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Hypolipidemic properties of cocoa and coffee by-products after simulated gastrointestinal digestion: A comparative approach †

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Abstract: New sustainable ingredients with beneficial properties for health are a main goal for the food industry. In this regard, the cocoa shell (CS) and the coffee pulp (CP), by-products from the coffee and cocoa industry produced worldwide in large amounts, are suitable candidates. We previously stated that these by-products are sources of phytochemicals and dietary fiber with potential hypolipidemic properties. This study aimed to assess the hypolipidemic properties of CS and CP after simulated gastrointestinal digestion. The capacities of the residual fraction of each digestion phase to bind bile salts and cholesterol and inhibit the lipase activity were measured to establish the *in vitro* hypolipidemic properties of both by-products. Furthermore, the digested fractions' effect on lipid accumulation was evaluated in the HepG2 cell line. From results, the CS showed a higher ability to bind cholesterol (4–24%) and bile salts (2–3%) in gastric and colonic phases. Meanwhile, during the gastrointestinal phase, CP showed a greater capacity to bind cholesterol (1–13%) and bile salts (2%). The capacity to inhibit lipase activity was more accentuated in the CS in gastrointestinal digestion (16%) whereas during gastric digestion in the CP (11%). Likewise, the digested fractions of both by-products (100 μg/mL) significantly alleviated the accumulation of fat (17–20%) in the HepG2 cell model after the stimulation of cells with palmitic acid. This comparative approach suggests that both by-products exhibit similar hypolipidemic properties after *in vitro* digestion. This research supports the revalorization of cocoa and coffee by-products as food ingredients and nutraceuticals with potential health benefits in preventing chronic metabolic diseases. due to their remarkable hypolipidemic properties.

Keywords: cocoa shell; coffee pulp; gastrointestinal digestion; hypolipidemic properties; hypocholesterolemic; novel ingredients

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1. Introduction

Cocoa and coffee are two of the most consumed products in the world. The annual world gross cocoa harvest for the 2019 period was 4726 thousand tons, and it is expected to increase by approximately 2.5% in 2020 [1]. Regarding coffee, world production reached 163.7 million 60 kg bags in the same period [2]. During cocoa bean and coffee cherry processing, approximately 90% of the products are discarded as wastes, which sometimes are not appropriately handled, causing high environmental impact [3–5]. The cocoa shell (CS) is the tegument that coats the cocoa bean and is obtained during its roasting process [5]. The cocoa shell is about 10–17% of the total weight of the cocoa bean,

representing approximately 800 tons of waste, which revalorization leads to economic and environmental benefits [1]. The cocoa shell is a source of carbohydrates (62%), proteins (10–27%), dietary fiber (DF) (59%), and valuable bioactive compounds (methylxanthines, phenolic compounds, among others) [6,7]. The coffee pulp (CP) is one of the coffee by-products obtained during the wet processing of coffee beans, and it represents 29% dry weight of the whole cherry [8,9]. Coffee pulp composition includes dietary fiber (57%), carbohydrates (20%), protein (11%), and fat (3%), as well as valuable bioactive compounds [3,10].

Although both by-products have been used for different purposes, such as animal feed or fertilizers, a promising green application is their utilization as new sustainable food ingredients with beneficial properties for health. The CS and the CP have been declared safe ingredients, and their characteristics and composition make them a potential source of nutrients and beneficial compounds [5,10]. Previous studies have established a relationship between phytochemicals from both the CS and the CP and the regulation of biomarkers of metabolic syndrome [3,7,11,12]. Recent research proposes that the digestion process positively influences the hypolipidemic properties of both by-products [13,14]. Therefore, the main objective of this study was to assess the *in vitro* hypolipidemic properties of the CS and the CP, and its biological activity in HepG2 cells, after *in vitro* simulated gastrointestinal digestion.

2. Materials and Methods

2.1. Reagents

Dulbecco's Modified Eagle Medium low glucose (1000 mg/mL) (DMEM) and 0.25% trypsin-EDTA were obtained from GE Healthcare Life Sciences. Fetal Bovine Serum (FBS), penicillin-streptomycin (100×) were acquired from Gibco Life Technologies. *o*-Phthalaldehyde, furfural, bile salts, lipase, Tween® 20, Bovine Serum Albumin (BSA), Palmitic Acid (PA), 2′,7′-Dichlorofluorescein diacetate (DCFDA), and Oil Red O were purchased from Sigma-Aldrich. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was purchased from Promega Corporation (Carlsbad, CA, USA). All other chemicals and reagents were obtained from Panreac (Barcelona, Spain), unless otherwise specified. The CS was kindly provided by Chocolates Santocildes (Castrocontrigo, León, Spain). The CP was kindly supplied by AORA Health (Spain).

2.2. Cocoa Shell and Coffee Pulp Flour and Aqueous Extracts Preparation

The by-products were processed in a mill to obtain the flours. Aqueous extracts were obtained according to the conditions described by Rebollo-Hernanz et al. [15,16].

2.3. INFOGEST Static In Vitro Simulated Digestion

In vitro gastrointestinal digestions were carried out following the INFOGEST method [17]. *In vitro* simulated colonic digestions were performed following the method previously described by Papillo et al. [18].

2.4. Cholesterol-Binding Capacity

To measure the binding capacity of the residual fraction from the flour digestion of both by-products, 0.1 g of the residual fraction from each of the sample digestion phases was incubated with 2 mL of egg yolk diluted 1:10 at different pH (pH 2 and pH 7), and incubated with agitation (2 h, 37 °C, 750 rpm), followed by centrifugation (800 × *g*, 15 min). Subsequently, 0.1 mL of the supernatant were mixed with 0.6 mL of pure acetic acid, 0.2 mL of H_2SO_4 (96%), and 0.1 mL of o-phthalaldehyde (0.6 mg/mL) and incubated with agitation (30 min, 60 °C, 750 rpm). The absorbance was read at 550 nm using a microplate reader (Cytation 5, Biotek) [19].

2.5. Bile Salts-Binding Capacity

For the measure of the residue's bile salts-binding capacity, 0,1 g of the residual fraction of gastric and intestinal flour digestion of both by-products were mixed with a solution of NaCl (15 M, pH 7.0) and sodium cholate (4.65 mM), incubated (3 h, 37 °C, 750 rpm), and centrifuged (800 × *g*, 15 min). After that, an aliquot of the supernatants (0.1 mL) was added to 0.6 mL, H_2SO_4 (45%), and 0.1 mL furfural (0.3%) and incubated with shaking until the color development (30 min, 65 ºC, 750 rpm). The absorbance was measured by triplicate at 620 nm using a microplate reader [19].

2.6. Inhibitory Activity against Pancreatic Lipase

To evaluate the inhibition ability against pancreatic lipase, 0.1 g of the residual fraction of each digestion phase of both by-products were mixed with olive oil (2 mL), lipase (2 mL, 0.75 mg/mL), bile salts (5 mL), and phosphate-buffered saline (PBS) 10X (5 mL, pH 7.2). The samples were incubated (37 °C, 1 h), then the reaction was stopped by placing the samples on ice for 5 min, followed by centrifugation (800 × *g*, 15 min). Supernatants of the oil-containing samples were then emulsified by the addition of Tween® 20 (0.2 mL). Finally, the lipase activity of each of the samples was titrated with NaOH (0.02 M) and phenolphthalein [20].

2.7. Sample preparation for HepG2 cells

The digested fractions of the cocoa shell flour (CSF) and extract (CSE) and of the coffee pulp flour (CPF) and extract (CPE) were diluted in PBS 1× and filtered with 0.45 µm cellulose acetate filters.

2.8. HepG2 cell cultures

HepG2 cells obtained from ATCC (Rockville, MD, USA) were cultured in DMEM low glucose (1 g/mL glucose) supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. To evaluate the cytotoxicity, HepG2 cells were seeded at 5×10^5 cells/mL in 96-well culture plates and incubated for 24 h. On day 2, cells were treated with CSF, CSE, CPF, and CPE (50–250 mg/mL) and left incubating at 37 °C and 5% CO_2 for 24 h. On day 3, cell viability assay was performed with the CellTiter 96® AQueous MTS Reagent Powder (Promega Corporation) according to the manufacturer's instructions.

2.8.1. Non-alcoholic Fatty Liver Disease induction in HepG2 cells

HepG2 cells were seeded onto a 0.32 cm^2 grown area 96 well flat bottom plate at a density of 10^5 cells/cm^2 and incubated for 24 h. On day 2, non-alcoholic fatty liver disease (NAFLD) was induced in HepG2 cells with PA (500 µM), the digested fractions (CSF, CSE, CPF, and CPE) were applied at a subtoxic concentration (100 µg/mL) for 24 h at 37 °C and 5% CO_2. To prepare the PA solution, PA was conjugated with DMEM containing 1% BSA. Control cells were treated with 1% BSA only. NAFLD induction was determined by measuring the intracellular reactive oxygen species (ROS) formation by adding DCFDA (25 µM). Fluorescence intensity was detected using a fluorescence microplate reader at an excitation/emission wavelength at 485 nm/530 nm, respectively. DCFDA was prepared according to the manufacturer's instructions.

2.8.2. Biological hypolipidemic activity

The accumulation of total lipids was performed as previously described by Rebollo-Hernanz et al. [21] using Oil Red O. The intracellular triglycerides (TAG) content was measured using a TAG colorimetric assay kit (Spinreact) according to manufacturer's instructions. Fatty Acid Synthase (FASN) Activity was carried out as previously described by Rebollo-Hernanz et al. [21].

2.8. Statistical analysis

All experiments were carried out in triplicate. All values are expressed as the mean ± standard deviation (SD) (n = 3). The effects of treatments were assessed by one-way ANOVA followed by post hoc Tukey test. Differences with values of $p < 0.05$ were defined as statistically significant. All analyses were performed using SPSS 24.0 (Lead Technologies, Chicago, IL, USA).

3. Results and Discussion

3.1. Comparison of the in vitro Hypolipidemic Properties of Cocoa Shell Flour and Coffee Pulp Flour Non-Digestible Fraction after in vitro Static Digestion

Figure 1A, B shows the capacity of the digestion residual fractions of CSF and CPF to bind cholesterol at pH 2 and 7, respectively. Non-digested (ND) CSF exhibited a higher cholesterol-binding capacity at pH 2 than ND-CFP. However, the digestion process affected flour cholesterol-binding capacity at pH 2, and non-significant differences were observed between the capacity of both by-products after digestion. As observed in **Figure 1B**, at pH 7, coffee and cacao by-products brought out a similar capacity to bind cholesterol before and after the digestion process, except for the CSF residue of the colonic digestion, which showed significantly higher cholesterol-binding capacity (24% $p < 0.05$) than the CPF one.

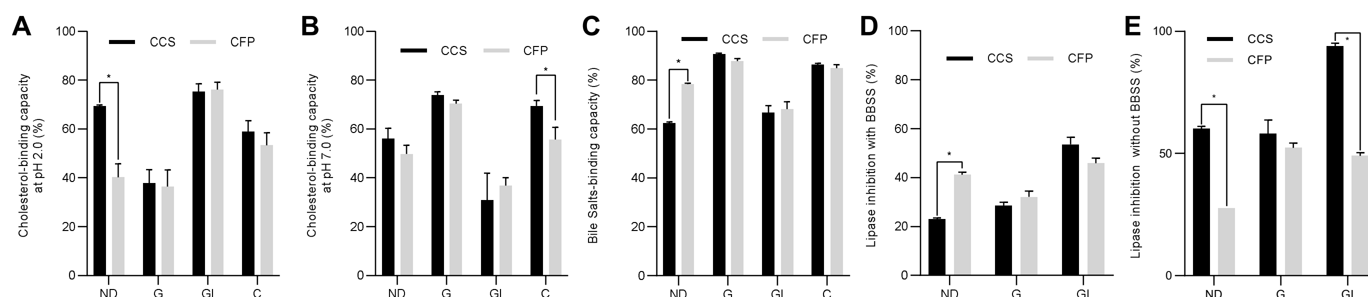

Figure 1. CSF and CPF hypolipidemic properties after the digestion process. (**A**) Cholesterol-binding capacity of CSF and CPF digested residual fractions at pH 2; (**B**) Cholesterol-binding capacity of CSF and CPF digested residual fractions at pH 7; (**C**) Bile Salts-binding capacity of CSF and CPF digested residual fractions; (**D**) *In vitro* pancreatic lipase inhibition activity of CSF and CPF with bile salts; (E) *In vitro* pancreatic lipase inhibition activity of CSF and CPF without bile salts. The results are expressed as mean ± SD (n = 3). Statistically significant ($p < 0.05$) differences between paired samples according to the T-test are represented by an asterisk (*). CSF: Cocoa Shell Flour; CPF: Coffee Pulp Flour; ND: Non-digested flour; G: Gastric residual fraction; GI: Intestinal residual fraction; C: Colonic residual fraction, BBSS: Bile salts.

The digestion process modified the ability of the non-digested flours to bind bile salts, and, after digestion, there were no significant differences between the capacity of both flours to bind salts (Figure 1C). On the other hand, the differences in inhibiting lipase activity in the presence and absence of bile salts found between the undigested flours of CSF and CPF disappeared after the digestion process (Figure 1D, 1E). However, in the absence of bile salts, the residue of the gastrointestinal (GI) phase of CSF digestion exhibited a higher capacity to inhibit the lipase activity than GI-CPF (102% $p < 0.05$) (Figure 1E).

Previous studies have demonstrated the fundamental role played by the DF in the reduction of bioaccessible cholesterol during the digestion process, by direct entrapment, due to a possible change in its properties during the digestion process [22]. DF has also been shown to decrease cholesterol absorption by reducing the formation of available micelles for its absorption by reducing the surfactant activity of bile salts. Likewise, the DF and the phenolic compounds present in these by-products have been related to the inhibition of pancreatic lipase [22,23]. From results and considering the composition in DF of

each by-product CS (59%) and CP (57%), it seems reasonable to think that their hypolipidemic properties should be similar throughout the digestion process.

3.2. Cocoa shell and Coffee Pulp were Not-Toxic and Regulated PA-stimulated ROS Production and Lipid Accumulation

MTS Cytotoxicity assay on HepG2 proliferation was carried out with CSE, CSF, CPE, and CPF digested fractions (50–250 μg/mL). The results showed that all the concentrations used were safe and non-cytotoxic. HepG2 cells were incubated for 24 h with PA (500 μM) to mimic NAFLD conditions. The PA stimulation significantly increased ROS production ($p < 0.05$) and triggered morphological changes in HepG2 cells, such as the appearance of lipid droplets inside the cells, which were significantly counteracted by the treatment with CSE, CSF, CPE, and CPF digested fractions (100 μg/mL) ($p < 0.05$) (**Figure 2**). Furthermore, digested fractions significantly decreased ($p < 0.05$) TAG levels (5-38%) and FASN activity (9-41%). Therefore, results suggest that both by-products can reverse the accumulation of lipid droplets and TAG inside the cells, and could modulate hepatic lipid metabolism via inhibition of *de novo* lipogenesis. *De novo* lipogenesis is elevated during NAFLD, and represents an important source of hepatic and circulating lipids, therefore, a decrease in this activity could lead to a decrease in their levels [24,25].Previous studies have shown that CPE and CSE could have antioxidant effects on HepG2 cell lines, probably due to their phytochemical content. Furthermore, these CSE components have been shown to protect mitochondrial function, closely related to markers associated with metabolic syndrome [11,12,26,27]. Both by-products may be recommended as therapeutic or preventive NAFLD treatments.

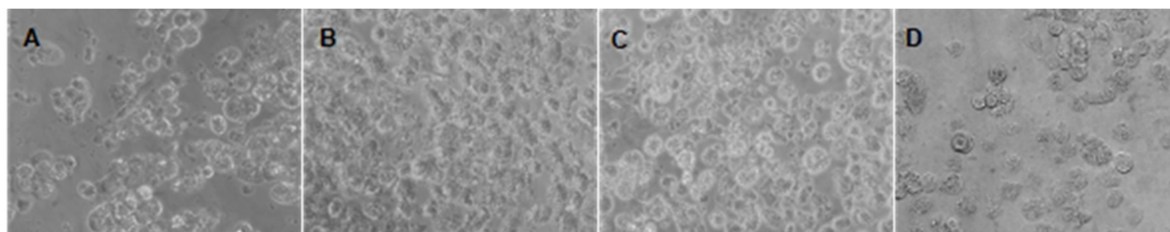

Figure 2. Illustrative representation of HepG2 morphologies after incubation in different conditions (**A**) Cells cultured in DMEM-BSA 1%; (**B**) cells cultured in DMEM-BSA 1% with PA (500 μM); (**C**) cells cultured in DMEM-BSA 1% with PA (500 μM) with treatment (Intestinal CPE, 100 μg/mL); (**D**) cells cultured in DMEM-BSA 1% with PA (500 μM) with treatment (Gastric CSE, 100 μg/mL).

4. Conclusions

This study indicates the CS and the CP exhibit similar valuable hypolipidemic properties after *in vitro* digestion and supports the revalorization of both by-products as novel healthy food ingredients with potential beneficial properties to prevent chronic metabolic diseases. Further investigations need to be completed to confirm their hypolipidemic properties *in vivo*.

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