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Bioaccessibility, bioactivity and cell metabolism of dark chocolate phenolic compounds after *in vitro* gastro-intestinal digestion

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ABSTRACT

The bioaccessibility of phenolic compounds after *in vitro* gastro-intestinal digestion of dark chocolate, dark chocolate enriched with Sakura green tea and dark chocolate enriched with turmeric powder was studied. The phenolic profile, assessed by accurate mass spectrometry analysis, was modified during *in vitro* gastro-intestinal digestion, with a considerable decrease of total and individual phenolic compounds. Phenolic acids showed the highest bioaccessibility with hydroxycinnamic acids displaying higher bioaccessibility (from 41.2% to 45.1%) respect to hydroxybenzoic acids (from 28.1% to 43.5%). Isomerisation of caffeoyl-quinic acids and galloyl-quinic acids as well as dimerization of (epi)gallocatechin were also observed after *in vitro* gastro-intestinal digestion. Antioxidant activity increased after the gastric step and rose further at the end of the digestion. Furthermore, *in vitro* digested phenolic-rich fractions showed anti-proliferative activity against two models of human colon adenocarcinoma cell lines. Cell metabolism of digested phenolic compounds resulted in the accumulation of coumaric and ferulic acids in the cell media.

1. Introduction

Cocoa and cocoa-based products, such as dark chocolate, are widely consumed in several countries and significantly contribute to the daily intake of antioxidants and phenolic compounds in adults and children (Rusconi & Conti, 2010). Recently, our research group comprehensively analysed the phenolic profile of dark chocolate (Martini, Conte, & Tagliazucchi, 2018). More than 140 individual phenolic compounds were identified by accurate mass spectrometry analysis. Flavan-3-ols are the most abundant phenolic compounds in dark chocolate, accounting for around the 64% of total phenolics (Martini et al., 2018).

There are several *in vivo* studies suggesting that cocoa-derived polyphenols may have beneficial effects on markers of cardiovascular disease risk (Del Rio et al., 2013). Short-term randomized clinical trials have demonstrated that dark chocolate intake reduced blood pressure, improved flow-mediated dilation and ameliorated the lipid profile in healthy and hypertensive subjects (Grassi, Lippi, Necozione, Desideri, & Ferri, 2005; Grassi, Necozione et al., 2005; Lin et al., 2016). These effects have been partially attributed to the high flavan-3-ols content of dark chocolate (Engler et al., 2004). Furthermore, dark chocolate intake has been shown to reduce the number of pre-neoplastic lesions in azoxymethane-induced colonic cancer in rats (Hong, Nulton, Shelechi, Hernández, & Nemposeck, 2013; Rodríguez-Ramiro, Ramos, López-Oliva et al., 2011). The protective effect of dark chocolate against colon cancer may be due to the biological activities of its phenolic compounds through the regulation of several signal transduction pathways and the modulation of gene expression (Carnésecchi et al., 2002; Granado-Serrano et al., 2010; Martín et al., 2010; Rodríguez-Ramiro, Ramos, Bravo, Goya, & Martín, 2011).

The bioavailability of phenolic compounds differs widely among the different classes. Some phenolic compounds are poorly absorbed (Del Rio et al., 2013) and/or are unstable under the gastro-intestinal tract conditions (Bouayed, Deußer, Hoffmann, & Bohn, 2012; Juániz et al., 2017). Indeed, dark chocolate phenolic compounds are entrapped in a solid food matrix and only the released compounds are potentially bioavailable and able to exert their beneficial effects in the gastro-intestinal tract or at systemic level (Tagliazucchi, Verzelloni, Bertolini, & Conte, 2010; Tagliazucchi, Verzelloni, & Conte, 2012). Therefore, studies carried out with cell culture models using pure phytochemicals (Carnésecchi et al., 2002) or cocoa/chocolate extracts (Rodríguez-Ramiro, Ramos, Bravo et al., 2011) are unrealistic unless the bioaccessibility and gastro-intestinal tract stability of the phenolic compounds have been well defined. Furthermore, *in vitro* studies did not take into account the stability of tested molecules in cell cultures and their metabolic fate within the cells (Aragonès, Danesi, Del Rio, & Mena, 2017).

This work aimed to investigate the effect of *in vitro* gastro-intestinal digestion on the bioaccessibility of phenolic compounds in dark

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chocolate and dark chocolate functionalized with Sakura green tea leaves or turmeric powder. In addition, the antioxidant and anti-proliferative activities of *in vitro* digested dark chocolates phenolic compounds against two models of human colonic cell lines were assessed. Finally, the last task was to identify and quantify the main metabolites derived from incubation of *in vitro* digested dark chocolate phenolic compounds with cells.

2. Materials and methods

2.1. Materials

Phenolic compound standards, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,4,6-tri(2-pyridyl)-S-triazine (TPTZ), Folin-Ciocalteau phenol reagent were purchased from Sigma (Milan, Italy). Methanol and formic acid were obtained from Carlo Erba (Milan, Italy). All MS/MS reagents were from Bio-Rad (Hercules, CA, U.S.A.). Chemicals and enzymes for the digestion procedure were purchased from Sigma-Aldrich (Milan, Italy). All the materials and chemicals for cell culture were from Euroclone (Milan, Italy). MTS cell proliferation assay kit was purchased from Promega (Milan, Italy). Solid phase extraction (SPE) columns (C18, 50 µm, 60 Å, 500 mg) were supplied by Waters (Milan, Italy). Three different types of chocolate (dark 70% cocoa (DC), dark 70% cocoa and 8% turmeric (TDC), dark 70% cocoa and 2% Sakura green tea (GTDC)) were bought from a local shop in Modena (Italy). The chocolates were all from the same manufacturer and had the same composition. The ingredients were cocoa mass, sugar, cocoa butter, soya lecithin and natural flavour vanilla. GTDC and TDC were enriched with 2% Sakura green tea leaves and 8% turmeric powder, respectively. Three chocolate bars for each sample were used in this study.

2.2. In vitro gastro-intestinal digestion of dark chocolates and preparation of the chemical extract

For the *in vitro* digestion, the protocol previously developed within the COST Action INFOGEST was followed (Minekus et al., 2014). The procedure consisted of three consecutive steps: oral, gastric and intestinal phases. The three steps were carried out in absence of light. Simulated salivary, gastric, and intestinal fluids (SSF, SGF and SIF) were employed for each step and prepared according to Minekus et al. (2014). Five grams of each type of dark chocolate were melted at 37 °C for 10 min and then 5 mL of the stock SSF solution and 150 U/mL of porcine α-amylase were added (oral phase of digestion). The samples were shaken for 5 min at 37 °C. The second step of the digestion (gastric phase) was carried out by adding to the bolus 10 mL of SGF. The pH was adjusted to 2.0 with 6 mol/L HCl and supplemented with porcine pepsin (2000 U/mL of simulated gastric fluid). After 2 h of incubation at 37 °C, the final intestinal step was carried out by adding 15 mL of SIF (prepared by mixing 10 mL of pancreatic fluid and 5 mL of bile salts). Then, the pH was adjusted to 7.0, supplemented with pancreatin and the samples were incubated at 37 °C for 2 h. All samples were immediately cooled on ice, centrifuged at 10000*g* for 20 min at 4 °C to eliminate insoluble materials and the supernatant frozen at −80 °C for further analysis. The digestions were performed in triplicate.

In addition, phenolic compounds were extracted from each dark chocolate (chemical extract) as reported in Martini et al. (2018). The extractions were performed in triplicate.

Dark chocolate chemical extracts and samples collected at the end of each stage of the *in vitro* digestion procedure were then used for total phenolic compounds and antioxidant activity determinations.

2.3. Identification and quantification of phenolic compounds by liquid chromatography mass spectrometry (LC-ESI-QTOF-MS/MS)

Dark chocolate chemical extracts and *in vitro* digested samples were analysed on Agilent HPLC 1200 Infinity (Agilent Technologies, Santa Clara, CA) equipped with a C18 column (HxSil C18 Reversed phase, 250 × 4.6 mm, 5 µm particle size, Hamilton Company, Reno, Nevada, USA) as reported in Martini et al. (2018). The mobile phases consisted of (A) H_2O/formic acid (99:1, v/v) and (B) acetonitrile/formic acid (99:1, v/v). After 0.5 min at 4% B, the gradient linearly rose up to 30% B in 60 min. The mobile phase composition was ramped up to 100% B in 1 min and maintained for 5 min in order to wash the column before returning to the initial condition. The flow rate was established at 1 mL/min. After passing to the column, the eluate was split and 0.3 mL/min were directed to a 6520 accurate Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA). Identification of phenolic compounds in all samples was carried out using full scan, data-dependent MS^2 scanning from m/z 100 to 1700. MS operating conditions, calibration curve equations, linearity ranges and limit of quantifications (LOQ) for the different standards are reported in Martini et al. (2018).

Quantitative results were expressed as µmol of compounds per 100 g of chocolate.

2.4. Total phenolic compounds and antioxidant activity assays

Folin-Ciocalteau assay was performed as reported by Singleton, Orthofer, and Lamuela-Raventós (1999). The results were expressed as µmol of gallic acid per 100 g of chocolate.

The antioxidant properties of dark chocolate chemical extracts and *in vitro* digested samples were evaluated performing two different assays. The ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) and ferric reducing power (FRAP) assays were performed according to the protocols described by Re et al. (1999) and Benzie, and Strain (1996), respectively. The ABTS scavenging capacity and FRAP values were expressed as mmol of trolox equivalent per 100 g of chocolate.

2.5. Preparation of dark chocolate phenolic-rich fractions

Samples collected at the end of the *in vitro* digestion were then passed through a SPE column preconditioned with 4 mL of acidified methanol (containing 0.1% of formic acid), followed by 5 mL of acidified water (containing 0.1% of formic acid). Elution was carried out with acidified water (6 mL) to eliminate the unbound material. Phenolic compounds were then desorbed by elution with 3 mL of acidified methanol. The obtained phenolic-rich extracts were diluted in the cell media and used for the anti-proliferative activity determination. Each sample was extracted in triplicate.

2.6. Cell cultures and anti-proliferative activity of in vitro digested dark chocolate phenolic-rich fractions

Human adenocarcinoma Caco-2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 1% antibiotic mix (streptomycin and penicillin) and 2 mmol/L L-glutamine. Caco-2 cells were used for experiments between passage 57 and 58. Human adenocarcinoma SW480 cells were cultured in Leibowitz medium supplemented with 10% FBS, 1% antibiotic mix (streptomycin and penicillin) and 2 mmol/L L-glutamine. SW480 cells were used for experiments between passage 33 and 34. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

Cells were seeded at 5×10^3/100 µL and 10×10^3/100 µL for Caco-2 and SW480, respectively, in 96-well plates 24 h before the assay to allow cell adhesion to the bottom of the wells.

For the anti-proliferative assays a colorimetric method for the sensitive quantification of viable cells was performed, using MTS assay kit. Different amounts of the *in vitro* digested phenolic-rich fractions were