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Evaluation of the Hypolipidemic Properties of Cocoa Shell after Simulated Digestion Using In Vitro Techniques and a Cell Culture Model of Non-Alcoholic Fatty Liver Disease †

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Abstract: Obesity is closely associated with the increasing prevalence of non-alcoholic fatty liver disease (NAFLD). Due to the lack of proper pharmacological treatments for NAFLD, finding novel ingredients is necessary to reduce its incidence. Cocoa shell is a cocoa byproduct verified as a safe ingredient and a potential source of health-promoting compounds. Hence, this study's main objective was to evaluate, after an in vitro simulated digestion, the hypolipidemic properties of the residual fraction of cocoa shell flour and the biological activity of the digested fractions of cocoa shell flour and extract in HepG2 cells. An in vitro static digestion (INFOGEST) of cocoa shell flour was used to establish the residual fraction's capacity to bind cholesterol and bile salts and inhibit lipase. The results showed that digestion promoted the ability to bind cholesterol and bile salts of a residual fraction from a cocoa shell up to 65.2% and 90.5%. Moreover, digestion improved (1.6-fold, $p < 0.05$) the ability to inhibit lipase activity. The digested fractions of the flour and extract from the cocoa shell (50–250 μg/mL) significantly ($p < 0.05$) reduced the accumulation of fat (17–42%), triglycerides (9–38%), and cholesterol (11–54%) in HepG2 cells after NAFLD induction with palmitic acid (500 μM). In conclusion, digestion positively impacted the hypolipidemic properties of cocoa shells in vitro and enhanced their biological activity in cell culture models. Since cocoa shells might be used as a safe, novel ingredient to prevent hyperlipidemia and regulate lipid metabolism, future animal and clinical investigations will be necessary to confirm the effects observed.

Keywords: cocoa by-products; cocoa shell; gastrointestinal digestion; hypolipidemic; hypocholesterolemic; non-alcoholic fatty liver disease

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1. Introduction

Obesity is defined as an excessive accumulation of adipose tissue. It is closely associated with multiple metabolic risk factors for a cardiovascular disease, including insulin resistance, diabetes, dyslipidemia, and a spectrum of liver abnormalities, known as non-alcoholic fatty liver disease (NAFLD) [1]. NAFLD has become one of the leading causes of chronic liver disease, representing the hepatic manifestation of the metabolic syndrome, ranging from simple steatosis (i.e., fat accumulation in more than 5% of the hepatocytes) to necroinflammation and fibrosis, leading to non-alcoholic steatohepatitis (NASH), which often results in a progression to cirrhosis, liver failure, and hepatocellular

carcinoma [2,3]. Many pharmacological treatments have been proposed for the management of NAFLD. However, none of them is approved in clinical use currently [4]. Due to the lack of proper pharmacological treatments for NAFLD, modifying the diet to prevent obesity and its comorbidities is the principal candidate. In this context, finding novel ingredients that may help us to reduce the incidence of these disorders is necessary.

During cocoa bean processing, approximately 90% is discarded as cocoa byproducts, which have a high potential for developing new products. The cocoa shell is the tegument that covers the cocoa bean. It is obtained during the cocoa bean roasting process, constituting about 10–17% of the total cocoa bean weight [5]. The cocoa shell is a rich source of carbohydrates (62%) and proteins (10–27%), as well as dietary fiber (DF) (59%) and valuable bioactive compounds (theobromine, caffeine, phenolic compounds, etc.), which can be used as an ingredient in the food industry, among other industries [6,7]. Furthermore, cocoa shells have recently been validated as a potential safe ingredient. It is a potential source of nutrients and health-promoting compounds used as nutraceuticals to manage chronic diseases [7]. Phytochemicals from cocoa shells have been associated with the regulation of biomarkers of metabolic syndrome (lipid accumulation, inflammation, oxidative stress, and insulin resistance) [8]. Therefore, this study's main objective was to evaluate, after an in vitro simulated digestion, the hypolipidemic properties of the residual fraction of cocoa shell flour and the biological activity of the digested fractions of cocoa shell flour and extract in HepG2 cells.

2. Materials and Methods

2.1. Materials

Dulbecco's Modified Eagle's Medium low glucose (1000 mg/mL) (DMEM) and 0.25% trypsin-EDTA were purchased from GE Healthcare Life S Life Technologies. [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was purchased from Promega Corporation (Carlsbad, CA, USA). *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 (St. Louis, MO, USA). All other chemicals and reagents were obtained from Panreac (Barcelona, Spain), unless otherwise specified.

2.2. Cocoa Flour and Aqueous Extracts Preparation

The cocoa shell was provided by Chocolates Santocildes (Castrocontrigo, León, Spain). Cocoa shell flour was obtained after milling and cocoa shell aqueous extracts described by Rebollo-Hernanz et al. [9].

2.3. INFOGEST Static In Vitro Simulation Digestion

Digestions were carried out based on the preliminary and optimized protocols described by Brodkorb et al. [10]

2.4. Cholesterol-Binding Capacity

For the measure of the residue's cholesterol-binding capacity, fresh egg yolks were diluted 1:10 (*v*/*w*), and the dilution was emulsified, divided, and adjusted to pH 2.0 and pH 7.0. Samples (0.1 g) were mixed with the diluted egg yolk (2 mL) and incubated with agitation (2 h, 37 °C), followed by an 800 *g* centrifugation (15 min). Consecutively, samples supernatants (0.1 mL) 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 while mixing for color development (30 min, 60 °C). Finally, triplicate-measured absorbance at 550 nm using a microplate reader (Cytation 5, Biotek).

2.5. Bile Salts-Binding Capacity

A mixture of NaCl (15 M, pH 7.0) and sodium cholate (4.65 mM) was added to 0.1 g of the residual fraction of gastric and intestinal flour digestion. The samples were incubated for 3 h at 37 °C while mixing, followed by an 800× *g* 15-min centrifugation. Hereafter, 0.6 mL of H_2SO_4 (45%) and 0.1 mL of furfural (0.3%) were added to 0.1 mL of the supernatant. The samples were incubated with oscillations for color development (30 min, 65 °C). Absorbance was measured by triplicate at 620 nm using a microplate reader.

2.6. Inhibitory Activity against Pancreatic Lipase

To determine the inhibitory activity against pancreatic lipase, a mixture consisting of olive oil (2 mL), lipase (2 mL, 0.75 mg /mL), bile salts (5 mL), and phosphate-buffered saline (PBS) 10× (5 mL, pH 7.2) was added to 0.1 g of the residual fraction of each flour digestion. Controls were carried with or without each compound (including the residual fraction). Samples were incubated (37 °C, 1 h). The reaction was stopped in the cold for 5 min, followed by centrifugation (800× *g*, 15 min). Hereafter, Tween® 20 (0.2 mL) was added to the supernatant of those samples that contained olive oil to emulsify the fats. Finally, each sample's lipase activity was measured using an acid–base titration with NaOH (0.02 M) and phenolphthalein.

2.7. Treatment Preparation for HepG2 Cells

The digested fractions of cocoa shell flour (CSF) and extract (CSE) were diluted in DMEM-free and filtered with 0.45-μm cellulose acetate filters.

2.8. Cell Culture Method

HepG2 cells were obtained from ATCC (Rockville, MD, USA). Cells were cultured in DMEM (1000 g/mL glucose) supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere.

2.8.1. Cell Viability

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 and CSE (50–250 mg/mL) for 24 h at 37 °C in a humidified atmosphere. On day 3, a cell viability assay was performed with CellTiter 96® AQueous MTS Reagent Powder according to the manufacturer's instructions.

2.8.2. NAFLD Induction in HepG2 Cells

HepG2 were seeded at 10^5 cells/cm^2 in 96-well culture plates and incubated for 24 h. On day 2, NAFLD was induced in HepG2 cells with PA (500 μM), and they were treated with CSF or CSE (100 μg/mL) for 24 h at 37 °C in a humidified atmosphere. To prepare the PA solution, PA was conjugated with DMEM containing 1% BSA. Control cells were treated with 1% BSA only.

2.8.3. Reactive Oxygen Species Formation in HepG2 Cells

After 24-h incubation with treatments, 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 of 485 nm/530 nm, respectively. DCFDA was prepared according to the manufacturer's instructions.

2.8.4. Biological Hypolipidemic Activity

The accumulation of total lipids and intracellular triglycerides was performed as previously described by Rebollo-Hernanz et al. [9] using Oil Red O and a colorimetric triglycerides assay kit (Spinreact), respectively, according to the manufacturer's instructions.

Cholesterol accumulation was measured as previously described by Ryu et al. [11] using a colorimetric cholesterol assay kit (Spinreact) according to the manufacturer's instructions.

2.9. Statistical Analysis

All experiments were carried out in triplicate. Results were expressed as the mean ± standard deviation (SD) ($n = 3$) and were assessed statistically by one-way analysis of variance (ANOVA) and post hoc Tukey's test. Differences were defined as statistically significant for values of $p < 0.05$. All analyses were performed using SPSS 24.0.

3. Results

3.1. Simulated In Vitro Digestion Enhanced the Hypolipidemic Properties of Cocoa Shell

As observed in Figure 1A, the digestion process affected the cholesterol-binding capacity in the cocoa shell. Simulating the physiological parameters, gastric digestion residues at pH 2 and gastrointestinal phase at pH 7, reduced the cholesterol-binding capacity. However, it improved during colonic digestion at both pH. On the other hand, the digestion of cocoa shell significantly ($p < 0.05$) improved bile salts-binding capacity during all digestion phases (Figure 1B). Furthermore, cocoa shell digestion positively enhanced the lipase inhibition during the gastric and gastrointestinal phases with or without the bile salts addition (Figure 1C).

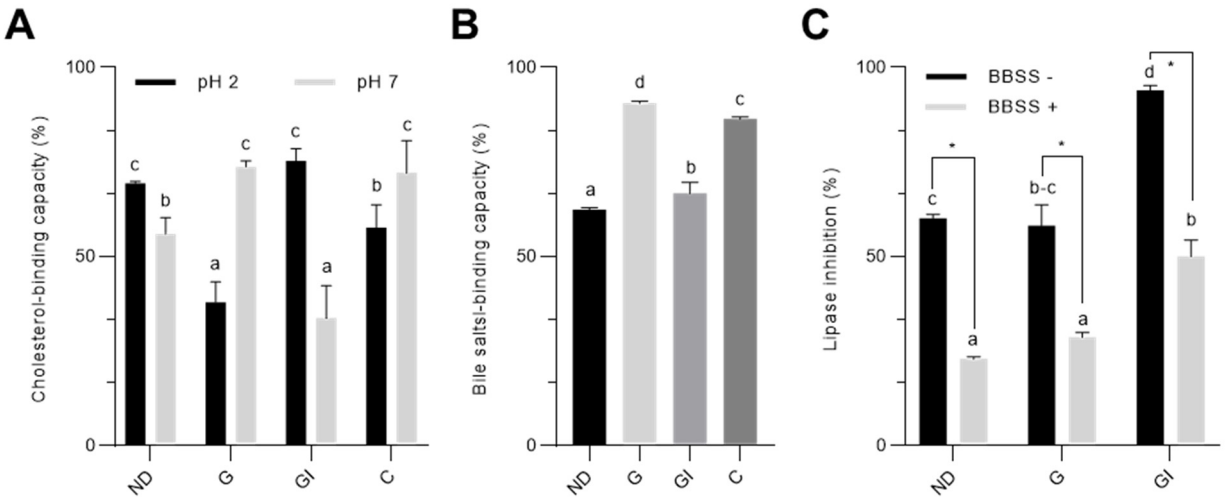

Figure 1. Effects of digestion on the cocoa shell hypolipidemic properties. (**A**) Cholesterol-binding capacity of cocoa shell digested residual fractions at pH 2.0 and pH 7.0. (**B**) Bile salts-binding capacity of cocoa shell digested residual fractions. (**C**) In vitro lipase inhibition capacity of cocoa shell digested residual fractions without bile salts (BBSS−) and containing them (BBSS+). The results are expressed as the mean ± SD. Bars with different letters significantly ($p < 0.05$) differ according to ANOVA and Tukey's multiple range test. Statistically significant ($p < 0.05$) differences between paired samples according to the *t*-test *a*re represented by an asterisk (*). ND: nondigested flour, G: gastric residual fraction, GI: intestinal residual fraction, and C: colonic residual fraction.

3.2. Cocoa Shell Was Not Toxic and Regulated PA-Stimulated ROS Formation and Lipid Accumulation

To simulate the NAFLD model on cell cultures, HepG2 cells were incubated for 24 h with PA (500 μM), and the ROS levels were measured to study the NAFLD induction. An MTS assay was carried out to determine the effects of different concentrations of CSE and CSF (50–250 μg/mL) on HepG2 proliferation, and the findings indicated that all the concentrations used were not cytotoxic and significantly ($p < 0.05$) reduced the toxic effects of PA. The results showed that PA significantly ($p < 0.05$) induced ROS formation. Moreover,

the HepG2 cell morphology changed after incubation with PA, favoring the formation of lipid drops (Figure 2). CSE and CSF (50–250 μg/mL) significantly ($p < 0.05$) reduced the accumulation of fat (17–42%) and modulated the triglyceride and cholesterol levels, lowering them by about 9–38% and 11–54% ($p < 0.05$), respectively, after the induction of NAFLD with PA.

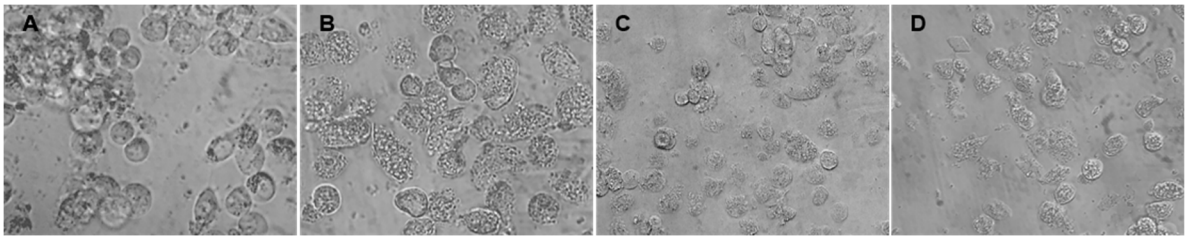

Figure 2. Illustrative representation of HepG2 morphologies after incubation in different conditions. (**A**) Cells cultured in Dulbecco's Modified Eagle's Medium-Bovine Serum Albumin (DMEM-BSA) 1%. (**B**) Cells cultured in DMEM-BSA 1% with Palmitic Acid (PA) (500 μM). (**C**) Cells cultured in DMEM-BSA 1% with PA (500 μM) treated with gastric cocoa shell extract (CSE) (100 μg/mL). (**D**) Cells cultured in DMEM-BSA 1% with PA (500 μM) treated with gastric CSE (200 μg/mL).

4. Discussion

Cocoa has been reported to have benefits for health [12]. The cocoa shell composition has been described to be similar to that of cocoa beans. It also presents hypolipidemic and hypoglycemic properties in vitro, which may be explained because of its chemical composition [5,7]. Cocoa shells are a rich source of DF [7]. DF is not absorbed nor digested during the human gastrointestinal digestion process but could be wholly or partially fermented [13]. DF has been reported to mediate lipid metabolism and cholesterol absorption. Previous studies have demonstrated that most NAFLD patients follow a diet poor in DF [14,15]. The residual fraction of the simulated gastric and gastrointestinal digestion of cocoa shell flour showed less cholesterol-binding capacity at physiological pH (2.0 and 7.0, respectively) than nondigested flour. Besides, the residual fraction of every phase-simulated digestion improved the bile salts-binding capacity. Previous studies have demonstrated that DF possesses a bile salts-binding ability and modulates the accumulation of circulating triglycerides by inhibiting the bile salt surfactant activity, which reduces micelle formations during intestinal digestion and, therefore, intestinal lipid absorption [16]. Likewise, DF can reduce cholesterol absorption. The residual fraction of every simulated digestion of cocoa shell flour showed higher pancreatic lipase inhibition than non-digested flour. The DF bile salts-binding capacity reduces micelle formation, explaining the decrease in lipase activity because of a reduction in lipid accessibility. It could also explain why bile salts added to the lipase inhibition method reduced the lipase inhibition capacity of cocoa shell flour [16]. Similarly, DF can entrap the enzyme (lipase) or the substrate (triglycerides) and reduce its activity [17]. These results suggest that cocoa shell digestion could enhance its hypolipidemic properties, which could play an essential role in treating or preventing chronic diseases associated with hyperlipidemia, such as NAFLD.

Studies in HepG2 cells showed that CSE and CSF reduced the lipid, cholesterol, and triglycerides accumulation after the induction of simulated NAFLD and could significantly reduce ROS levels, probably due to the antioxidant properties of the cocoa shell polyphenols [18]. Some authors suggested that oxidative stress and mitochondrial dysfunction could be critical factors during NAFLD progression [19]. Previous studies in our group proved the effects of cocoa shell phytochemicals on protecting mitochondrial function and regulating metabolism in hepatocytes [20]. The results obtained suggest the potential role of the cocoa shell in preventing this disease. From this perspective, and based on the high DF and phenolic compound contents of cocoa shell, evaluating the effects of its digestion on the hypolipidemic properties and biological activity is necessary to reassign them as novel ingredients that could have health benefits in some diseases like NAFLD and could also have a positive impact on the environment. Further investigations

on the bio-accessible and the nondigestible fractions of the cocoa shell and their in vivo functional properties should be completed to confirm its health properties.

5. Conclusions

Digestion positively impacted the cocoa shell's hypolipidemic properties, leading to enhanced biological activity in vitro and in cell culture models. Since the cocoa shell might be used as a safe, novel ingredient to prevent hyperlipidemia and regulate lipid metabolism, future animal and clinical investigations will be necessary to confirm the effects observed in vitro.

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