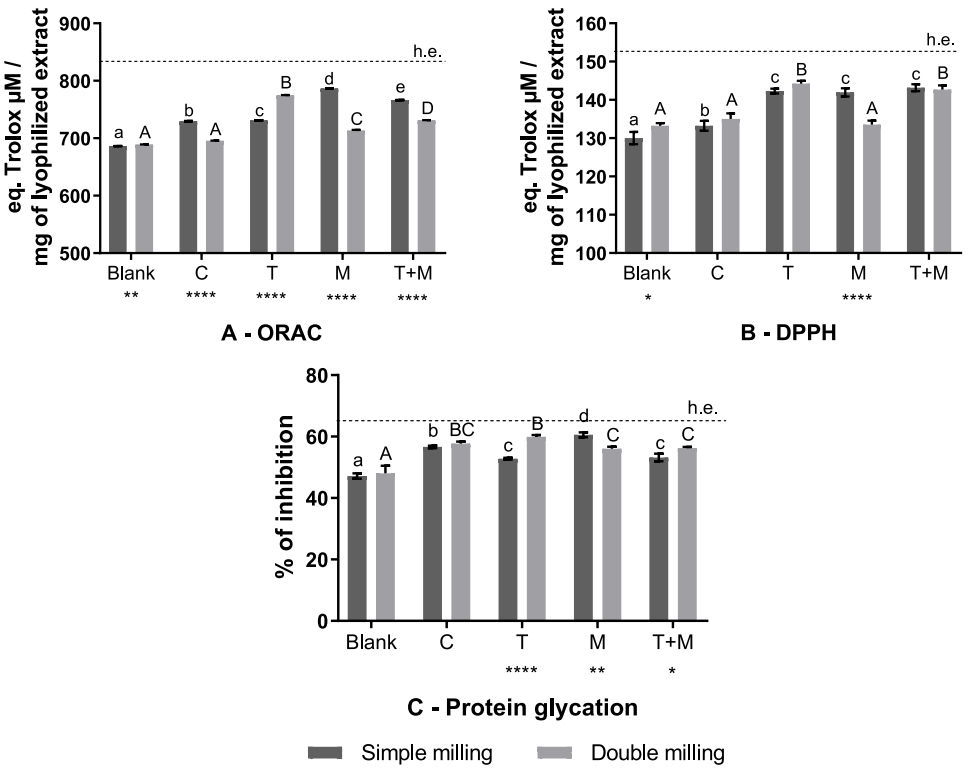

Figure 2. Bioactivity of hydroalcoholic and LTV extracts assisted or not assisted by enzymes, with single or double milling. Values were expressed as % inhibition of protein glycation (A) and as μg of gallic acid/mg of lyophilized extract for oxygen radical absorption capacity (B, ORAC) and for DPPH (C), as mean ± standard deviation, and were analyzed by two-way ANOVA, followed by the Tukey test $p < 0.05$. Different lowercase letters mean differences within the "single milling" group. Different capital letters signify differences within the "double milling" group. Asterisks mean differences between milling processes (**$p < 0.01$; ***$p < 0.005$; ****$p < 0.0001$) Caption: h.e.: hydroalcoholic extract, LTV: low-temperature vacuum, Blank: aqueous extract without enzymatic treatment, C: aqueous extract with the addition of 10 U of cellulose/g of dry matter, T: aqueous extract with addition of 10 U of tannase/g of dry matter, M: aqueous extract with the addition of 10 U of a commercial mixture of pectinase, hemicellulase, and β-glucanase/g of dry matter, and T+M: aqueous extract with addition of 5 U of tannase/g of dry matter + 5 U of commercial mixture of pectinase, hemicellulase, and β-glucanase/g of dry matter.

can represent an eco-friendly alternative for recovery of phenolic compounds from byproducts.

Among the LTV extracts, the enzymatic treatment proved to be efficient for a greater extraction of phenolic compounds ($p_e < 0.0001$). In the LTV extracts produced with simple milling, all enzymatic treatments showed a higher content of total phenolics in relation to the blank extract with simple milling (without any enzymatic treatment). Among the double milling extracts, all enzymatic treatments were also efficient, except for the cellulose treatment. The type of milling also influenced the amount of total phenolics obtained ($p_m < 0.0001$), and the double milling tends to extract a higher amount of phenolics compared to the single milling. This result was expected, since the particle size increases the contact surface of the residue with the solvent, improving the recovery of phenolic compounds;[13] enzymatic treatment can also contribute to increasing the extraction of compounds in different types of plants, breaking up the cell walls of the matrix, and releasing the compounds bound to it, as well as increasing the biological properties of these, through structural modification, increasing absorption or biological activity.[19,22]

Different studies for recovering phenolic compounds from coffee byproducts (coffee husk, pulp, mucilage, and parchment) using conventional and emerging technologies were reported, being the chlorogenic acid the main phenolic compound.[11] About green coffee beans particularly, Pimpley and Murthy studied the integrated approach of green technologies like enzymatic hydrolysis-, steam-, microwave-, and ultrasound-assisted methods for extraction of phenolics and their results showed that the combined effect of enzyme- and steam-assisted extraction improved the total polyphenols, flavonoids, phenolic profile, and antioxidant capacity significantly.[26] Using coffee husks and pulp as a substrate for fermentation, García and collaborators also observed that the fermentation process increased the number of polyphenols extracted, especially chlorogenic acid, caffeic acid, and rutin.[27] In fact, a literature review conducted by Janissen and Huynh reported that the concentration of phenolic compounds and chlorogenic acid in coffee products depends directly on the solvent used in extraction, the extraction technique, and also other factors such as the origin and variety of coffee and quality of the raw material used in extraction.[28]

In this work, chlorogenic acid was the major phenolic compound identified by HPLC/DAD in the green coffee extracts obtained (Figure 1B). However, all LTV extracts had a lower amount of chlorogenic acid detected when compared to the hydroalcoholic extract (mean of all LTV extracts: 4.69 ± 1.50; h.e: 17.7 ± 0.07 g/100 g of lyophilized extract). Nonetheless, among the LTV samples, both the enzymatic treatment and the type of milling had an impact on the detected amount of chlorogenic acid ($p_e < 0.0001$; $p_m < 0.0001$), and the enzymatic treatment and double milling favored a greater extraction of chlorogenic acid, following the

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same profile of results observed for the amount of total phenolic compounds.

Coffee and its byproducts are recognized for their high content of chlorogenic acid, and the amount of this compound found in coffee pulp is similar to that found in green beans.[11] Chlorogenic acid was also the compound found in greater abundance in the study published by Ramón-Gonçalves and collaborators[29] extracting phenolics from Arabica coffee, using ethanol:water solution as a solvent. The study conducted by Inácio and collaborators[30] observed that enzyme-assisted extraction and ultrasound, combined or not, also resulted in extracts with mostly chlorogenic acid, followed by caffeic acid and dihydroxybenzoic acid. In the present study, the profile found was more varied, with phenolic compounds of bioactive importance such as caffeine, theophylline, and gallocatechins. Caffeine (Figure 1C) also had lower levels in the aqueous extracts compared to the hydroalcoholic extract (average of all LTV extracts: 1.46 ± 0.21; h.e: 2.72 ± 0.03 g/100 g of lyophilized extract). The enzymatic treatment favored the extraction of a higher amount of caffeine (p_e < 0.0001), especially among the samples with double milling. However, although the type of mill affected the amount of caffeine detected (p_m < 0.0001), at some points, the double mill led to a lower amount of caffeine extracted, different from what was expected.

Regarding the minority phenolic compounds found, the LTV extracts showed equal or higher levels of theophylline (Figure 1D), compared to the hydroalcoholic extract (average of all LTV extracts: 0.076 ± 0.008; h.e: 0.071 ± 0.002 g/100 g of lyophilized extract), with emphasis on the extracts produced with double milling and enzymatic extraction with tannase and commercial mixture (up to 0.091 ± 0.001 g/100 g of lyophilized extract). Catechin (Figure 1E), epicatechin (Figure 1F), and epicatechin gallate (Figure 1G) showed similar profiles. The LVT treatments without enzymatic treatment (of both types of milling) showed lower levels than the hydroalcoholic extract (catechin: 0.090 ± 0.001 g/100 g of lyophilized extract; epicatechin: 0.056 ± 0.013 g/100 g of lyophilized extract; epicatechin gallate: 0.677 ± 0.002 g/100 g of lyophilized extract), but the enzymatic treatment favored the extraction of higher levels of these phenolic compounds, with emphasis on the following treatments: double milling + enzymatic treatment with tannase and commercial mixture for catechin (0.160 ± 0.001 g/100 g of lyophilized extract); simple milling + enzymatic treatment with commercial mixture for epicatechin (0.090 ± 0.001 g/100 g of lyophilized extract); and simple milling + enzymatic treatment with cellulase for epicatechin gallate (0.777 ± 0.018 g/100 g of lyophilized extract).

These phenolic compounds found in the analyzed samples are important for the formation of the characteristic aroma and flavor of coffee, in addition to also promoting beneficial effects on health, presenting antioxidant, anti-inflammatory, and antihyperglycemic characteristics, among others.[11] In particular, chlorogenic acid is known for several benefits, including anticancer, antioxidant, and antiglycating properties.[7]

3.2. Effect of Processing on the Antioxidant and Antiglycating Capacity of Phenolic Extracts of Green Coffee. The antioxidant capacity of all extracts obtained was evaluated by using two *in vitro* techniques: ORAC (Figure 2A) and DPPH (Figure 2B), and the results were similar to that observed in the total phenolic compound analysis. This was expected since the relationship between polyphenols and antioxidant activity has been studied for years and the antioxidant activity of a specific phenolic compound is related to the number of available hydroxyl groups present in the chemical structure. Therefore, the manner these compounds neutralize free radicals will depend on their relative concentrations in the sample matrix.[31] The antioxidant activity by ORAC was positively correlated (data not shown) with the amount of total phenolic compounds (R^2 = 0.7344, p = 0.0008), as was the DPPH (R^2 = 0.7850, p = 0.0003), showing that the greater the amount of phenolic compounds extracted in this matrix, the greater the *in vitro* antioxidant activity.

The LTV extracts showed a similar profile for ORAC and DPPH, and all of them showed lower antioxidant capacity than the hydroalcoholic extract (ORAC: average of all LTV extracts: 730.0 ± 35.8; h.e.: 832.5 ± 0.8 eq. Trolox μM/mg of lyophilized extract; DPPH: average of all LTV extracts: 137.9 ± 5.4; h.e.: 153.5 ± 0.7 eq Trolox μM/mg of lyophilized extract). The enzymatic extraction combined with the different mills favored a greater antioxidant capacity in most combinations. It is currently known that the enzymatic extraction can change the final phenolic profile of a product, transforming the polyphenols in their glycosylated form, ester, or polymers into their aglycone form, which presents greater benefits to human health. Thus, enzymatic extraction generally tends to favor the phenolic profile of a product, maintaining or improving its health benefits. Although our results demonstrated that the use of enzymes during the extraction of phenolics did not change the phenolic profile of the extracts, as seen in other work,[19,32,33] it increased the total phenolic compounds extracted (Figure 1A) and also improved the biological activity *in vitro* (Figure 2), when compared with extracts that were not assisted by enzymes (called blank).

Other studies have also evaluated the effect of different extraction methods on antioxidant capacity.[13] Inácio and collaborators investigated different methods of extracting phenolic compounds from Arabica coffee residues using ultrasonic extraction; enzyme-assisted extraction; and enzyme-assisted extraction under the action of ultrasound. The highest extraction yield was obtained by the ultrasound method, with an increase in the antioxidant activity in these extracts, measured by the ORAC and ABTS methods.[30]

The protein glycation profile by the BSA/FRU method (Figure 2C) followed the tendency observed in the antioxidant capacity, with the hydroalcoholic extract showing a higher percentage of inhibition (65.2 ± 0.2%) than the LTV extracts without enzymatic inhibition (mean blanks: 47.6 ± 0.6%) and the same amount recovered using enzymes. In this case, the type of milling affected the final result in different ways: the use of double milling favored the increase in protein glycation inhibition when associated with tannase (59.9 ± 0.6%), but it decreased when associated with commercial mixing (56.1 ± 0.1%).

The BSA/MGO and ARG/MGO models were also used, but there were no positive results (data not shown). One hypothesis as to why green coffee extracts showed positive results only in the BSA/FRU model is the fact that this model evaluates the glycating capacity of the reactants at all stages of protein glycation, while the other models evaluate the antiglycating capacity only in the intermediate stages of protein glycation.[24,34]

Under conditions of hyperglycemia, reducing sugars can initiate glycation reactions of some proteins of the circulatory system, such as albumin, a protein that is present in high

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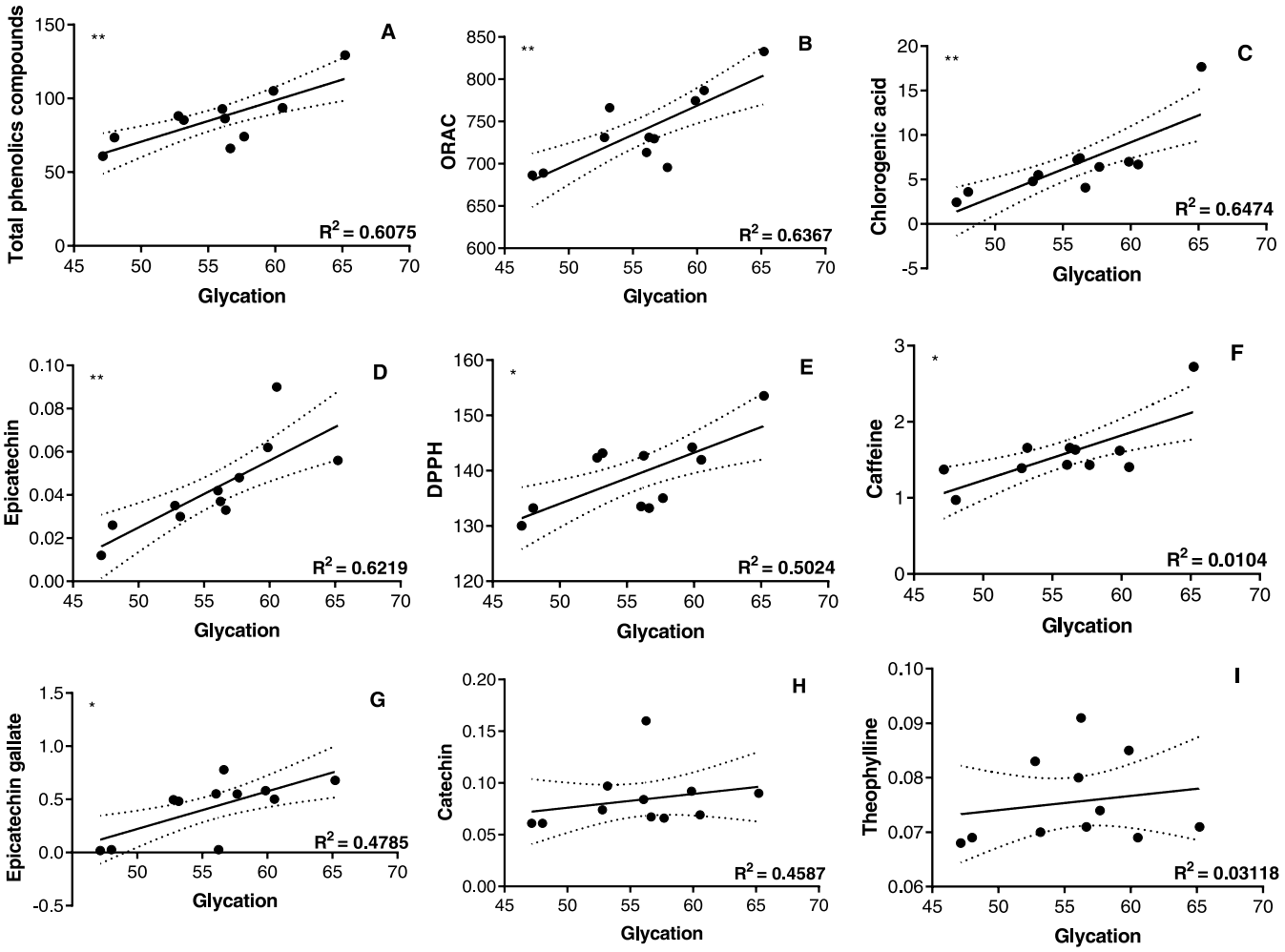

Figure 3. Correlation between protein glycation and other parameters (A, total phenolics; B, antioxidant activity by ORAC; C, chlorogenic acid content; D, epicatechin content; E, antioxidant activity by DPPH; F, caffeine content; G, content of epicatechin gallate; H, catechin content; I, theophylline content). Asterisks indicate significant difference (*$p < 0.05$; **$p < 0.001$).

concentrations in serum and is highly sensitive to glycation.[35] The level of glycated albumin in normoglycemic individuals varies between 1 and 16%, but in diabetic people, this value can be 2−3 times higher, reaching up to 90% of glycated albumin in patients with severe diabetes.[36,37] The BSA/FRU model is also important for evaluating the level of protein glycation at an accelerated level, since fructose and its metabolites are important precursors of the intracellular formation of AGEs, being a more potent inhibitor of glycation when compared to glucose.[38,39]

3.3. Correlation of Antiglycant Capacity with the Phenolic Profile and Antioxidant Capacity. In order to better understand the mechanism of protein glycation inhibition observed in the extracts obtained from green coffee, a correlation analysis was performed between the results of protein glycation and other analyses (phenolic profile and antioxidant capacity). As can be seen in Figure 3, in this analysis model, the inhibition of protein glycation was positively correlated ($p < 0.01$) with the factors antioxidant activity by ORAC, total phenolic compounds, chlorogenic acid content, and epicatechin and less significantly with antioxidant activity measured by DPPH, caffeine content, and epicatechin gallate. The contents of catechin and theophylline were not correlated with the inhibition of protein glycation. These results show a relationship between the amount of phenolics and antioxidant capacity with the inhibition of protein glycation, a fact corroborated by previous studies.[37]

Fernandes and collaborators evaluated the antiglycating capacity of the phenolic extract of citrus residues, rich in flavonoids, and observed a high correlation between the amount of total phenolics, antioxidant capacity, and antiglycating activity, while the inhibition of AGEs was greater than 60%.[9] The peanut and grape residue also showed high antiglycating capacity, and the major phenolic compounds identified were catechin and procyanidin B2.[34,40] In addition, administration of peanut residue extract to murine macrophage RAW264.7 cells stimulated with AGEs inhibited oxidative stress, reducing the production of nitric oxide as well as the cytokines interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α).[41]

The phenolic compounds found in the green coffee extract that seem to have the strongest and most positive impact on the inhibition of protein glycation are chlorogenic acid and epicatechin. Chlorogenic acid is particularly known for its strong AGE-inhibiting action *in vitro*.[42] Ramón-Gonçalves and collaborators evaluated the role of chlorogenic acid and coffee silverskin extract in the formation of AGEs and observed significant reductions of 90 and 49% in a protein−MGO model system,[29] evidencing the role of chlorogenic acid as one of the compounds responsible for the action of coffee and its

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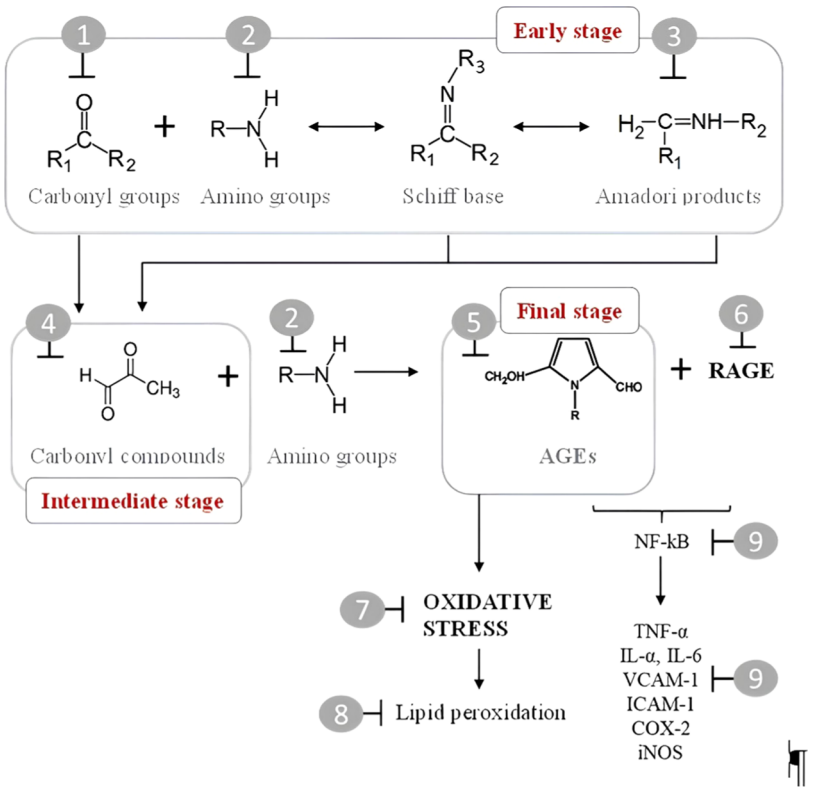

Figure 4. Possible mechanisms of the antiglycating action of polyphenol. Polyphenol may act as (1) inhibiting the autoxidation of sugars and lowering blood glucose levels; (2) binding with amino groups, masking amino acid residues and protecting proteins against conformational transition from their native structure, charge variation at the beginning of glycation, and cross-linking reactions in the final stage; (3) reducing the formation of Amadori products; (4) capturing reactive carbonyl compounds by conjugation reactions, forming mono- and di- adducts, and competing for reactive protein sites; (5) reducing the formation of fluorescent and nonfluorescent AGEs; (6) reducing RAGE expression and activity; (7) protecting the cell against oxidative stress, eliminating free radicals, and increasing the ratio of glutathione in the reduced form (GSH)/glutathione in the oxidized form (GSSG); (8) reducing LDL oxidation, blood lipid peroxidation levels, and acting in the maintenance of the mitochondrial membrane; and (9) inhibiting the activity of pro-inflammatory cytokines. Figure adapted from Melo.[45] Abbreviations: AGEs: advanced glycation end products; RAGE: receptor for AGE; NF-kB: nuclear factor kappa B; TNF-α: tumor necrosis factor α; IL-1α: interleukin 1 α; IL-6: interleukin 6; VCAM-1: vascular cell adhesion molecule 1; ICAM-1: intercellular adhesion molecule 1; COX-2: cyclooxygenase-2; and iNOS: inducible nitric oxide synthase.

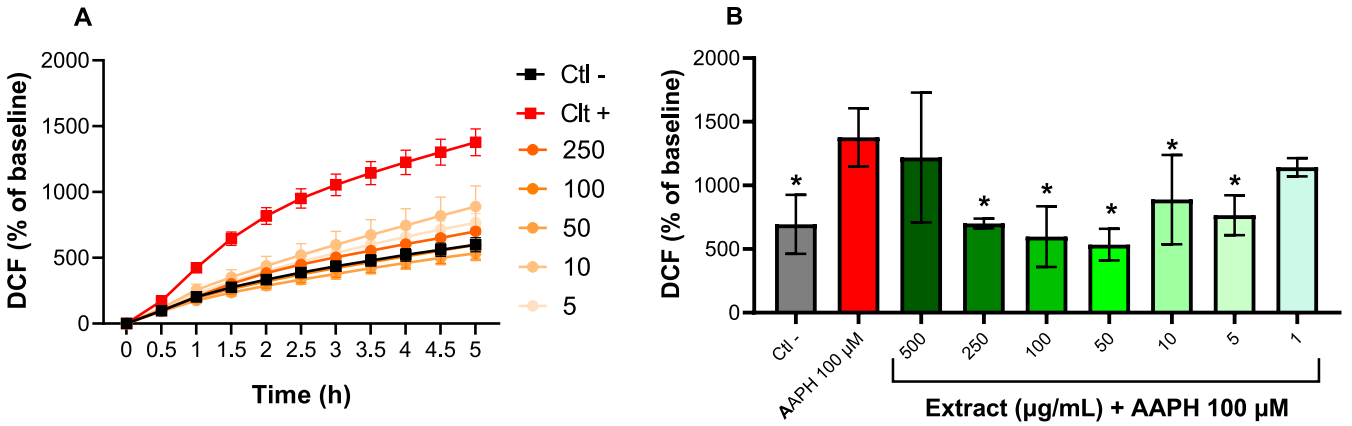

Figure 5. Production of reactive oxygen species (ROS) in intracellular kinetics in control Caco-2 cells, the positive control (treated with 100 μM AAPH), and treated with hydroalcoholic extract of green and/or defective coffee at different concentrations and 100 μM AAPH. Values represented as kinetics (A) and as final time (B), as mean ± standard deviation, and analyzed by one-way ANOVA, followed by Dunnet's test. Asterisks represent statistical significance in relation to the positive control ($p < 0.05$).

products in the reduction of AGEs. In his study evaluating the properties of coffee fractions, Verzelloni and collaborators also showed that coffee contains molecules with antiglycant activity *in vitro*, especially chlorogenic acid.[43]

Phenolic compounds can prevent the production of AGEs by different mechanisms of action. They can physically protect the protein from the glycation process, through the interaction with side chains of protein amino residues blocking the reaction sites with carbonyl-reactive groups and generating

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antioxidant compounds, making glycation targets on the protein molecule inaccessible to take part in the glycation reaction.[37,44] Besides that, they can act on the reactive carbonyl groups of reducing sugars, inhibiting the formation of AGE intermediates and decreasing the expression of the pro-inflammatory receptor for AGEs (RAGE) and the subsequent cellular inflammatory cascade. Another hypothesis is that the phenolic compounds can scavenge ROS produced during the glycation process, thus inhibiting glycation and disfavoring the formation of AGEs.[37] A summary of the possible mechanisms of action of polyphenols under AGEs is represented in Figure 4.

3.4. Effect of the Green Coffee Extract in ROS Generation in Caco-2 Cells. To better understand the role of the green coffee extract in the ROS generation, the antioxidant capacity of the hydroalcoholic extract of green coffee was evaluated in an *in vitro* model of Caco-2 intestinal cells. This analysis was performed only with the hydroalcoholic extract, since it presented higher levels of total phenolics as well as better antioxidant capacity in vitro by ORAC and DPPH, as presented in the results above.

As can be seen in Figure 5, cells stimulated with 100 μM AAPH (positive control) showed a 98% increase in ROS generation compared to control cells (p = 0.0017). The hydroalcoholic extract of green coffee inhibited this generation of ROS induced by AAPH, when used at concentrations of 250 to 5 μg/mL, including maintaining the generation of ROS at levels similar to those of control cells (without induction). The extract at a concentration of 50 μg/mL was the most effective, reducing the generation of ROS by 61%, compared to the positive control (p = 0.0001), showing the potential antioxidant action of the hydroalcoholic extract of green coffee in stressed cells.

Oxidative stress is described as a dangerous imbalance between the production of ROS and antioxidant defense mechanisms, leading to potential tissue dysfunction and damage. During glycation, an excess of ROS, among other molecules, can be formed, being capable of reacting and damaging cells and other proteins.[39] Higher formation of ROS, cross-linking of proteins, and interaction of these AGEs with their receptors and/or binding proteins with subsequent release of pro-inflammatory cytokines growth factors and adhesion molecules are some toxic effects linked with accumulation of AGEs that can lead to health problems such as diabetes, cardiovascular diseases, cancer, and others.[46]

4. CONCLUSIONS

The extracts obtained through the LTV technique showed lower total phenolic content and less *in vitro* biological activity (antioxidant and antiglycant) when compared to the conventional hydroalcoholic extract; however, the double milling and the enzymatic treatment seem to recover this potential in the LTV extracts, showing that it is possible to obtain an eco-friendly phenolic-rich extract from green coffee residue, presenting an opportunity of using it as an upcycled product, with potential application both in the food industry and in cosmetics.

Among the emerging extraction techniques used, we consider that the LTV coupled with double milling and the enzyme tannase presented the best performance, as it presented a greater capacity for the recovery of total phenolics, a parameter that reflects the biological antioxidant and antiglycating activity. However, it is important to emphasize that the detection of some specific phenolic compounds, such as chlorogenic acid and caffeine, was below that obtained in the hydroalcoholic extract. Furthermore, for the extraction of specific phenolic compounds, other types of combinations may be more appropriate (i.e., for catechin: LTV + double milling + tannase and commercial mix of enzymes; for epicatechin gallate: LTV + simple milling + cellulose). Furthermore, we have to consider that the LTV extraction was carried out under industrial-scale conditions, in a pilot plant, demonstrating once again the possibility of scaling up the phenolic compound's extraction from green coffee beans without the use of organic solvents.

The generation of intracellular ROS in Caco-2 intestinal cells was evaluated only for the hydroalcoholic extract, emphasizing its important antioxidant activity, even at low concentrations, in addition to being noncytotoxic, which is an important factor for this extract to be used in the development of functional food ingredients.

■ ASSOCIATED CONTENT

Data Availability Statement

Data are provided within the manuscript. If any other data are needed for consultation, they will be made available.

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Notes

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