Cocoa Procyanidins with Different Degrees of Polymerization Possess Distinct Activities in Models of Colonic Inflammation

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Abstract

Procyanidins are available in the diet from sources such as cocoa and grapes. Procyanidins are unique in that they are comprised of repeating monomeric units and can exist in various degrees of polymerization. The degree of polymerization plays a role in determining the biological activities of procyanidins. However, generalizations cannot be made regarding the correlation between procyanidin structure and bioactivity, because the size-activity relationship appears to be system-dependent. Our aim was to screen fractions of procyanidins with differing degrees of polymerization *in vitro* for anti-inflammatory activities in models of colonic inflammation. Monomeric, oligomeric, and polymeric cocoa procyanidin fractions were screened using cell models of disrupted membrane integrity and inflammation in human colon cells. High molecular weight polymeric procyanidins were the most effective at preserving membrane integrity and reducing secretion of interleukin-8 in response to inflammatory stimuli. Conversely, oligomeric procyanidins appeared to be the least effective. These results suggest that polymeric cocoa procyanidins may be the most effective for preventing loss of gut barrier function and epithelial inflammation, which are critical steps in the pathogenesis of metabolic endotoxemia, inflammatory bowel disease, and colon cancer. Therefore, further investigations of the potential health-protective benefits of cocoa procyanidins with distinct degrees of polymerization, particularly high molecular weight procyanidins, are warranted.

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Keywords

Cocoa; procyanidins; degree of polymerization; inflammation; colon; permeability

1. INTRODUCTION

Flavan-3-ols, also referred to as flavanols or procyanidins (PCs), are a subclass of flavonoids comprised of monomers [(±)-catechin (C), (−)-epicatechin (EC), etc.], oligomers, and polymers. Flavanols are characterized by their degree of polymerization (DP, the number of monomeric residues in an oligomer or polymer) [1] and the mean DP (mDP), the average DP of all flavanols in a matrix. PCs are widely available in the diet, from sources such as cocoa, grapes, apples, and berries [2]. Examples of flavan-3-ols found in cocoa are shown in Figure 1. PCs have been widely studied for their biological activities related to prevention or amelioration of acute and chronic diseases. DP appears to play a role in determining PC efficacy in models of disease including inflammation [3–7] and cancer [8–14]. Unfortunately, no broad generalizations can be made regarding the correlation between PC structure and bioactivity because the DP-activity relationship appears to be system-dependent. In some cases, activity is directly proportional to DP; in other cases the reverse is true [15]. Moreover, in some systems, there appears to be an "optimum DP", above and below which activity is reduced.

Bioavailability, which is roughly inversely proportional to DP, represents one of the factors further complicating the interpretation of PC biological activity. Reported oral bioavailability is generally <10% for monomers (although up to 55% has been reported for cocoa catechins) [16, 17], much lower for small procyanidins, and essentially 0% for large procyanidins [17–20]. *In vitro* assays that predict a positive DP-activity relationship may not translate to *in vivo* feeding studies, where poor bioavailability may severely limit tissue exposure and subsequent bioactivity of large PCs. Interestingly, however, the relative bioactivity of PCs *in vivo* does not necessarily correspond to their relative bioavailability [21, 22].

In vitro screening assays may be most useful for predicting PC activities in the lumen or epithelium of the gastrointestinal tract, where direct contact with higher concentrations of PCs is possible. Such activities include, but are not limited to, inhibition of luminal and brush border digestive enzymes, modulation of intestinal barrier function and endotoxin uptake, and modulation of intestinal inflammation, proliferation, and apoptosis. These activities are critical mediators of obesity, diabetes, cancer, and inflammatory diseases that can be modulated by PCs [23, 24].

Cocoa is one of the most flavanol-rich food products and contains PCs of a wide range of DP [1, 25–29]. Recently, we successfully fractionated a cocoa PC extract into monomer-, oligomer-, and polymer-rich fractions and reported that the oligomeric fraction appears to provide enhanced protection against diet-induced obesity and type-2 diabetes compared to monomeric or polymeric procyanidins [22]. These data prompted us to screen these fractions of similarly sized cocoa procyanidins *in vitro* for anti-inflammatory activities in models of

colonic inflammation. We hypothesized that distinct cocoa procyanidin fractions (i.e. groups of similar PCs) containing distinct mDPs would exhibit distinct anti-inflammatory activities.

2. MATERIALS AND METHODS

2.1 Cocoa Procyanidin Fractions

A procyanidin-rich cocoa extract (CE) and fractions with distinct flavanol compositions (monomer-, oligomer-, and polymer-rich fractions) were produced from commercially available cocoa powder as described previously [22]. The concentrations and enrichment of specific cocoa procyanidins in each fraction is presented in Supplementary Table 1. The normal-phase HPLC flavanol profiles of CE and cocoa PC fractions are shown in Supplementary Figure 1. Additionally, a cocoa polymer fraction with greater enrichment of high MW PCs (92% by weight of DP7+) was a generous gift from The Hershey Co. (Hershey, PA the fraction was originally prepared for Hershey by Planta Analytica, Danbury, CT). The composition of this fraction is shown in Table 1.

2.2 Normal-phase HPLC Analysis

The PC composition of the DP7+ fraction was evaluated by normal-phase HPLC profiling [22, 30]. Analyses were performed on an Agilent Technologies (Santa Clara, CA) 1260 Infinity HPLC equipped with a solvent degasser, quaternary pump, an autosampler with temperature control, a thermostat column compartment, and a fluorescence detector (FLD). Separations were carried out using a Develosil Diol column (100 Å, 250 × 4.6 mm, 5 μm particle size) equipped with a Luna HILIC guard column (4 × 3.0 mm ID SecurityGuard cartridge and cartridge holder) (both from Phenomenex, Torrance, CA). The column temperature was 35°C. Binary gradient elution employing 2% acetic acid (v/v) in ACN (phase A) and 2% acetic acid (v/v) and 3% ddH$_2$O (v/v) in MeOH (phase B) was performed at a flow rate of 1 mL/min. The gradient was as follows: 93% A at 0 min, 93% A at 3 min, 62.4% A at 60 min, 0.0% A at 63 min, 0.0% A at 70 min, 93.0% A at 76 min, 7.0% B at 0 min, 7.0% B at 3 min, 37.6% B at 60 min, 100.0% B at 63 min, 100.0% B at 70 min, and 7.0% B at 76 min. FLD excitation and emission wavelengths were 230 nm and 321 nm, respectively. The DP7+ fraction was prepared at 10 mg/mL in acetone: water: acetic acid (70:28:2, v/v/v) immediately prior to analysis. Samples were held at 5 °C in the autosampler before injection. Injection volume was 5 μL. Mixtures of authentic standards consisting of monomers (DP 1: C, EC, ECG), PC oligomers (dimers-hexamers), and PC polymers (heptamers-decamers) were prepared and used as a reference for comparison of elution profiles as described previously [22].

2.3 Cell Culture Conditions

Caco-2 and HT-29 human colon cancer cells (American Type Culture Collection, Manassas, VA) were maintained in sub-confluence in Dulbecco's Modification of Eagle's medium (DMEM) or McCoy's 5A medium, respectively. All medium was supplemented with 10% fetal bovine serum, 100 IU/mL penicillin and 100 μg/mL streptomycin at 37°C under 5% CO$_2$ atmosphere. Cells were subcultured by trypsinization.

2.4 Colon Permeability Assay

The ability of cocoa fractions to mitigate colon permeability *in vitro* was examined by measuring the apical to basolateral flux of fluorescein isothiocyanate–dextran (FITC-D) across differentiated Caco-2 cell monolayers as described previously [31]. In brief, Caco-2 cells were seeded in polycarbonate transwell inserts ($0.33cm^2$ area and 0.4 μm pore size, Corning Life Sciences, Tewksbury, MA) and allowed to reach confluence and differentiate for 21 d. Only monolayers with a transepithelial electrical resistance (TEER) of greater than 500 Ω cm^2 were used for experiments [32]. Differentiated monolayers were treated with final concentrations of 100 μg/mL cocoa extract, 100 μg/mL cocoa procyanidin fractions, 10–25 μg/mL DP7+ cocoa fraction, or vehicle only (dimethylsulfoxide, DMSO) 2 h prior to addition of 2% dextran sodium sulfate (DSS, avg. MW = 40,000 Da, MP Biomedicals, Solon, OH) to the media to induce loss of epithelial membrane integrity [33]. Cells were then co-incubated for an additional 48 h. Fluorescein isothiocyanate (FITC)-labeled dextran (MW = 4000 Da, Sigma-Aldrich, St. Louis, MO) was added to the apical compartment at a final concentration of 1 mg/mL, and cells were incubated for 6 h. Basolateral media (50 μL) were removed every 30 min and fluorescence determined using a Fluoroskan Ascent FL fluorescent plate reader (λ_{ex} = 493 nm, λ_{em}= 517 nm, Thermo Scientific, Waltham, MA). The rate of increase in basolateral fluorescence was determined and normalized to the values derived from monolayers not treated with DSS. Representative FITC flux rates in cells are shown in Supplementary Figure 2.

2.5 Colon Inflammation Assay

HT-29 cells (1×10^6/well) were plated in 24-well plates and allowed to reach 80% confluence. Cocoa extract or PC fractions were added to each well at final concentrations of 100 μg/mL, or 10–25 μg/mL for the DP7+ cocoa fraction. After pretreatment for 24 h, cells were stimulated with 5 ng/mL, tumor necrosis factor (TNF)-α (Peprotech, Inc., Rocky Hill, NJ) for 6 h. Interleukin (IL)-8 levels in the medium were determined by ELISA (R&D Systems, Minneapolis, MN).

2.6 Data and Statistical Analysis

Statistical analyses were performed using Prism (v 6.0, GraphPad Software, La Jolla, CA). Data were analyzed by one-way ANOVA with Tukey's HSD post-hoc test to compare all treatment means. Statistical significance was defined as *P* < 0.05.

3. RESULTS

3.1 DP7+ Fraction Composition

The PC profile of the DP7+ fraction was analyzed by normal-phase HPLC, and is shown in Figure 2. This fraction is highly enriched for high MW (late-eluting) PCs compared to low MW (early-eluting) PCs [22]. This fraction is even more highly enrich for high MW PCs compared to the polymer-rich fraction prepared from cocoa extract (see Supplementary Figure 1), which we characterized previously [22].

3.2 Epithelial Permeability Assay

The ability of cocoa PCs to protect intestinal epithelial cells from DSS-induced loss of membrane integrity is shown in Figure 3. DSS significantly increased apical → basolateral flux of FITC-dextran compared to vehicle (DMSO) treated cells. CE and all cocoa PC fractions significantly inhibited DSS-induced loss of barrier function. The polymer-rich fraction possessed the greatest protective activity, followed by the monomer-rich fraction. CE and the oligomer-rich fraction were least protective. The results for CE and the monomer-rich fraction correlate with our previous study, in which we found that these were the least and most effective at reducing serum levels of endotoxin, respectively, in high fat-fed mice [22]. By contrast, the data from the oligomer- and polymer-rich fractions do not correlate well with our previous study.

3.3 Inflammation Assay

The anti-inflammatory activities of cocoa PCs are shown in Figure 4. Stimulation of cells with TNF-α induced a significant increase in IL-8 production compared to unstimulated cells. CE reduced IL-8 secretion to levels that were similar to unstimulated cells. Among the individual cocoa PC fractions, only the polymer-rich fraction significantly reduced IL-8 secretion compared to vehicle. This result is consistent with data showing that polymeric PCs more effectively inhibited NF-κB activation and secretion of pro-inflammatory eicosanoids (PGE$_2$) and cytokines (TNF-α) in endotoxin-stimulated mouse macrophages compared to oligomeric PCs [3]. This also agrees with data showing that only cocoa PCs with DP   4 were able to stimulate secretion of anti-inflammatory IL-4 in human peripheral blood mononuclear cells [6]. In terms of inflammatory-associated cancer, the present result is consistent with *in vitro* studies showing that cytotoxicity and anti-cancer activities of PCs are positively correlated with DP [10–12, 14].

3.4 Epithelial Permeability Assay (DP 7+)

In order to further confirm the impacts of high MW cocoa PCs in these models, a more concentrated extract (92% by weight PCs with DP 7+) was assayed for activity using the same models. Due to the greater enrichment for DP 7-12 compared to the polymer fraction previously assayed, lower concentrations (10–25 μg/mL) were employed for the DP 7+ fraction. The ability of this fraction to protect intestinal epithelial cells from DSS-induced loss of membrane integrity is shown in Figure 5. Similar to the previous experiment, the DP 7+ enriched fraction inhibited the effects of DSS on membrane integrity in a dose dependent-fashion (10 μg/mL partly inhibited the effects of DSS, while 25 μg/mL completely blocked the effects of DSS).

3.5 Inflammation Assay (DP 7+)

The anti-inflammatory activities of the DP 7+ fraction are shown in Figure 6. The DP 7+ enriched fraction blunted increases in IL-8 secretion induced by TNF-α stimulation. The effects of the DP 7+ fraction were dose-dependent: 10 μg/mL did not significantly decrease IL-8 secretion compared to vehicle, while 25 μg/mL significantly decreased IL-8 secretion compared to vehicle control but not to the level of IL-8 in unstimulated cells. It should be noted that the basal (unstimulated) and stimulated vehicle control levels of IL-8 in the cells

were very different between the first and second experiments (25 vs. 0.1 ng/mL for unstimulated, 120 vs. 45 ng/mL for stimulated vehicle control, Figure 4 vs. Figure 6). These differences may be due to differences in cell passage number and/or degradation of the TNF-α stock used for stimulation. Nevertheless, TNF-α induced a significant increase in IL-8 in both experiments, which was inhibited to varying degrees by the cocoa extract or cocoa fractions.

4. DISCUSSION

The results of the present study suggest that high MW polymeric cocoa PCs may be the most effective for preventing loss of gut barrier function and epithelial inflammation, which are critical steps in the pathogenesis of metabolic endotoxemia, inflammatory bowel disease, and colon cancer. Previous studies have shown that cocoa possesses anti-cancer and anti-inflammatory activities in the colon [34–37], but the present data indicate that the polymers are the most effective of the PC components of cocoa. These activities may be particularly translatable to *in vivo* situations, as they are epithelial activities that do not require systemic bioavailability for activity. Therefore, polymeric PCs may effectively modulate these key mechanistic targets, resulting in improved disease prevention or reduced disease severity. This may imply that enriching the polymeric PC content of cocoa (by altering horticultural practices, fermentation, drying, roasting, processing, etc.) or developing specific PC polymer-rich matrices may result in products with enhanced anti-inflammatory activities. Additional research is required in appropriate animal models of metabolic endotoxemia (high-fat feeding), colon inflammation (DSS-induced inflammation, or bacterial colitis in IL-10 null mouse models), and colon cancer [azoxymethane (AOM) or AOM+DSS models] in order to further elucidate the potential activities of polymeric PCs. However, such *in vivo* studies are difficult due to the time and cost associated with isolating sufficient quantities of individual PCs or purified PC fractions to conduct lengthy animal studies [22]. Efficient fractionation or other strategies are thus needed to facilitate these studies as well as translation to human clinical work.

Interestingly, although cocoa oligomers appeared to be the least effective at protecting epithelial membrane integrity and preventing inflammation in the present study, this fraction was the most effective at preventing weight gain and development of glucose intolerance and insulin resistance in mice fed a high fat diet in our previous study [22]. This highlights the fact that the activities of PCs, and the relationship between DP and activity, are highly context-dependent. Therefore, interventions using custom PC profiles to prevent or ameliorate chronic diseases should be carefully tailored to match composition to the desired activity. Nevertheless, this study and our previous work [38, 39] highlight the potential bioactivities of larger PCs, which have received little attention (compared to the monomeric and dimeric PCs) due to complexities associated with their qualitative and quantitative analysis, as well as scarcity of authentic standards. This work highlights the need for novel approaches to elucidate the activities of these complex compounds. Future work is needed to isolate, purify, characterize and test purified individual compounds within the high MW polymeric fraction, although this approach is difficult with existing technologies.

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Supplementary Material

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Acknowledgments

Abbreviations

C	(±)-catechin
EC	(−)-epicatechin
AOM	azoxymethane
CE	cocoa extract
DP	degree of polymerization
DSS	dextran sodium sulfate
DMSO	dimethylsulfoxide
FITC	fluorescein isothiocyanate
LU	fluorescence units
IL	interleukin
mDP	mean degree of polymerization
M	monomer-rich fraction
O	oligomer-rich fraction
P	polymer-rich fraction
PCs	procyanidins
TNF	tumor necrosis factor

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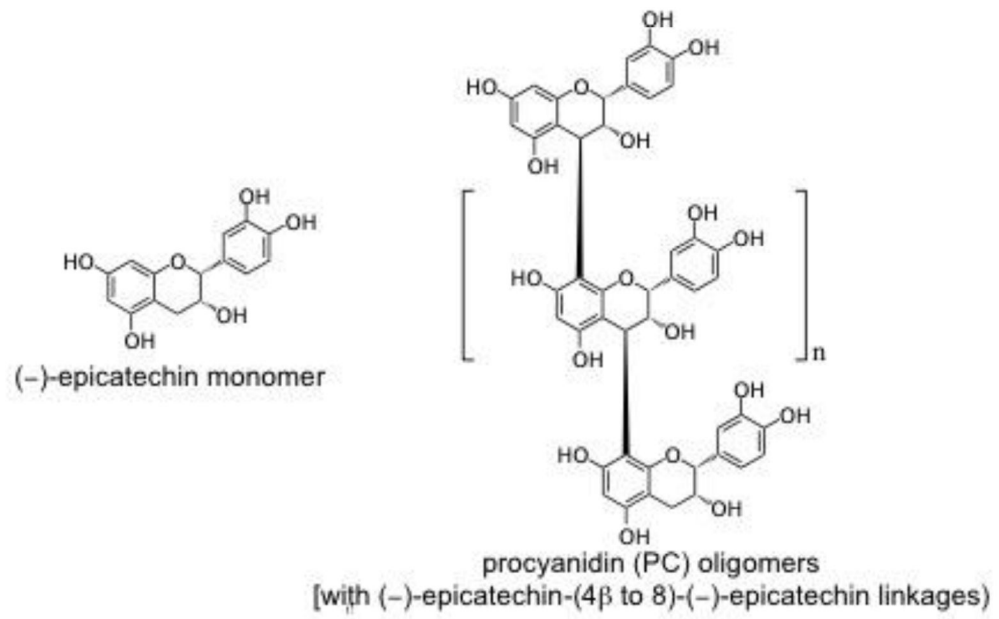
Figure 1.
Structures of selected representative flavan-3-ols found in cocoa: (−)-epicatechin monomer and procyanidin oligomers comprised of (−)-epicatechin monomers linked (4β → 8).
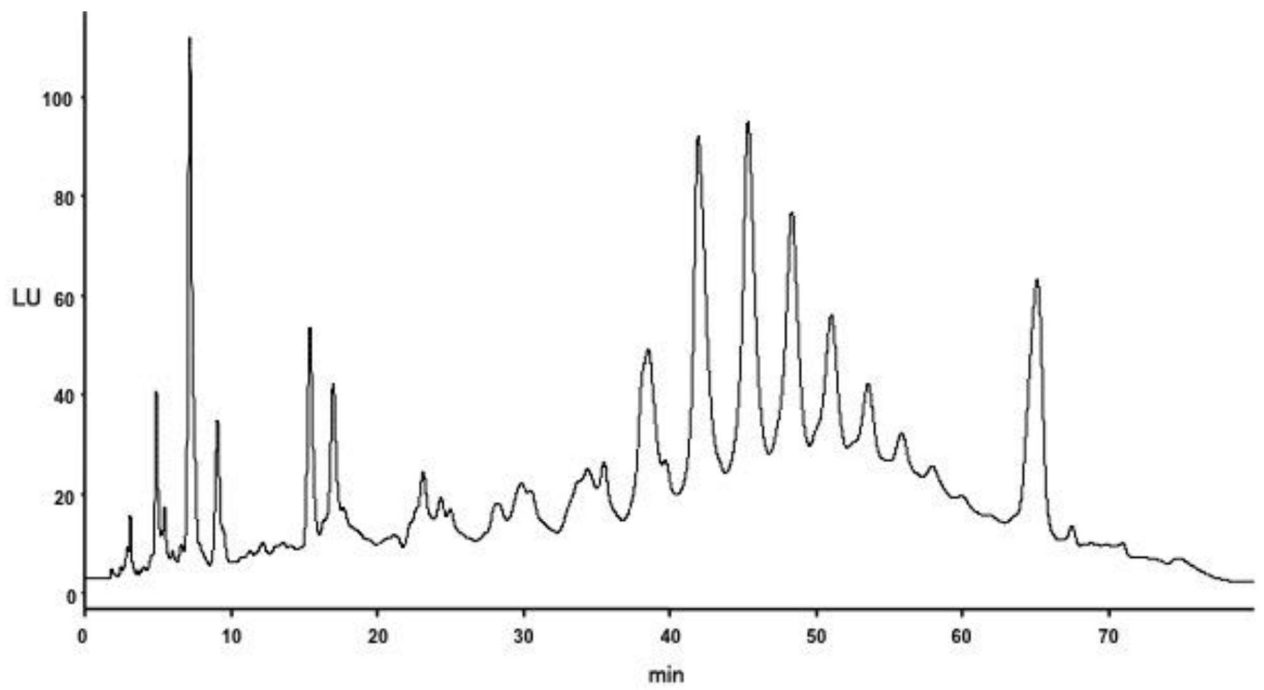
Figure 2.
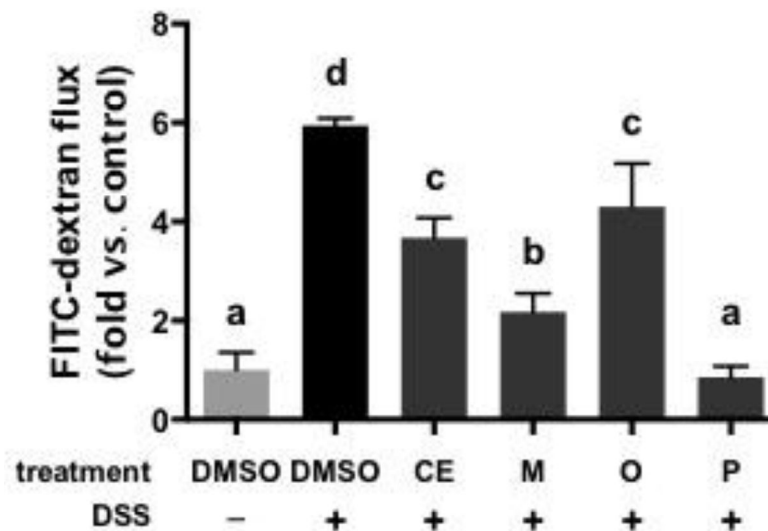Normal-phase HPLC chromatogram (with fluorescence detection) of a DP7+ cocoa PC
fraction.

Figure 3.
Impact of cocoa extract (CE) and cocoa procyanidin fractions (monomer-, oligomer- and polymer-rich fractions: M, O, and P, respectively) (100 µg/mL) on the barrier integrity of differentiated Caco-2 cells exposed to dextran sodium sulfate (DSS), as measured by transmembrane permeability to FITC-dextran. Data represent the mean of two independent experiments ($n = 3$ per experiment) ± SEM. Different superscript letters indicate $P < 0.05$ by one way ANOVA with Tukey's HSD post-test.
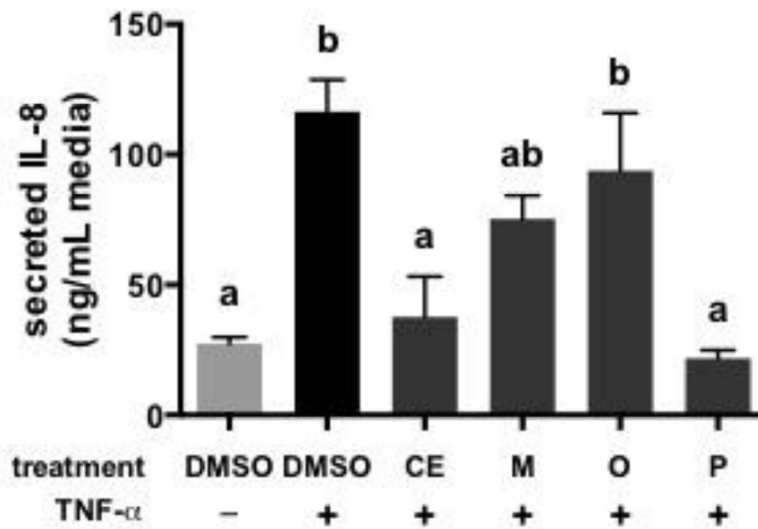
Figure 4.
Impact of cocoa extract (CE) and cocoa procyanidin fractions (monomer-, oligomer- and polymer-rich fractions: M, O, and P, respectively) on the secretion of IL-8 by HT-29 human colon cancer cells stimulated with TNF-α. Data represent the mean of two independent experiments ($n = 2$ per experiment) ± SEM. Different superscript letters indicate $P < 0.05$ by one-way ANOVA with Tukey's HSD post-test.
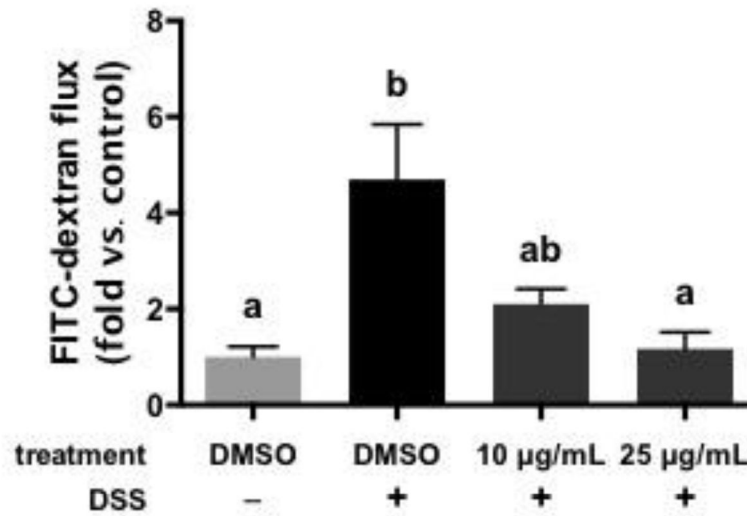
Figure 5.
Impact of a high molecular weight polymeric cocoa procyanidin extract (DP 7+, 10 or 25 µg/mL) on the barrier integrity of differentiated Caco-2 cells exposed to dextran sodium sulfate (DSS), as measured by transmembrane permeability to FITC-dextran. Data represent the mean ± SEM ($n = 3$). Different superscript letters indicate $P < 0.05$ by one way ANOVA with Tukey's HSD post-test.
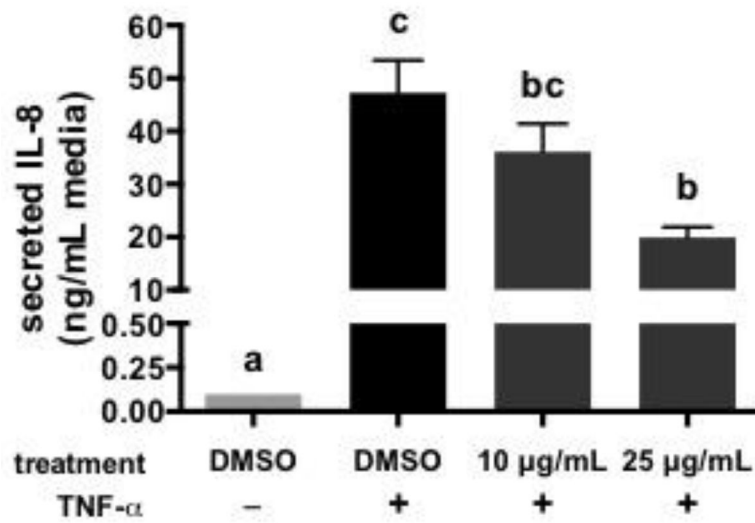
Figure 6.
Impact of a high molecular weight polymeric cocoa procyanidin extract (DP 7+, 10 or 25 µg/mL) on the secretion of IL-8 by HT-29 human colon cancer cells stimulated with TNF-α. Data represent the mean ± SEM ($n = 3$). Different superscript letters indicate $P < 0.05$ by one-way ANOVA with Tukey's HSD post-test.

Table 1

Composition of a cocoa extract fraction enriched for high molecular weight polymeric procyanidins (DP 7+)

DP	% (w/w)
7	21
8	20
9	20
10	15
11	9
12	7
other	8