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.

Keywords: mass spectrometry, *in vitro* digestion, cell metabolism, functional foods, Caco-2, SW480
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). 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, 2005a; Grassi et al., 2005b; 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 et al., 2011a). 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, 2011b).

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, et al., 2011b) 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 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 minutes 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 10000g 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) H₂O/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,
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, & 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, & 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% CO₂. Cells were seeded at 5x10³/100 μL and 10x10³/100 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 diluted in cell culture media and added to the cell plates for 24 h. At the end of the treatments, the medium was refreshed with 180 μL of culture medium and 20 μL of MTS reagent were added to each well. After 4 h of incubation at 37°C, the absorbance was measured at the wavelength of 490 nm using a microplate reader and results were expressed as IC₅₀. IC₅₀ was defined as the concentration of phenolic compounds required to inhibit 50% cell proliferation and expressed as μmol of total phenolic compounds/100 g of chocolate. The IC₅₀ values were determined using nonlinear regression analysis and fitting the data with the log (inhibitor) vs. response model generated by GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). The amount of phenolic compounds was determined by LC-ESI-QTOF MS/MS analysis as described in section 2.3.

2.7. Liquid chromatography coupled to mass spectrometry (LC-MS/MS) analysis of cell media

Caco-2 and SW480 cell lines were incubated with in vitro digested dark chocolate (DC) at a concentration corresponding to IC₅₀. After 24 h of incubation at 37°C, cell culture supernatants
were collected and analysed by LC-MS/MS to determine the stability and the metabolism of the
dark chocolate phenolic compounds in the cell media. Cell media were extracted according to Sala
et al. (2015) and investigated according to Martini, Conte, & Tagliazucchi (2017). Briefly, samples
were analysed using a HPLC Agilent 1200 Series system equipped with an Agilent 6300 ion trap
mass spectrometer. Separations were performed using a C18 column (HxSil C18 Reversed phase,
250×4.6 mm, 5 μm particle size, Hamilton Company, Reno, Nevada, USA), with an injection
volume of 40 μL and elution flow rate of 1 mL/min. The mobile phase composition, the gradient
and MS operating conditions are the same as reported in Martini et al. (2017). MS experiments were
performed in ESI negative ion mode. Identification of phenolic compounds and metabolites in all
samples was carried out using full scan, data-dependent MS$^2$ scanning from m/z 100 to 1700.

2.8. Statistic

All data are presented as mean ± SD for three replicates for each prepared sample. One-way
analysis of variance (one-way ANOVA) with Tukey’s post-hoc test was applied using Graph Pad
prism 6.0 (GraphPad software, San Diego, CA, U.S.A.). The differences were considered
significant with $P<0.05$. 

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3. Result and discussion

3.1. In vitro bioaccessibility of phenolic compounds in different types of dark chocolate

In our previous work we identified and quantified by high-resolution mass spectrometry 141, 155 and 142 phenolic compounds in dark chocolate (DC), dark chocolate enriched with Sakura green tea (GTDC) and dark chocolate enriched with turmeric powder (TDC), respectively (Martini et al., 2018). In this work, we present data regarding the release and bioaccessibility of dark chocolate total and individual phenolic compounds following in vitro gastro-intestinal digestion. Figure 1 shows the impact of in vitro gastro-intestinal digestion on total phenolic compounds. The chemical extract of GTDC showed a significant higher amount ($P<0.05$) of total polyphenols ($20090.58 \pm 760.92 \mu \text{mol gallic acid equivalent/100 g of dark chocolate}$) respect to TDC ($17887.63 \pm 556.33 \mu \text{mol gallic acid equivalent/100 g of dark chocolate}$) and DC ($15425.27 \pm 660.47 \mu \text{mol gallic acid equivalent/100 g of dark chocolate}$). After salivary phase, only 8.9%, 7.8% and 10.2% of total phenolic compounds were released from the food matrices in DC, GTDC and TDC, respectively. The amount of bioaccessible total phenolic compounds increased by 31%, 26.5% and 20.1% in DC, GTDC and TDC, respectively, after two hours of gastric digestion (Figure 1). The incubation with pancreatic solution further increased the bioaccessibility of total compounds in the different samples but to a different extent (Figure 1). At the end of the entire phase of digestion, the 68.7%, 68.2% and 40.1% of total phenolic compounds from DC, GTDC and TDC, respectively, were bioaccessible, while the remaining were degraded or not extracted from the solid matrices. From a quantitative point of view, GTDC showed a significant higher amount ($P<0.05$) of total bioaccessible polyphenols ($13709.31 \pm 377.47 \mu \text{mol gallic acid equivalent/100 g of dark chocolate}$) respect to DC ($10599.88 \pm 213.43 \mu \text{mol gallic acid equivalent/100 g of dark chocolate}$). TDC showed the lowest amount ($P<0.05$) of total bioaccessible phenolic compounds ($7172.14 \pm 512.02 \mu \text{mol gallic acid equivalent/100 g of dark chocolate}$). These results are in agreement with previously reported data showing that the gastro-intestinal tract behaved as an extractor promoting the release
of phenolic compounds from solid food matrices (Blancas-Benitez, Pérez-Jiménez, Montalvo-
González, González-Aguilar, & Sáyago-Ayerdi, 2018; Tagliazucchi et al., 2010; Tagliazucchi et al.,
2012). However, other studies found a decrease in bioaccessible total phenolic compounds during
the intestinal digestion (Bouayed et al., 2012; Lingua, Wunderlin, & Baroni, 2018). The different
results can be related to the higher stability of dark chocolate phenolic compounds to the intestinal
conditions respect to the other foods tested or to a different food matrix effect. However, it should
be taken into account that the Folin-Ciocalteau assay is strongly subject to interferences, especially
from sugars and vitamin C (Singleton et al., 1999). On the other hand, dark chocolate is rich in
Maillard reaction products that react in a concentration-dependent manner with the Folin-Ciocalteau
reagent, possibly resulting in an overestimation of bioaccessible total phenolic compounds
(Verzelloni, Tagliazucchi, & Conte, 2007).

Figure 2 and Tables 1-6 show how the in vitro gastro-intestinal digestion modified the phenolic
compounds profile in the samples from a qualitative and quantitative point of view. The MS data of
the individual phenolic compounds are reported in Martini et al. (2018). A total of 78, 122 and 86
phenolic compounds were identified by accurate mass spectrometry analysis after in vitro gastro-
intestinal digestion of DC, GTDC and TDC, respectively. This means that 45%, 21% and 39% of
individual phenolic compounds were not bioaccessible in DC, GTDC and TDC, respectively. A
significant lower amount of phenolic compounds was observed in all the samples after simulated
gastro-intestinal digestion respect to the chemical extracts. In TDC, only 17.6% of the total amount
of phenolic compounds was released from the food matrix or not degraded during digestion. In DC
and GTDC, the amount of bioaccessible total phenolic compounds at the end of the digestion was
23.0% and 23.2%, respectively (Figure 2H and Table 6).

The apparent lowest bioaccessible value of phenolic compounds in TDC was ascribed to the poor
bioaccessibility (0.24%) of curcuminoids (Table 4).
Among the different phenolic classes, phenolic acids showed the highest bioaccessibility (Figure 2B and 2G) with hydroxycinnamic acids displaying higher bioaccessibility (from 41.2% to 45.1%) than hydroxybenzoic acids (from 28.1% to 43.5%). These compounds were efficiently released from the food matrices and stable under gastro-intestinal conditions. When the effect of gastro-intestinal digestion in coffee and cardoon was studied, chlorogenic acids were proved to be quite stable (Juániz et al., 2017; Monente et al., 2015). Similarly, Tagliazucchi et al. (2010) and Bouayed et al. (2012) found that caffeic and coumaric acids were quite stable during in vitro gastro-intestinal digestion. On the other hand, Bouayed et al. (2012) observed a bioaccessibility of 31.6%-56.5% of hydroxycinnamic acids in selected apple varieties following in vitro gastro-intestinal digestion.

Hydroxycinnamic acid-aspartate derivatives were the most bioaccessible hydroxycinnamic acids in the tested dark chocolates (Table 2). Ferulic acid (the most abundant hydroxycinnamic acid in dark chocolates) was detected in lower concentration in the intestinal environment respect to hydroxycinnamic acid-aspartate derivatives (Table 2). Coumaric acid was the only hydroxycinnamic acid recovered in the intestinal media at higher concentrations than its initial content in the samples. Coumaric acid and in general simple hydroxycinnamic acids are known to be strongly bound to fibers, such as cellulose, hemicellulose, lignin and pectin (Juaniz et al., 2016 and 2017). However, Blancas-Benitez et al. (2015) found that hydroxycinnamic and hydroxybenzoic acids were efficiently released from mango dietary fiber during in vitro gastro-intestinal digestion. Indeed, a loss of an OH-group in the phenolic ring of di-hydroxycinnamic acid isomers resulting in the formation of coumaric acid could be hypothesized, as already suggested by Juaniz et al. (2017).

In addition, some isomerization reactions took place during in vitro gastro-intestinal digestion. Isomerization from 5-caffeoylquinic acid to 3-caffeoylquinic acid and 4-caffeoylquinic acid is highly pH-dependent and may occur during the intestinal step of the digestion process (alkaline pH) (Bermúdez-Soto, Tomás-Barberán, & García-Conesa, 2007; Juaniz et al., 2017). This may explain...
the appearance of 4-caffeoylquinic acid in all the dark chocolates after *in vitro* gastro-intestinal digestion (*Table 7*). Similarly, other isomerization reactions might take place among galloylquinic acid isomers as observed in GTDC subjected to *in vitro* digestion (*Table 5*).

Flavan-3-ols were the dominant class of phenolic compounds in the tested dark chocolates. However, due to their low bioaccessibility, hydroxycinnamic acids dominated the phenolic profile in *in vitro* digested chocolates, with the only exception of GTDC (*Figure 2A* and *Table 1*). While the monomeric flavan-3-ols appeared to be in some way bioaccessible, the recovered amount of procyanidins was extremely low and most of them were not found in the intestinal environment. (Epi)gallocatechin isomers were only detected after *in vitro* digestion of GTDC probably because they were present in higher concentration in GTDC respect to the other dark chocolate samples. The high instability of catechins and procyanidins had been reported earlier (Bouayed et al. 2012). In a previous study, procyanidin B2 was almost completely degraded into the monomeric epicatechin during gastric digestion (Kahle et al., 2011). The degradation of procyanidin B2, epicatechin and catechin into unknown degradation products in artificial intestinal conditions was also observed (Kahle et al., 2011; Zhu et al., 2002; Bouayed et al. 2012). In another study, epigallocatechin and epigallocatechin gallate were found to be sensitive to gastro-intestinal digestion with less than 10% recovery after *in vitro* digestion of green tea (Green, Murphy, Schulz, Watkins, & Ferruzzi, 2007).

Some new compounds appearing in the intestinal environment may be indicative of catechin monomers degradation (*Table 7*). For example, trihydroxybenzene may be originated from the B-ring of (epi)gallocatechin and epigallocatechin gallate. Indeed, after *in vitro* digestion of GTDC two new compounds were detected and identified as (epi)gallocatechin homodimers (theasinensin isomer and P2 analogue) (Neilson et al., 2007). Finally, the highest bioaccessibility of (epi)gallocatechin isomers and the higher content of gallic acid observed after digestion of GTDC, respect to the contents found in the chemical extract, could be explained as a consequence of hydrolysis of epigallocatechin gallate.
3.2 Effect of in vitro digestion on antioxidant activities

In order to study how the antioxidant activity of the dark chocolate samples was modified throughout the digestive process, antioxidant activity was determined by FRAP and ABTS assays at each stage of the in vitro gastro-intestinal digestion and in the chemical extracts (Figure 3A and B).

The GTDC chemical extract was the sample with the highest activity for both the assays (15.4 ± 1.4 mmol trolox/100 g chocolate in ABTS and 15.4 ± 0.8 mmol trolox/100 g chocolate in FRAP). In general, it was observed that the two different assays gave similar trends for the distinct tested samples during the gastro-intestinal digestion. For all the tested samples, the antioxidant activity after the salivary phase was significantly lower than the antioxidant activity of the chemical extracts, in accordance with the low total phenolic content extracted after this step. Antioxidant activity increased after the gastric step of the digestion and further rose after the intestinal step. The 91.5%, 74.0% and 80.1% of DC, GTDC and TDC antioxidant activities, respectively, was observed after intestinal digestion respect to the chemical extracts with the ABTS assay. The FRAP assay recovered lower antioxidant activity than those observed with the ABTS assay and equal to 37.6%, 35.1% and 38.0% of DC, GTDC and TDC, respectively. Beside phenolic compounds, dark chocolate also contains other well-known antioxidants such as Maillard reaction products that can be formed during chocolate high temperature processes such as drying, roasting and conching (Quiroz-Reyes, & Fogliano, 2018). Differences between FRAP and ABTS values could be explained by considering that Maillard reaction products show a high chain-breaking activity despite their low reducing potential (Di Mattia, Sacchetti, Mastrocola, & Serafini, 2017). At the end of the gastro-intestinal digestion, GTDC displayed the highest amount of antioxidant activity in both the assays (11.4 ± 0.1 mmol trolox/100 g chocolate in ABTS and 5.4 ± 0.5 mmol trolox/100 g chocolate in FRAP).
These results are consistent with previous studies, where an increase in antioxidant activity was observed during digestion of grapes (Tagliazucchi et al., 2010), fruits (Tagliazucchi et al., 2012) and fruit extracts (Pavan, Sancho, & Pastore, 2014). However, other authors reported a large decrease in antioxidant activity after digestion of different foods (Garbetta et al., 2018; Lingua et al., 2018; Wang, Amigo-Benavent, Mateos, Bravo, & Sarriá, 2017). Multiple factors such as assay conditions, solubility and matrix effect may affect the antioxidant activity of foods and phenolic compounds during in vitro digestion. In any case, the antioxidant potential of dietary phenolic compounds in the intestinal tract, independently from their bioavailability, could offer protection by scavenging reactive oxygen species and reducing the oxidative stress at the intestinal cells level (Lingua et al., 2018 Tagliazucchi et al., 2010).

3.3 Anti-proliferative activity of in vitro digested dark chocolate and dark chocolate enriched with Sakura green tea or turmeric powder phenolic-rich fractions on human colon adenocarcinoma cell lines

The anti-proliferative activity of DC, GTDC and TDC phenolic-rich fractions extracted at the end of the in vitro gastro-intestinal digestion on the growth of human colon adenocarcinoma Caco-2 and SW-480 cells was investigated. Caco-2 and SW480 cells were incubated with different concentrations of phenolic-rich extracts ranging from 7 to 170 μmol/100 g of chocolate for 24 h. As shown in Figure 4, the inhibition was similar between DC and GTDC phenolic-rich fractions against Caco-2 cells. This result was not surprising, considering the similar phenolic profile of the two dark chocolates after in vitro gastro-intestinal digestion. However, when TDC phenolic-rich fraction was tested against Caco-2 cell line, a significantly lower IC50 value (which means a higher anti-proliferative activity) was found in comparison with the other two tested dark chocolates. All of the samples showed a higher anti-proliferative activity against SW480 respect to Caco-2.
According to the literature, there are no reports regarding the anti-proliferative activity of dark chocolate after in vitro digestion. Previous in vitro studies have shown anti-proliferative properties of procyanidin and procyanidin-enriched extract isolated from cocoa powder in colon cancer Caco-2 cells (Carnesecchi et al., 2002; Martin and Ramos, 2017). However, 24 h treatment of Caco-2 and SW480 with catechin, epicatechin or procyanidin B2 did not affect cell growth, suggesting that other compounds rather than flavan-3-ols can be responsible for the observed anti-proliferative effect of phenolic-rich fractions extracted at the end of the in vitro gastro-intestinal digestion (Ramos, Rodríguez-Ramiro, Martín, Goya, & Bravo, 2011). The highest effect of TDC phenolic-rich fraction against Caco-2 cell line can be ascribed to the anti-proliferative activity of curcuminoids or a synergistic effect between curcuminoids and other phenolic compounds (Iwuchukwu, Tallarida, & Nagar, 2011).

3.4. In vitro metabolism of digested dark chocolate phenolic-rich fraction in cell cultures

In order to verify the cell metabolism of dark chocolate phenolic compounds, Caco-2 and SW480 media were analysed by LC-MS ion trap after incubation (24 h) with in vitro digested dark chocolate (at concentration corresponding to IC_{50}). Different metabolic reactions, including (de)hydroxylation, (de)hydrogenation, and conjugation with methyl, glucuronide, sulphate, and glutathione moieties were monitored. Some parent compounds and newly formed metabolites were detected in both cell types and reported in Table 8.

In addition to the parent compounds catechin and epicatechin, two newly formed metabolites were tentatively identified. Methyl-(epi)catechin was found in the cell media of both the cell lines whereas dimethyl-(epi)catechin was found only in Caco-2 medium. Previous studies identified methyl-epicatechin and sulphate-epicatechin as the main metabolites in Caco-2 experiments with a prevalence of methylation (Aragonès et al., 2017; Sanchez-Bridge et al., 2015). The lack of identification of sulphate metabolites of (epi)catechin could be due to their low concentration in the
media (i.e. they could be formed but were below the limit of detection) or to the inhibition of the specific enzymes as a consequence of the presence of other phenolic compounds. Sanchez-Bridge et al. (2015) showed that the co-administration of epicatechin with flavonols, flavones and isoflavones reduced the metabolism of epicatechin (especially sulphation) in Caco-2. Despite the appearance in vivo of glucuronidated epicatechin metabolites, we did not find these substituted metabolites under our experimental conditions. Previous studies suggested the absence of specific uridine 5’-diphospho-glucuronosyl-transferase isoforms able to form glucuronic acid conjugate of epicatechin in Caco-2 cells (Actis-Goretta et al., 2013; Sanchez-Bridge et al., 2015).

The main hydroxycinnamic acid derivatives found after in vitro gastro-intestinal digestion of dark chocolate were the conjugated forms with amino acids (such as aspartate and tyrosine, Table 2). With the exception of trace amounts of feruloyl-aspartate (found in the media of both cell lines), we were not able to identify these compounds after incubation with the two cell lines. Diversely, we found ferulic and coumaric acids in the media of both cell lines and caffeic acid only after incubation with SW480 cells. A sulphated form of coumaric acid and dihydro-ferulic acid were tentatively identified as newly formed metabolites in the cell culture media after incubation with SW480 and Caco-2, respectively. The concentration of coumaric acid increased from 5.10 ± 0.12 after in vitro digestion to 86.14 ± 3.19 and 96.80 ± 4.96 μmol/100 g of chocolate after 24 h of incubation with Caco-2 and SW480, respectively (Table 2 and Table 8). The increased amount of coumaric acid may derive from hydroxylation of cinnamic acid (which was present in the dark chocolate after in vitro gastro-intestinal digestion but not in the cell culture media, data not shown), dehydroxylation of caffeic acid or dehydrogenation of dihydro-coumaric acid, as already suggested by Poquet, Clifford, & Williamson (2008) for dihydro-ferulic acid. Alternatively, a hydrolysis of coumaroyl-aspartate and/or coumaroyl-tyrosine, catalysed by membrane-bound carboxypeptidases, may be hypothesized. Indeed, coumaric acid has been found particularly stable when incubated with Caco-2 or rat hepatic cells (Kahle et al., 2011; Kern et al., 2003). After 24 h of incubation with
Caco-2 an increased amount of ferulic acid respect to the concentration found at the end of the digestion was detected (Table 2 and Table 8). Methylation of caffeic acid by catechol-O-methyltransferase may account for the increase in ferulic acid concentration (Kern et al., 2003). This conclusion is supported also by the evidence of the disappearance of caffeic acid from the medium. Indeed, methylation of di-hydro-caffeic acid may account for the appearance of di-hydro-ferulic acid in the medium, as already suggested in Caco-2 cells by Poquet et al. (2008). The same conclusions cannot be drawn for SW480. In the medium of this cell line we found some residual caffeic acid and the amount of ferulic acid did not increase during incubation (Table 2 and Table 8). Indeed, we did not identify di-hydro-ferulic acid in the medium of SW480. This evidence suggested that caffeic and di-hydro-caffeic acid were not substrates for the catechol-O-methyltransferase in SW480, despite its presence as indicated by the appearance of methylated (epi)catechin as reported above. Therefore, hydroxycinnamic acids metabolism under our experimental conditions resulted in the accumulation of coumaric and ferulic acids in cell media with only minor phase II metabolism. Figure 5 reported the hypothetical pathways of hydroxycinnamic acids metabolism leading to the accumulation of coumaric and ferulic acids. Quercetin-hexoside and quercetin-pentoside were tentatively identified after 24 h of incubation with SW480 cell line, despite their low concentration in the sample after in vitro gastro-intestinal digestion. This is indicative of their relative stability in cell culture medium as already suggested by Xiao, & Högger (2015). Instead, quercetin-hexoside was not identified after 24 h of incubation with Caco-2. The aglycone quercetin, which was not present in dark chocolate after in vitro gastro-intestinal digestion, appeared after incubation with Caco-2, suggesting that this cell line was able to de-glycosylate quercetin-hexoside releasing the corresponding aglycone. De-glycosylation of flavonoid glycosides can be catalysed by the action of membrane-bound lactase phloridzin hydrolase and/or cytosolic β-glucosidase (Németh et al., 2003). Some previous studies failed to detect de-glycosylation of quercetin-glucoside by using Caco-2 cells (del Mar Contreras, Borrás-
However, Caco-2 cells express both lactase phloridzin hydrolase and cytosolic $\beta$-glucosidase (Németh et al., 2003). This discrepancy can be due to the shorter incubation time in the previous studies (1-2 h vs 24 h in our study). Quercetin was not identified in SW480 cell culture medium, suggesting that this cell line was not able to hydrolyse quercetin-hexoside. Finally, one methylated derivative of ellagic acid was tentatively identified only in the SW480 cell medium.

4. Conclusions

Bioactivity of phenolic compounds is primarily conditioned by their bioaccessibility in the gastrointestinal tract, and secondly by their cellular uptake and internal transformation. The present study determined the amounts of bioaccessible dark chocolate phenolic compounds after gastro-intestinal digestion. We have demonstrated that gastro-intestinal digestion modified the phenolic profile in the samples from a qualitative and quantitative point of view.

We have also demonstrated that Caco-2 and SW480 cell lines showed metabolic activity resulting in a partial modification of dark chocolate phenolic compounds leading to the accumulation of coumaric and ferulic acids in the cell media. The observed anti-proliferative activity could be related to the accumulation of these simple hydroxycinnamic acids. The presence of ferulic acid and quercetin in Caco-2 cell medium at higher concentration than in SW480 cell medium or the presence of caffeic acid only in SW480 cell medium may suggest the intrinsic differences between the two cell lines and the metabolic mechanisms involved. Further studies are necessary in order to confirm the proposed pathways of metabolism of hydroxycinnamic acids during incubation with cell lines and their potential anti-proliferative activity.

The addition of green tea leaves or turmeric powder in dark chocolate recipe lead to a modification of dark chocolate healthy properties. Functionalization with green tea leaves resulted in a higher
amount of flavan-3-ols and flavonols after \textit{in vitro} digestion than dark chocolate, achieving a more efficient antioxidant activity. Similarly, the addition of turmeric powder may lead to an increased anti-proliferative activity against adenocarcinoma cell lines respect to DC and GTDC. In this way, the potential healthy effect of dark chocolate consumption could be maximized, reducing the amount of energy and calories introduced with chocolate itself and resulting in a lower intake to achieve the same biological effects.
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Figure captions

Figure 1. Changes in total phenolic content during *in vitro* gastro-intestinal digestion. Total phenolic content was determined with the Folin-Ciocalteau assay and expressed as μmol of gallic acid equivalents/100 g of chocolate. Light grey columns represent the changes detected during *in vitro* digestion of dark chocolate. Grey columns represent the changes detected during *in vitro* digestion of dark chocolate enriched with Sakura green tea leaves. Dark grey columns represent the changes detected during *in vitro* digestion of dark chocolate enriched with turmeric powder. Results are expressed as mean ± standard deviation. Different letters refer to statistically significant differences (*P*<0.05) in total phenolic compounds content among samples.

Figure 2. Bioaccessibility of individual phenolic compounds identified and quantified by LC-ESI-QTOF MS/MS grouped by classes. (A) Flavan-3-ols; (B) hydroxycinnamic acids; (C) flavonols; (D) other phenolics; (E) flavones; (F) ellagitannins; (G) hydroxybenzoic acids; (H) sum of the different classes. DC identify dark chocolate; GTDC identify dark chocolate enriched with Sakura green tea leaves; TDC identify dark chocolate enriched with turmeric powder. Black columns represent the amount of the individual classes found in the chemical extract whereas grey columns the amount at the end of the digestion.

Figure 3. Changes in antioxidant activity during *in vitro* gastro-intestinal digestion. Antioxidant capacity (expressed as mmol of trolox equivalent/100 g of chocolate), measured by ABTS (A) and FRAP (B) assays. Light grey columns represent the changes detected during *in vitro* digestion of dark chocolate. Grey columns represent the changes detected during *in vitro* digestion of dark chocolate enriched with Sakura green tea leaves. Dark grey columns represent the changes detected during *in vitro* digestion of dark chocolate enriched with turmeric powder digestion. Results are expressed as mean ± standard deviation. Values in the same graph with different lowercase letters are significantly different (*P* < 0.05).
Figure 4. Anti-proliferative activity of phenolic-rich fractions extracted at the end of the *in vitro* digestion. DC identify dark chocolate; GTDC identify dark chocolate enriched with Sakura green tea leaves; TDC identify dark chocolate enriched with turmeric powder. IC$_{50}$ is defined as the concentration of phenolic compounds required to inhibit 50% of cell proliferation. The amount of phenolic compounds was determined by LC-ESI-QTOF MS/MS analysis as described in material and methods. Dark grey columns represent the IC$_{50}$ versus Caco-2 cells. Grey columns represent the IC$_{50}$ versus SW480 cells. Values in with different lowercase letters are significantly different ($P < 0.05$).

Figure 5. Proposed pathways for hydroxycinnamic acids metabolism after incubation with Caco-2 and SW480 cell lines of dark chocolate phenolic-rich fractions extracted at the end of the *in vitro* digestion. COMT: catechol-O-methyl transferase; CPase: carboxypeptidase; RA: reduction; DHY: de-hydroxylation; HYD: hydroxylation; SULT: sulfotransferase. Compounds in dark grey boxes were detected both in *in vitro* digested samples and after incubation with cells; compounds in light grey boxes were detected only in *in vitro* digested samples; compounds in white boxes were detected only after incubation with cells. The unbroken arrows indicate previously demonstrated pathways whereas dotted arrows indicate pathways hypothesized in this study. Steps not found in SW480 are indicated. Please note that caffeic acid was found only after incubation with SW480 whereas di-hydro-ferulic acid was found only after incubation with Caco-2.
Table 1. Quantitative results (μmol/100 g of chocolate) for flavan-3-ols identified in dark chocolate after chemical extraction and after gastro-intestinal digestion. Values represent means ± standard deviation of triplicate determination.

| Compound | Dark chocolate 70% | Sakura green tea | Turmeric | Dark chocolate 70% |
|----------|-------------------|-----------------|----------|-------------------|
|          | Chemical extraction | After digestion | Chemical extraction | After digestion | Chemical extraction | After digestion |
| **Flavan-3-ols** | | | | |
| Catechin | 228.28 ± 6.86a | 42.00 ± 6.65b | 240.07 ± 18.28a | 47.18 ± 2.12b | 245.28 ± 3.97a | 40.21 ± 5.40b |
| Epicatechin | 701.00 ± 26.48a | 124.75 ± 7.79b | 1047.21 ± 40.17c | 175.59 ± 6.55d | 753.21 ± 27.86a | 126.66 ± 8.23b |
| Galloccatechin | 1.48 ± 0.03a | n.d. | 7.21 ± 0.48b | 6.58 ± 0.20b | < LOQ | n.d. |
| Epigallocatechin | 54.93 ± 0.34a | n.d. | 102.62 ± 6.00b | 53.64 ± 0.78a | 52.10 ± 2.76a | n.d. |
| Catechin-O-sulphate isomer | 7.10 ± 0.45a | 4.21 ± 0.23b | 5.97 ± 0.69a | 3.75 ± 0.13a | 10.59 ± 1.07c | 4.81 ± 0.11d |
| Epicatechin-O-sulphate isomer | 24.66 ± 1.21a | 14.13 ± 1.01b | 24.69 ± 0.41a | 14.65 ± 0.93b,d | 35.28 ± 2.86c | 16.10 ± 0.61d |
| Catechin-3-O-trihydroxybenzene | 16.97 ± 1.72a,d | 1.11 ± 0.01b | 16.07 ± 0.34a | 1.68 ± 0.06c | 20.48 ± 1.93d | 1.08 ± 0.01b |
| Epicatechin-3-O-trihydroxybenzene | 12.45 ± 0.21a | 1.01 ± 0.05b | 15.79 ± 0.90c | 1.33 ± 0.02d | 16.69 ± 1.45c | 0.65 ± 0.02a |
| Catechin-7-O-trihydroxybenzene | 8.97 ± 0.52a | 0.82 ± 0.02b | 13.21 ± 1.93c | 1.10 ± 0.08b | 11.69 ± 0.48c | 0.40 ± 0.01d |
| Epicatechin-C-pentoside isomer | 1.45 ± 0.17a | 0.74 ± 0.01b | 1.03 ± 0.24a | 0.86 ± 0.01b | 2.86 ± 0.38c | 0.90 ± 0.03b |
| Catechin-3-O-gallate* | n.d. | n.d. | < LOQ | < LOQ | n.d. | n.d. |
| Epicatechin-3-O-gallate* | n.d. | n.d. | 31.45 ± 0.45a | 1.16 ± 0.11b | n.d. | n.d. |
| (Epi)catechin-3-O-hexoside isomer | 43.59 ± 0.69a | 10.95 ± 0.52b | 33.72 ± 4.62c | 13.16 ± 0.62d | 12.10 ± 1.79d | 7.13 ± 0.42e |
| (Epi)catechin-C-hexoside isomer | 6.59 ± 0.14a | 4.90 ± 0.19b | 5.59 ± 0.34c | 6.17 ± 0.27a,c | 4.55 ± 0.66b | 6.74 ± 0.34a |
| (Epi)catechin-7-O-hexoside | 14.17 ± 1.00a | 3.64 ± 0.16b | 14.69 ± 1.03a | 4.30 ± 0.17c | 18.07 ± 0.10d | 1.41 ± 0.02e |
| (Epi)catechin-C-hexoside isomer | 14.10 ± 0.72a | 8.11 ± 0.54b | 12.00 ± 2.76a | 9.67 ± 0.37c | 12.83 ± 0.01a | 9.02 ± 0.40b,c |
| (Epi)catechin-3-O-hexoside isomer | 4.69 ± 0.72a | 2.18 ± 0.10b | 6.24 ± 0.76a | 3.10 ± 0.16c | 5.10 ± 0.28a | 1.81 ± 0.01d |
| Epigallocatechin-3-O-gallate* | n.d. | n.d. | 115.66 ± 7.45a | 3.20 ± 0.34b | n.d | n.d. |
| Galloccatechin-3-O-hexoside | 0.31 ± 0.03a | 1.41 ± 0.02b | 0.21 ± 0.01a | 1.52 ± 0.02b | 0.52 ± 0.03c | 1.49 ± 0.02b |
| Epigallocatechin-3-O-hexoside | 0.31 ± 0.03a | 1.27 ± 0.01b | 0.24 ± 0.01a | 1.80 ± 0.05c | 0.59 ± 0.03d | 1.41 ± 0.01e |
| Procyanidin dimer A type | 3.79 ± 0.21a | n.d. | 4.52 ± 0.28a | n.d. | 4.17 ± 0.24a | n.d. |
| Procyanidin dimer B type isomer | 29.83 ± 2.90a | 2.09 ± 0.23b | 28.93 ± 1.17a | 2.08 ± 0.05b | 36.52 ± 5.10a | 1.48 ± 0.11c |
| Procyanidin dimer B type isomer | 10.52 ± 0.76a,c | 1.12 ± 0.24b | 9.28 ± 0.14a | < LOQ | 12.48 ± 0.66c | 1.04 ± 0.13b |
| Procyanidin dimer B type isomer | 128.38 ± 11.86a | n.d. | 180.86 ± 18.76b | 2.29 ± 0.15c | 121.10 ± 0.14a | n.d. |
| Procyanidin dimer B type isomer | 117.52 ± 6.83a | 19.56 ± 0.54b | 155.14 ± 3.97c | 24.26 ± 1.03d | 131.90 ± 9.52a | 23.60 ± 1.66d |
| Procyanidin dimer B type isomer | 18.34 ± 1.03a | n.d. | 22.97 ± 2.21b | n.d. | 23.90 ± 2.55b | < LOQ |
| Procyanidin dimer B type isomer | 35.07 ± 0.45a | n.d. | 42.59 ± 1.21b | 1.92 ± 0.11c | 45.24 ± 3.07b | < LOQ |
| Compound                                           | n.d. | n.d. | 0.41 ± 0.03<sup>a</sup> | n.d. | n.d. | n.d. |
|----------------------------------------------------|------|------|--------------------------|------|------|------|
| (Epi)catechin-3-O-dihexoside isomer (Epi)gallocatechin-7-O-glucuronide isomer | n.d. | n.d. | 2.28 ± 0.03<sup>a</sup> | n.d. | < LOQ | 1.52 ± 0.03<sup>b</sup> | < LOQ |
| (Epi)catechin-3-O-dihexoside isomer (Epi)gallocatechin derivative isomer | n.d. | n.d. | 1.14 ± 0.03<sup>a</sup> | n.d. | < LOQ | 0.83 ± 0.10<sup>b</sup> | n.d. |
| (Epi)catechin-3-O-gallate-7-O-glucuronide isomer (Epi)catechin-3-O-gallate derivative isomer | n.d. | n.d. | 1.07 ± 0.07<sup>a</sup> | n.d. | < LOQ | 0.79 ± 0.07<sup>b</sup> | n.d. |
| Procyanidin dimer A type pentoside isomer          | 4.90 ± 0.03<sup>a</sup> | n.d. | 4.45 ± 0.83<sup>a</sup> | n.d. | 5.79 ± 0.14<sup>b</sup> | < LOQ |
| Procyanidin dimer A type pentoside isomer          | 3.76 ± 0.03<sup>a</sup> | n.d. | 4.03 ± 0.28<sup>a</sup> | n.d. | 3.59 ± 0.14<sup>a</sup> | < LOQ |
| Procyanidin dimer A type pentoside isomer          | 6.03 ± 0.10<sup>a</sup> | n.d. | 7.72 ± 0.72<sup>b</sup> | n.d. | 6.28 ± 0.38<sup>a</sup> | < LOQ |
| Procyanidin dimer A type pentoside isomer          | 5.07 ± 0.38<sup>a</sup> | n.d. | 9.45 ± 0.38<sup>b</sup> | n.d. | 4.14 ± 0.21<sup>a</sup> | < LOQ |
| Procyanidin dimer A type hexoside isomer           | 5.10 ± 0.21<sup>a</sup> | n.d. | 5.24 ± 0.79<sup>a,b</sup> | n.d. | 6.59 ± 0.24<sup>b</sup> | < LOQ |
| Procyanidin dimer A type hexoside isomer           | 3.48 ± 0.10<sup>a</sup> | 0.42 ± 0.01<sup>b</sup> | 4.28 ± 0.38<sup>c</sup> | 0.45 ± 0.01<sup>b</sup> | 5.45 ± 0.17<sup>d</sup> | n.d. |
| Procyanidin dimer A type hexoside isomer           | 7.10 ± 0.31<sup>a</sup> | n.d. | 10.66 ± 1.28<sup>b</sup> | < LOQ | 8.03 ± 0.17<sup>a</sup> | < LOQ |
| Procyanidin dimer A type hexoside isomer           | 5.07 ± 0.10<sup>a</sup> | n.d. | 8.90 ± 0.52<sup>b</sup> | < LOQ | 7.10 ± 0.10<sup>c</sup> | < LOQ |
| Procyanidin dimer B type hexoside isomer           | 2.76 ± 0.14<sup>a</sup> | 1.78 ± 0.08<sup>b</sup> | 3.07 ± 0.24<sup>a</sup> | 1.93 ± 0.03<sup>d</sup> | 3.52 ± 0.34<sup>a</sup> | 1.68 ± 0.08<sup>b</sup> |
| Procyanidin dimer B type hexoside isomer           | 3.59 ± 0.07<sup>a</sup> | 0.92 ± 0.01<sup>b</sup> | 3.66 ± 0.14<sup>a</sup> | 1.22 ± 0.07<sup>c</sup> | 4.21 ± 0.14<sup>d</sup> | 1.03 ± 0.07<sup>a</sup> |
| Procyanidin dimer B type hexoside isomer           | 1.76 ± 0.28<sup>a</sup> | 1.08 ± 0.02<sup>b</sup> | 1.90 ± 0.10<sup>a</sup> | 1.29 ± 0.02<sup>b</sup> | 1.86 ± 0.14<sup>a</sup> | 1.21 ± 0.02<sup>b</sup> |
| Procyanidin dimer B type derivative isomer         | 6.41 ± 0.10<sup>a</sup> | 2.88 ± 0.09<sup>b</sup> | 6.48 ± 1.62<sup>a</sup> | 2.75 ± 0.15<sup>d</sup> | 7.83 ± 0.45<sup>a</sup> | 1.75 ± 0.11<sup>c</sup> |
| Procyanidin dimer A type isomer                    | 2.41 ± 0.17<sup>a</sup> | n.d. | 2.31 ± 0.14<sup>a</sup> | n.d. | 3.34 ± 0.14<sup>b</sup> | n.d. |
| Procyanidin dimer B type derivative isomer         | 3.21 ± 0.17<sup>a</sup> | n.d. | 3.17 ± 0.10<sup>a</sup> | < LOQ | 3.83 ± 0.38<sup>a</sup> | n.d. |
| Procyanidin dimer B type isomer                    | 14.83 ± 1.07<sup>a</sup> | 0.34 ± 0.02<sup>b</sup> | 24.59 ± 1.76<sup>c</sup> | 0.73 ± 0.01<sup>d</sup> | 17.10 ± 1.41<sup>a</sup> | n.d. |
| Procyanidin dimer B type isomer                    | 11.07 ± 0.24<sup>a</sup> | 0.20 ± 0.01<sup>b</sup> | 14.48 ± 0.86<sup>b</sup> | < LOQ | 12.59 ± 0.38<sup>b</sup> | n.d. |
| Procyanidin dimer B type isomer                    | 38.00 ± 1.55<sup>a</sup> | n.d. | 52.28 ± 3.10<sup>b</sup> | n.d. | 40.00 ± 2.21<sup>a</sup> | < LOQ |
| Procyanidin dimer B type isomer                    | 30.69 ± 0.59<sup>a</sup> | 0.46 ± 0.01<sup>b</sup> | 60.31 ± 4.62<sup>c</sup> | n.d. | 33.24 ± 0.17<sup>a</sup> | n.d. |
| Procyanidin dimer B type isomer                    | 12.41 ± 0.66<sup>a</sup> | n.d. | 12.66 ± 0.62<sup>a</sup> | n.d. | 12.07 ± 0.76<sup>a</sup> | n.d. |
| Procyanidin trimer B type isomer | 7.07 ± 0.28<sup>a</sup> | n.d. | 7.41 ± 0.24<sup>a</sup> | n.d. | 10.03 ± 0.52<sup>b</sup> | n.d. |
| Procyanidin trimer B type isomer | 3.83 ± 0.10<sup>a</sup> | n.d. | 5.79 ± 0.28<sup>b</sup> | n.d. | 4.76 ± 0.07<sup>c</sup> | n.d. |
| Procyanidin trimer A type hexoside isomer | 2.41 ± 0.21<sup>a</sup> | n.d. | 3.41 ± 0.10<sup>b</sup> | n.d. | 2.90 ± 0.24<sup>a</sup> | n.d. |
| Procyanidin trimer A type hexoside isomer | 1.86 ± 0.07<sup>a</sup> | n.d. | 3.34 ± 0.10<sup>b</sup> | n.d. | 2.41 ± 0.14<sup>c</sup> | n.d. |
| Procyanidin tetramer A type isomer | 4.97 ± 0.03<sup>a</sup> | n.d. | 4.76 ± 0.24<sup>a</sup> | < LOQ | 4.90 ± 0.28<sup>a</sup> | < LOQ |
| Procyanidin tetramer A type isomer | 6.97 ± 0.45<sup>a</sup> | n.d. | 12.69 ± 1.97<sup>b</sup> | n.d. | 7.03 ± 0.55<sup>a</sup> | < LOQ |
| Procyanidin tetramer A type isomer | 6.97 ± 0.10<sup>a</sup> | n.d. | 13.93 ± 2.83<sup>b</sup> | < LOQ | 9.76 ± 0.28<sup>c</sup> | < LOQ |
| Procyanidin tetramer A type isomer | 2.72 ± 0.24<sup>a,b</sup> | n.d. | 3.83 ± 1.00<sup>a</sup> | < LOQ | 2.28 ± 0.21<sup>b</sup> | n.d. |
| Procyanidin tetramer B type isomer | 1.28 ± 0.03<sup>a</sup> | n.d. | 6.28 ± 0.17<sup>b</sup> | n.d. | 1.28 ± 0.17<sup>a</sup> | n.d. |
| Procyanidin tetramer B type isomer | 4.62 ± 0.14<sup>a</sup> | n.d. | 6.24 ± 0.76<sup>b</sup> | n.d. | 6.38 ± 0.17<sup>b</sup> | n.d. |
| Procyanidin tetramer B type isomer | 3.21 ± 0.10<sup>a</sup> | n.d. | 6.28 ± 0.24<sup>b</sup> | n.d. | 5.90 ± 0.55<sup>b</sup> | < LOQ |
| Procyanidin tetramer B type isomer | 4.10 ± 0.28<sup>a</sup> | n.d. | 8.90 ± 0.10<sup>b</sup> | n.d. | 5.69 ± 0.03<sup>c</sup> | n.d. |
| Procyanidin pentamer B type isomer | 2.07 ± 0.07<sup>a</sup> | n.d. | 1.90 ± 0.14<sup>a</sup> | n.d. | 2.31 ± 0.02<sup>a</sup> | n.d. |
| Procyanidin pentamer B type isomer | 2.83 ± 0.14<sup>a</sup> | n.d. | 5.34 ± 0.48<sup>b</sup> | n.d. | 3.41 ± 0.01<sup>c</sup> | n.d. |
| Procyanidin pentamer B type isomer | 4.03 ± 0.28<sup>a</sup> | n.d. | 11.97 ± 0.97<sup>b</sup> | n.d. | 6.03 ± 0.14<sup>c</sup> | n.d. |
| Procyanidin hexamer A type isomer | < LOQ | n.d. | < LOQ | n.d. | < LOQ | n.d. |
| Procyanidin hexamer B type isomer | 1.07 ± 0.03<sup>a</sup> | n.d. | 3.38 ± 0.07<sup>b</sup> | n.d. | 1.28 ± 0.07<sup>a</sup> | n.d. |
| Procyanidin hexamer B type isomer | 1.50 ± 0.14<sup>a</sup> | n.d. | 4.66 ± 0.14<sup>b</sup> | n.d. | 1.00 ± 0.03<sup>c</sup> | n.d. |
| Procyanidin hexamer B type isomer | 0.76 ± 0.07<sup>a</sup> | n.d. | 6.38 ± 0.69<sup>b</sup> | n.d. | 1.59 ± 0.14<sup>c</sup> | n.d. |

< LOQ means the compound was detected but it was below the limit of quantification; n.d. means not detected
* mean the compounds were detected only in green tea dark chocolate.
Different superscript letters within the same row indicate that the values are significantly different (P<0.05).
Flavan-3-ols were quantified as epicatechin equivalent.
Data from chemical extraction were from Martini et al. (2018).
Table 2. Quantitative results (μmol/100 g of chocolate) for hydroxycinnamic acids identified in dark chocolate after chemical extraction and after gastro-intestinal digestion. Values represent means ± standard deviation of triplicate determination.

| Compound                        | Dark chocolate 70% | Sakura green tea dark chocolate 70% | Turmeric dark chocolate 70% |
|---------------------------------|--------------------|-----------------------------------|-----------------------------|
|                                 | Chemical extraction | After digestion | Chemical extraction | After digestion | Chemical extraction | After digestion |
| **Hydroxycinnamic acids**       |                    |                    |                        |                |                     |                |
| Coumaric acid                   | 2.07 ± 0.06<sup>a</sup> | 5.10 ± 0.12<sup>b</sup> | 2.26 ± 0.30<sup>a</sup> | 5.02 ± 0.20<sup>b</sup> | 3.35 ± 0.06<sup>c</sup> | 5.34 ± 0.42<sup>b</sup> |
| Di-hydro-coumaric acid          | 49.57 ± 0.85<sup>a</sup> | 31.29 ± 2.09<sup>b</sup> | 27.87 ± 0.49<sup>c</sup> | 17.06 ± 0.94<sup>d</sup> | 68.17 ± 2.50<sup>e</sup> | 38.31 ± 2.10<sup>b</sup> |
| Di-hydroxyxycinnamic acid isomer | 5.61 ± 0.06<sup>a</sup> | 4.96 ± 0.27<sup>b</sup> | 6.95 ± 0.12<sup>c</sup> | 7.31 ± 0.32<sup>c</sup> | 14.21 ± 0.18<sup>d</sup> | 6.41 ± 0.31<sup>e</sup> |
| Di-hydroxyxycinnamic acid isomer | 2.93 ± 0.12<sup>a</sup> | 1.05 ± 0.21<sup>b</sup> | 2.20 ± 0.06<sup>c</sup> | 1.15 ± 0.02<sup>d</sup> | 3.48 ± 0.06<sup>e</sup> | 1.05 ± 0.16<sup>f</sup> |
| Caffeic acid                    | 6.59 ± 0.06<sup>a</sup> | 2.16 ± 0.06<sup>b</sup> | 10.24 ± 0.12<sup>c</sup> | 2.82 ± 0.14<sup>d</sup> | 6.65 ± 0.12<sup>e</sup> | 2.78 ± 0.18<sup>f</sup> |
| Di-hydroxyxycinnamic acid isomer | 2.93 ± 0.06<sup>a</sup> | 1.35 ± 0.10<sup>b</sup> | 2.07 ± 0.01<sup>c</sup> | 1.13 ± 0.08<sup>d</sup> | 3.66 ± 0.24<sup>e</sup> | 1.82 ± 0.04<sup>f</sup> |
| Di-hydroxyxycinnamic acid isomer | 3.54 ± 0.06<sup>a</sup> | 0.46 ± 0.01<sup>b</sup> | 3.84 ± 0.18<sup>c</sup> | 1.02 ± 0.02<sup>d</sup> | 3.54 ± 0.18<sup>e</sup> | 0.57 ± 0.01<sup>f</sup> |
| Di-hydroxyxycinnamic acid isomer | 11.46 ± 0.98<sup>a</sup> | 1.79 ± 0.06<sup>b</sup> | 1.46 ± 0.01<sup>c</sup> | < LOQ | 5.37 ± 0.24<sup>d</sup> | 1.47 ± 0.05<sup>e</sup> |
| Ferulic acid                    | 315.62 ± 19.28<sup>a</sup> | 27.89 ± 3.21<sup>b</sup> | 299.43 ± 12.01<sup>c</sup> | 16.92 ± 1.25<sup>d</sup> | 285.05 ± 7.01<sup>e</sup> | 17.37 ± 0.93<sup>f</sup> |
| Coumaroyl aspartate             | 95.85 ± 1.40<sup>a</sup> | 69.18 ± 2.65<sup>b</sup> | 85.79 ± 0.30<sup>c</sup> | 65.26 ± 3.42<sup>d</sup> | 102.62 ± 1.04<sup>e</sup> | 79.55 ± 1.22<sup>f</sup> |
| Di-hydroxyxycinnamic aspartate isomer | 52.38 ± 3.17<sup>a</sup> | 22.70 ± 1.37<sup>b</sup> | 41.83 ± 0.98<sup>c</sup> | 21.47 ± 1.04<sup>d</sup> | 42.20 ± 0.24<sup>e</sup> | 25.11 ± 0.48<sup>f</sup> |
| Di-hydroxyxycinnamic aspartate isomer | 218.78 ± 17.62<sup>a</sup> | 119.70 ± 8.28<sup>b</sup> | 204.15 ± 1.52<sup>c</sup> | 121.05 ± 11.06<sup>d</sup> | 201.95 ± 3.54<sup>e</sup> | 130.79 ± 3.69<sup>f</sup> |
| Frucoyl aspartate                | 42.16 ± 0.31<sup>a</sup> | 36.91 ± 2.09<sup>b</sup> | 48.96 ± 0.36<sup>c</sup> | 26.49 ± 1.25<sup>d</sup> | 46.19 ± 2.11<sup>e</sup> | 38.66 ± 1.36<sup>f</sup> |
| Ferulic acid-4-O-pentoside       | 2.06 ± 0.01<sup>a</sup> | 1.24 ± 0.04<sup>b</sup> | 2.47 ± 0.01<sup>c</sup> | 1.85 ± 0.11<sup>d</sup> | 2.42 ± 0.10<sup>e</sup> | 1.27 ± 0.02<sup>f</sup> |
| Di-deoxyclovamide (Coumaroyl-DOPA) | 28.41 ± 1.28<sup>a</sup> | 14.85 ± 1.02<sup>b</sup> | 28.96 ± 0.12<sup>c</sup> | 15.32 ± 1.06<sup>d</sup> | 27.87 ± 1.46<sup>e</sup> | 16.82 ± 1.07<sup>f</sup> |
| 3-Coumaroylquinic acid cis<sup>*</sup> | n.d. | n.d. | 9.21 ± 0.10<sup>a</sup> | 6.59 ± 0.66<sup>b</sup> | n.d. | n.d. |
| 3-Coumaroylquinic acid trans<sup>*</sup> | n.d. | n.d. | 4.15 ± 0.10<sup>a</sup> | 3.84 ± 0.40<sup>b</sup> | n.d. | n.d. |
| 4-Coumaroylquinic acid cis<sup>*</sup> | n.d. | n.d. | 12.50 ± 0.61<sup>a</sup> | 8.47 ± 0.66<sup>b</sup> | n.d. | n.d. |
| 4-Coumaroylquinic acid trans<sup>*</sup> | n.d. | n.d. | 21.46 ± 0.55<sup>a</sup> | 17.62 ± 1.41<sup>b</sup> | n.d. | n.d. |
| Mono-deoxyclovamide (Caffeoyl-DOPA / Coumaroyltyrosine) isomer | 3.54 ± 0.01<sup>a</sup> | 1.78 ± 0.11<sup>b</sup> | 2.93 ± 0.24<sup>c</sup> | 1.38 ± 0.20<sup>d</sup> | 3.05 ± 0.01<sup>e</sup> | 1.47 ± 0.03<sup>f</sup> |
| Mono-deoxyclovamide (Caffeoyl-DOPA / Coumaroyltyrosine) isomer | 17.87 ± 0.12<sup>a</sup> | 9.06 ± 0.78<sup>b</sup> | 13.72 ± 0.61<sup>c</sup> | 9.16 ± 0.57<sup>d</sup> | 12.87 ± 0.79<sup>e</sup> | 9.12 ± 0.21<sup>f</sup> |
|                  |   |   |   |   |   |
|------------------|---|---|---|---|---|
| 5-Caffeoylquinic acid |   |   |   |   |   |
| tyrosine isomer   |   |   |   |   |   |
|                  |   |   |   |   |   |
| 3-Caffeoylquinic acid |   |   |   |   |   |
| tyrosine isomer   |   |   |   |   |   |
|                  |   |   |   |   |   |
| Clovamide (caffeoyl-tyrosine) isomer |   |   |   |   |   |
|                  |   |   |   |   |   |
| Clovamide (caffeoyl-tyrosine) isomer |   |   |   |   |   |

<LOQ means the compound was detected but it was below the limit of quantification; n.d. means not detected

* mean the compounds were detected only in green tea dark chocolate.

Different superscript letters within the same row indicate that the values are significantly different (P<0.05).

Hydroxycinnamic acids were quantified as coumaric acid or ferulic acid equivalent.

Data from chemical extraction were from Martini et al. (2018).
Table 3. Quantitative results (μmol/100 g of chocolate) for flavonols identified in dark chocolate after chemical extraction and after gastro-intestinal digestion. Values represent means ± standard deviation of triplicate determination.

| Compound                                      | Dark chocolate 70% Chemical extraction | Dark chocolate 70% After digestion | Sakura green tea dark chocolate 70% Chemical extraction | Sakura green tea dark chocolate 70% After digestion | Turmeric dark chocolate 70% Chemical extraction | Turmeric dark chocolate 70% After digestion |
|-----------------------------------------------|----------------------------------------|-----------------------------------|------------------------------------------------------|---------------------------------------------------|-----------------------------------------------|---------------------------------------------|
| *Flavonols*                                   |                                        |                                   |                                                      |                                                    |                                               |                                             |
| Quercetin                                     | 1.77 ± 0.02a                           | n.d.                              | 2.66 ± 0.05b                                         | < LOQ                                             | 2.25 ± 0.02c                                 | < LOQ                                       |
| Quercetin-3-O-pentoside                       | 5.43 ± 0.34a                           | 0.15 ± 0.01b                      | 4.59 ± 0.23c                                         | 0.29 ± 0.01b                                     | 6.80 ± 0.66d                                 | 0.20 ± 0.01b                                 |
| Kaempferol-7-O-hexoside                       | 0.23 ± 0.01a                           | n.d.                              | 0.39 ± 0.02a                                         | n.d.                                              | 0.25 ± 0.02a                                 | n.d.                                        |
| Kaempferol-3-O-galactoside                    | 0.07 ± 0.01a                           | n.d.                              | 0.46 ± 0.01b                                         | < LOQ                                             | < LOQ                                        | n.d.                                        |
| Kaempferol-3-O-glucoside                      | 0.08 ± 0.01a                           | < LOQ                             | 0.61 ± 0.05b                                         | < LOQ                                             | < LOQ                                        | n.d.                                        |
| Quercetin-3-O-rhamnoside                      | 0.08 ± 0.01a                           | n.d.                              | 0.82 ± 0.03b                                         | < LOQ                                             | < LOQ                                        | n.d.                                        |
| Dihydro-kaempferol-7-O-hexoside               | 0.41 ± 0.02a                           | n.d.                              | 0.34 ± 0.02a                                         | n.d.                                              | 0.38 ± 0.02a                                 | n.d.                                        |
| Quercetin-3-O-galactoside                     | 0.80 ± 0.02a                           | 0.10 ± 0.01b                      | 2.74 ± 0.14c                                         | n.d.                                              | 4.00 ± 0.07d                                 | 0.09 ± 0.01b                                 |
| Quercetin-3-O-glucoside                       | 3.21 ± 0.03a                           | 0.28 ± 0.01b                      | 3.75 ± 0.11a                                         | 0.49 ± 0.03b                                     | 5.00 ± 0.44c                                 | 0.24 ± 0.01b                                 |
| Myricetin-3-O-galattoside                     | 0.49 ± 0.01a                           | n.d.                              | 3.85 ± 0.07a                                         | 0.39 ± 0.01a                                     | < LOQ                                        | n.d.                                        |
| Myricetin-3-O-glucoside                       | 0.75 ± 0.02a                           | n.d.                              | 3.02 ± 0.23b                                         | 0.52 ± 0.03a                                     | < LOQ                                        | n.d.                                        |
| Kaempferol-3-O-rutinoside                     | 0.43 ± 0.01a                           | < LOQ                             | 1.07 ± 0.03a                                         | 0.41 ± 0.01a                                     | < LOQ                                        | n.d.                                        |
| Quercetin-3-O-rutinoside                      | n.d.                                   | n.d.                              | 6.89 ± 0.31a                                         | 1.19 ± 0.08b                                     | n.d.                                        | n.d.                                        |
| Myricetin-3-O-rutinoside                      | n.d.                                   | n.d.                              | 0.95 ± 0.02a                                         | 0.33 ± 0.03a                                     | n.d.                                        | n.d.                                        |
| Myricetin-3-O-[O-galloyl] hexoside             | 0.18 ± 0.01a                           | n.d.                              | 1.02 ± 0.03b                                         | 0.11 ± 0.01a                                     | < LOQ                                        | n.d.                                        |
| Kaempferol-7-O-rhamnoside-3-O-rutinoside       | 0.05 ± 0.01a                           | n.d.                              | 0.15 ± 0.02a                                         | 0.04 ± 0.01a                                     | < LOQ                                        | n.d.                                        |
| Quercetin-7-O-rhamnoside-3-O-rutinoside        | 0.23 ± 0.01a                           | n.d.                              | 0.33 ± 0.03a                                         | 0.16 ± 0.01a                                     | < LOQ                                        | n.d.                                        |
| Kaempferol-7-O-hexoside-3-O-rutinoside isomer  | 0.93 ± 0.01a                           | n.d.                              | 2.08 ± 0.03b                                         | 0.95 ± 0.04a                                     | < LOQ                                        | n.d.                                        |
| Kaempferol-7-O-hexoside-3-O-rutinoside isomer  | 0.72 ± 0.01a                           | n.d.                              | 2.26 ± 0.02b                                         | 1.16 ± 0.06c                                     | < LOQ                                        | n.d.                                        |
| Quercetin-7-O-hexoside-3-O-rutinoside isomer   | 1.84 ± 0.01a                           | n.d.                              | 3.67 ± 0.02b                                         | 1.62 ± 0.13c                                     | < LOQ                                        | n.d.                                        |
| Quercetin-7-O-hexoside-3-O-rutinoside isomer   | 3.43 ± 0.21a                           | n.d.                              | 5.98 ± 0.43b                                         | 2.93 ± 0.16a                                     | < LOQ                                        | n.d.                                        |
| Myricetin-7-O-hexoside-3-O-rutinoside          | 0.20 ± 0.01a                           | n.d.                              | 0.38 ± 0.03a                                         | 0.16 ± 0.01a                                     | < LOQ                                        | n.d.                                        |

< LOQ means the compound was detected but it was below the limit of quantification; n.d. means not detected.
* mean the compounds were detected only in green tea dark chocolate.
Different superscript letters within the same row indicate that the values are significantly different ($P<0.05$).
Flavonols were quantified as quercetin-3-\textit{O}-rutinoside equivalent.
Data from chemical extraction were from Martini et al. (2018).
Table 4. Quantitative results (μmol/100 g of chocolate) for flavones, ellagitannins, curcuminoids and other phenolics identified in dark chocolate after chemical extraction and after gastro-intestinal digestion. Values represent means ± standard deviation of triplicate determination.

| Compound                          | Dark chocolate 70% | Sakura green tea dark chocolate 70% | Turmeric dark chocolate 70% |
|-----------------------------------|--------------------|-------------------------------------|-----------------------------|
|                                   | Chemical extraction | After digestion | Chemical extraction | After digestion | Chemical extraction | After digestion |
| **Other phenolics**               |                    |                      |                            |                      |                            |                |
| Vanillin                          | 8.64 ± 0.32<sup>a</sup> | 2.24 ± 0.19<sup>b</sup> | 2.97 ± 0.18<sup>c</sup> | 2.10 ± 0.12<sup>c</sup> | 3.03 ± 0.13<sup>c</sup> | 1.51 ± 0.08<sup>d</sup> |
| Phloretin-C-hexoside isomer       | 0.25 ± 0.01<sup>a</sup> | < LOQ                  | 0.26 ± 0.01<sup>a</sup> | 0.07 ± 0.01<sup>b</sup> | 1.03 ± 0.05<sup>c</sup> | n.d.           |
| Eriodictyol-7-O-hexoside          | 0.16 ± 0.02<sup>a</sup> | < LOQ                  | 0.31 ± 0.02<sup>b</sup> | 0.58 ± 0.01<sup>c</sup> | 0.87 ± 0.05<sup>d</sup> | n.d.           |
| Cinchonain isomer                 | 6.24 ± 0.69<sup>a</sup> | 0.66 ± 0.02<sup>b</sup> | 3.90 ± 0.03<sup>c</sup> | 0.87 ± 0.06<sup>b</sup> | 7.21 ± 0.10<sup>a</sup> | 0.73 ± 0.01<sup>b</sup> |
| Cinchonain isomer                 | 2.66 ± 0.21<sup>a</sup> | n.d.                  | 2.72 ± 0.07<sup>a</sup> | n.d.                  | 2.59 ± 0.07<sup>a</sup> | n.d.           |
| Naringenin-C-hexoside-7-O-hexoside isomer | 0.38 ± 0.01<sup>a</sup> | < LOQ                  | 1.03 ± 0.03<sup>b</sup> | 0.73 ± 0.01<sup>c</sup> | < LOQ                  | n.d.           |
| Eriodictyol-C-hexoside-7-O-hexoside isomer | 0.25 ± 0.01<sup>a</sup> | n.d.                  | 0.46 ± 0.02<sup>b</sup> | 0.45 ± 0.01<sup>b</sup> | < LOQ                  | n.d.           |
| **Flavones**                      |                    |                      |                            |                      |                            |                |
| Apigenin-C-hexoside isomer        | 0.16 ± 0.02<sup>a</sup> | < LOQ                  | 0.69 ± 0.02<sup>a</sup> | 0.19 ± 0.01<sup>a</sup> | 0.31 ± 0.0<sup>a</sup> | n.d.           |
| Apigenin-C-hexoside-C-pentoside isomer | 1.56 ± 0.02<sup>a</sup> | 0.12 ± 0.01<sup>b</sup> | 3.46 ± 0.11<sup>c</sup> | 0.35 ± 0.02<sup>d</sup> | 1.36 ± 0.05<sup>a</sup> | 0.12 ± 0.01<sup>b</sup> |
| Apigenin-C-hexoside-2''-O-rhamnoside isomer | 2.15 ± 0.52<sup>a</sup> | n.d.                  | 1.46 ± 0.21<sup>b</sup> | 0.30 ± 0.01<sup>c</sup> | 2.80 ± 0.70<sup>a</sup> | n.d.           |
| Apigenin-6,8-di-C-hexoside isomer | 0.28 ± 0.02<sup>a</sup> | n.d.                  | 0.77 ± 0.02<sup>a</sup> | < LOQ                  | 0.38 ± 0.02<sup>a</sup> | 0.35 ± 0.03<sup>a</sup> |
| Apigenin-6,8-di-C-hexoside isomer | 0.33 ± 0.02<sup>a</sup> | 0.29 ± 0.01<sup>a</sup> | 0.89 ± 0.01<sup>b</sup> | 1.00 ± 0.05<sup>b</sup> | 0.54 ± 0.02<sup>c</sup> | n.d.           |
| Apigenin-C-hexoside-2''-O-hexoside isomer | 0.41 ± 0.01<sup>a</sup> | n.d.                  | 0.77 ± 0.04<sup>b</sup> | 0.40 ± 0.01<sup>a</sup> | < LOQ                  | n.d.           |
| **Ellagitannins**                 |                    |                      |                            |                      |                            |                |
| Ellagic acid                      | 185.96 ± 11.85<sup>a</sup> | 11.55 ± 0.05<sup>b</sup> | 176.95 ± 3.38<sup>a</sup> | 10.27 ± 0.74<sup>b</sup> | 167.22 ± 10.93<sup>a</sup> | 9.62 ± 0.88<sup>b</sup> |
| Ellagic acid-galloyl-hexose        | 15.40 ± 0.89<sup>a</sup> | n.d.                  | 36.85 ± 1.42<sup>a</sup> | 4.34 ± 0.14<sup>c</sup> | 13.54 ± 0.50<sup>a</sup> | n.d.           |
| HHDP-galloyl-hexose               | 52.28 ± 3.97<sup>a</sup> | n.d.                  | 81.62 ± 3.34<sup>a</sup> | 18.32 ± 0.56<sup>c</sup> | 46.72 ± 2.28<sup>a</sup> | n.d.           |
| **Curcuminoids**                 |                    |                      |                            |                      |                            |                |
| Bisdemethoxy-curcumin             | n.d.                | n.d.                  | n.d.                       | n.d.                  | 398.45 ± 7.45<sup>a</sup> | 0.81 ± 0.08<sup>b</sup> |
| Demethoxy-curcumin                | n.d.                | n.d.                  | n.d.                       | n.d.                  | 284.97 ± 4.59<sup>a</sup> | 1.48 ± 0.03<sup>b</sup> |
| Curcumin                          | n.d.                | n.d.                  | n.d.                       | n.d.                  | 257.07 ± 1.62<sup>a</sup> | n.d.           |

< LOQ means the compound was detected but it was below the limit of quantification; n.d. means not detected.

** mean the compounds were detected only turmeric dark chocolate.

Different superscript letters within the same row indicate that the values are significantly different (P<0.05).

Flavones were quantified as quercetin-3-O-rutinoside equivalent.

Ellagitannins were quantified as ellagic acid equivalent.

Curcuminoids were quantified as curcumin equivalent.

Data from chemical extraction were from Martini et al. (2018).
Table 5. Quantitative results (μmol/100 g of chocolate) for hydroxybenzoic acids identified in dark chocolate after chemical extraction and after gastro-intestinal digestion. Values represent means ± standard deviation of triplicate determination.

| Compound | Dark chocolate 70% | Sakura green tea dark chocolate 70% | Turmeric dark chocolate 70% |
|----------|---------------------|-------------------------------------|-----------------------------|
|          | Chemical extraction | After digestion                      | Chemical extraction         | After digestion | Chemical extraction | After digestion | Chemical extraction | After digestion |
| Hydroxybenzoic acid isomer | 1.36 ± 0.06<sup>a</sup> | 0.58 ± 0.09<sup>b</sup> | 1.30 ± 0.26<sup>a</sup> | 0.45 ± 0.06<sup>b</sup> | 1.75 ± 0.13<sup>c</sup> | 0.60 ± 0.02<sup>b</sup> |
| Hydroxybenzoic acid isomer | 0.84 ± 0.06<sup>a</sup> | 0.60 ± 0.05<sup>a</sup> | 0.52 ± 0.06<sup>a</sup> | 0.63 ± 0.04<sup>a</sup> | 2.66 ± 0.13<sup>b</sup> | 0.83 ± 0.01<sup>a</sup> |
| Hydroxybenzoic acid isomer | 35.52 ± 0.19<sup>a</sup> | 10.72 ± 0.71<sup>b</sup> | 41.95 ± 0.39<sup>c</sup> | 10.11 ± 0.85<sup>b</sup> | 48.70 ± 3.05<sup>c</sup> | 10.55 ± 0.47<sup>c</sup> |
| Hydroxybenzoic acid isomer | 2.01 ± 0.06<sup>a</sup> | 0.72 ± 0.09<sup>b</sup> | 8.57 ± 0.13<sup>c</sup> | 0.99 ± 0.04<sup>b</sup> | 2.14 ± 0.13<sup>a</sup> | 0.89 ± 0.12<sup>b</sup> |
| Hydroxybenzoic acid isomer | 4.03 ± 0.26<sup>a</sup> | 1.00 ± 0.09<sup>b</sup> | 6.10 ± 0.71<sup>c</sup> | 1.93 ± 0.03<sup>d</sup> | 5.00 ± 0.19<sup>c</sup> | 0.98 ± 0.12<sup>b</sup> |
| Protocatechuic acid | 69.87 ± 1.95<sup>a</sup> | 34.75 ± 2.09<sup>b</sup> | 59.16 ± 0.58<sup>c</sup> | 21.58 ± 0.86<sup>d</sup> | 118.90 ± 6.30<sup>e</sup> | 29.02 ± 1.76<sup>b</sup> |
| Vanillic acid isomer | 2.53 ± 0.2<sup>a</sup> | 2.58 ± 0.11<sup>a</sup> | 2.40 ± 0.06<sup>a</sup> | 2.88 ± 0.04<sup>a</sup> | 2.92 ± 0.13<sup>a</sup> | 3.11 ± 0.11<sup>a</sup> |
| Vanillic acid isomer | 4.87 ± 0.84<sup>a</sup> | 1.01 ± 0.09<sup>b</sup> | 3.57 ± 0.32<sup>a</sup> | 1.25 ± 0.05<sup>a</sup> | 7.27 ± 0.06<sup>c</sup> | 1.14 ± 0.08<sup>b</sup> |
| Gallic acid<sup>*</sup> | n.d. | n.d. | 0.91 ± 0.01<sup>a</sup> | 1.00 ± 0.02<sup>a</sup> | n.d. | n.d. |
| Syringic acid | 0.84 ± 0.13<sup>a</sup> | 0.63 ± 0.01<sup>a</sup> | 1.69 ± 0.19<sup>b</sup> | 0.96 ± 0.07<sup>a</sup> | 0.91 ± 0.13<sup>a</sup> | 0.95 ± 0.03<sup>a</sup> |
| Protocatechuic acid-4-O-hexoside | 0.65 ± 0.06<sup>a</sup> | n.d. | 2.01 ± 0.19<sup>b</sup> | 1.22 ± 0.09<sup>a</sup> | 1.23 ± 0.01<sup>c</sup> | 0.68 ± 0.01<sup>a</sup> |
| Vanillic acid-4-O-hexoside isomer | 1.62 ± 0.06<sup>a</sup> | 1.70 ± 0.07<sup>a</sup> | 1.30 ± 0.13<sup>a</sup> | 1.65 ± 0.20<sup>a</sup> | 1.95 ± 0.06<sup>a</sup> | 2.49 ± 0.18<sup>b</sup> |
| Vanillic acid-4-O-hexoside isomer | 11.49 ± 0.06<sup>a</sup> | 5.95 ± 0.24<sup>b</sup> | 10.84 ± 0.14<sup>a</sup> | 6.02 ± 0.19<sup>a</sup> | 17.08 ± 0.06<sup>c</sup> | 6.68 ± 0.41<sup>b</sup> |
| Vanillic acid-4-O-hexoside isomer | 12.47 ± 0.13<sup>a</sup> | 2.09 ± 0.13<sup>b</sup> | 7.86 ± 0.06<sup>c</sup> | 2.43 ± 0.26<sup>a</sup> | 11.23 ± 0.01<sup>d</sup> | 2.31 ± 0.14<sup>b</sup> |
| Galloyl glucose isomer<sup>*</sup> | n.d. | n.d. | 1.88 ± 0.10<sup>a</sup> | 1.75 ± 0.09<sup>a</sup> | n.d. | n.d. |
| Galloyquinic acid isomer<sup>*</sup> | n.d. | n.d. | 11.95 ± 0.52<sup>a</sup> | < LOQ | n.d. | n.d. |
| Galloyquinic acid isomer<sup>*</sup> | n.d. | n.d. | 13.57 ± 0.32<sup>a</sup> | 18.74 ± 0.98<sup>b</sup> | n.d. | n.d. |
| Syringic acid-4-O-hexoside isomer | 6.10 ± 0.26<sup>a</sup> | < LOQ | 5.26 ± 0.24<sup>a</sup> | < LOQ | 6.95 ± 0.06<sup>b</sup> | n.d. |
| Syringic acid-4-O-hexoside isomer | 4.81 ± 0.13<sup>a</sup> | 4.20 ± 0.27<sup>a</sup> | 4.61 ± 0.26<sup>a</sup> | 4.55 ± 0.28<sup>a</sup> | 6.75 ± 0.71<sup>b</sup> | 4.98 ± 0.24<sup>a</sup> |
| Vanillic acid derivative | 2.01 ± 0.01<sup>a</sup> | 2.71 ± 0.12<sup>b</sup> | 3.18 ± 0.06<sup>c</sup> | 2.38 ± 0.01<sup>b</sup> | 3.64 ± 0.26<sup>c</sup> | 1.97 ± 0.11<sup>a</sup> |

< LOQ means the compound was detected but it was below the limit of quantification; n.d. means not detected.
* mean the compounds were detected only in green tea dark chocolate.

Different superscript letters within the same row indicate that the values are significantly different (P<0.05).
Hydroxybenzoic acids were quantified as protocatechuic acid equivalent.
Data from chemical extraction were from Martini et al. (2018).
Table 6. Quantitative results (μmol/100 g of chocolate) for phenolic compounds grouped by classes identified in dark chocolate after chemical extraction and after gastro-intestinal digestion. Values represent means ± standard deviation of triplicate determination.

| Compound                  | Dark chocolate 70% | Sakura green tea dark chocolate 70% | Turmeric dark chocolate 70% |
|---------------------------|--------------------|-------------------------------------|-----------------------------|
|                           | Chemical extraction| After digestion                      | Chemical extraction         |
| Total flavan-3-ols        | 1732.09 ± 30.97<sup>a</sup> | 252.07 ± 10.34<sup>b</sup> | 2563.56 ± 50.12<sup>c</sup> | 389.99 ± 7.07<sup>d</sup> | 1857.63 ± 31.02<sup>a</sup> | 251.62 ± 10.03<sup>b</sup> |
| Total hydroxycinnamic acids | 921.67 ± 29.28<sup>a</sup> | 379.05 ± 9.94<sup>b</sup> | 899.48 ± 14.55<sup>a</sup> | 385.20 ± 12.00<sup>b</sup> | 893.29 ± 9.91<sup>a</sup> | 401.85 ± 4.79<sup>b</sup> |
| Total flavonols           | 21.33 ± 0.35<sup>a</sup> | 0.53 ± 0.02<sup>b</sup> | 48.00 ± 0.64<sup>c</sup> | 10.75 ± 0.24<sup>d</sup> | 18.70 ± 0.79<sup>e</sup> | 0.53 ± 0.01<sup>b</sup> |
| Total other phenolics     | 18.57 ± 0.35<sup>a</sup> | 2.90 ± 0.19<sup>b</sup> | 11.65 ± 0.19<sup>c</sup> | 4.80 ± 0.14<sup>d</sup> | 13.70 ± 0.16<sup>e</sup> | 2.24 ± 0.08<sup>b</sup> |
| Total flavones            | 4.89 ± 0.53<sup>a</sup> | 0.41 ± 0.01<sup>b</sup> | 8.03 ± 0.24<sup>c</sup> | 2.24 ± 0.05<sup>d</sup> | 5.39 ± 0.71<sup>e</sup> | 0.47 ± 0.01<sup>b</sup> |
| Total ellagitannins       | 253.64 ± 12.53<sup>a,d</sup> | 11.55 ± 0.05<sup>b</sup> | 295.43 ± 14.96<sup>a</sup> | 32.93 ± 0.94<sup>c</sup> | 227.48 ± 21.17<sup>d</sup> | 9.62 ± 0.88<sup>a</sup> |
| Total hydroxybenzoic acids | 161.04 ± 2.20<sup>a</sup> | 70.04 ± 2.25<sup>b</sup> | 188.64 ± 0.19<sup>c</sup> | 80.51 ± 1.64<sup>d</sup> | 239.09 ± 7.05<sup>e</sup> | 67.19 ± 1.91<sup>b</sup> |
| Total curcuminoids        | n.d.                | n.d.                               | n.d.                         | n.d.                         | 940.48 ± 8.90<sup>a</sup> | 2.29 ± 0.09<sup>b</sup> |
| Total phenolic compounds  | 3113.22 ± 44.48<sup>a</sup> | 716.55 ± 14.52<sup>b</sup> | 3954.78 ± 52.43<sup>c</sup> | 906.45 ± 14.06<sup>d</sup> | 4195.76 ± 36.26<sup>e</sup> | 735.81 ± 11.31<sup>b</sup> |

Different superscript letters within the same row indicate that the values are significantly different ($P<0.05$).  
Data from chemical extraction were from Martini et al. (2018).
Table 7. Mass spectral and quantitative data of newly formed phenolic compounds identified in different dark chocolates after in vitro gastro-intestinal digestion. DC identify dark chocolate; GTDC identify dark chocolate enriched with Sakura green tea leaves; TDC identify dark chocolate enriched with turmeric powder. Data are expressed as μmol/100 g of chocolate.

| Compounds                   | Molecular formula | Exp [M-H] | Calc [M-H] | ppm    | Fragment ions                  | DC        | GTDC      | TD         |
|-----------------------------|-------------------|-----------|------------|--------|--------------------------------|-----------|-----------|------------|
| 4-Caffeoylquinic acid<sup>a</sup> | C<sub>16</sub>H<sub>18</sub>O<sub>9</sub> | 353.0951  | 353.0950   | -0.05  | 179.0433, 173.0049            | 1.10 ± 0.09 | 1.04 ± 0.02 | 1.20 ± 0.04 |
| Trihydroxybenzene<sup>b</sup>  | C<sub>6</sub>H<sub>6</sub>O<sub>3</sub>  | 125.0311  | 125.0317   | 4.71   | 81.0290                        | 0.19 ± 0.01 | 0.45 ± 0.03 | 0.21 ± 0.02 |
| Theasinensin isomer<sup>c</sup>  | C<sub>30</sub>H<sub>26</sub>O<sub>14</sub> | 609.1335  | 609.1322   | -2.04  | 471.0657, 453.0536, 427.0777, 333.0453, 167.0299 | n.d.      | 0.73 ± 0.04 | n.d.       |
| Epigallocatechin dimer isomers<sup>d</sup> (P2-analogue)<sup>f</sup> | C<sub>29</sub>H<sub>24</sub>O<sub>13</sub> | 579.1198  | 579.1216   | 3.26   | 405.0840, 167.0453, 125.0292  | n.d.      | <LOQ      | n.d.       |

<LOQ means the compound was detected but it was below the limit of quantification.
n.d. means not detected
<sup>a</sup>quantified as coumaric acid equivalent
<sup>b</sup>quantified as protocatechuic acid equivalent
<sup>c</sup>quantified as epicatechin equivalent
Table 8. Phenolic compounds identified in the cell media after 24 h of incubation with Caco-2 and SW480 of dark chocolate phenolic-rich fraction extracted at the end of the in vitro digestion. Data are expressed as μmol/100 g of chocolate.

| Compounds                        | [M-H] | Fragment ions | Caco-2     | SW480     |
|----------------------------------|-------|---------------|------------|-----------|
| Catechin                         | 289   | 245           | < LOQ      | < LOQ     |
| Epicatechin                      | 289   | 245           | < LOQ      | < LOQ     |
| Methyl-(epi)catechin             | 303   | 288           | < LOQ      | < LOQ     |
| Di-methyl-(epi)catechin          | 317   | 287           | < LOQ      | n.d.      |
| Ferulic acid<sup>a</sup>         | 193   | 178           | 34.91 ± 1.67 | 9.87 ± 0.27 |
| Feruloyl-aspartate<sup>a</sup>   | 308   | 290, 264, 246, 220 | < LOQ | 1.69 ± 0.02 |
| Dihydro-ferulic acid<sup>a</sup>| 195   | 177, 136      | 4.25 ± 0.25 | n.d.      |
| Coumaric acid<sup>b</sup>        | 163   | 119           | 86.14 ± 3.19 | 96.80 ± 4.96 |
| Coumaroyl-sulphate               | 243   | 163, 158, 119 | n.d.       | < LOQ     |
| Dihydro-coumaric acid<sup>b</sup> | 165  | 147, 119      | n.d.       | 9.41 ± 0.58 |
| Caffeic acid<sup>c</sup>         | 179   | 135           | n.d.       | 1.00 ± 0.06 |
| Quercetin-glucoside<sup>d</sup>  | 463   | 301, 179, 151 | n.d.       | 0.31 ± 0.01 |
| Quercetin-pentoside<sup>d</sup>  | 433   | 301, 179, 151 | < LOQ      | 0.16 ± 0.01 |
| Quercetin<sup>d</sup>           | 301   | 271, 255      | 0.06 ± 0.01 | n.d.      |
| Methyl-ellagic acid<sup>e</sup>  | 315   | 257, 229      | n.d.       | 0.40 ± 0.02 |

<LOQ means the compound was detected but it was below the limit of quantification.

n.d. means not detected

<sup>a</sup>quantified as ferulic acid equivalent
<sup>b</sup>quantified as coumaric acid equivalent
<sup>c</sup>quantified as caffeic acid equivalent
<sup>d</sup>quantified as quercetin-3-O-glucoside equivalent
<sup>e</sup>quantified as ellagic acid equivalent