

Article

The Impact of Sea Buckthorn (*Hippophae rhamnoides* L.) and Cornelian Cherry (*Cornus mas* L.) Plant Extracts on the Physiology of Gastrointestinal Tract Cell In Vitro Model in the Context of Metabolic Diseases

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Abstract: The aim of this study was to evaluate the impact of ethanol extracts from sea buckthorn and Cornelian cherry fruits and leaves on physiology of gastrointestinal tract cells. We used three cell lines relevant to the types of cells, which are exposed to bioactive compounds after oral administration, namely intestinal absorptive cells (Caco-2/HT-29 MTX model), hepatocytes (HepG2 cells) and immunocompetent cells (RAW 264.7 and P388D1 monocytes). The contents of antioxidant and bioactive polyphenols, such as cinnamic, caffeic and *p*-coumaric acids, rutin, myricetin, resveratrol, quercetin, apigenin and kaempferol, were assessed in the extracts using HPLC chromatography. The application of the extracts to Caco-2/HT-29-MTX cultures increased enterocyte differentiation markers (alkaline phosphatase and villin1 level) and goblet cell markers (mucins) over a fortnight. The extracts reduced lipid droplet size in hepatocytes challenged with hyperglycaemic glucose concentration, insulin and palmitate. Sea buckthorn leaf, fruit and Cornelian cherry leaf extracts blocked oxidative burst in the PMA-stimulated monocytes, while the sea buckthorn leaf and Cornelian cherry fruit extracts downregulated lipopolysaccharide-induced NO and IL-1 β , respectively. The results indicate that the tested extracts modulate the behaviour of cells in the gastrointestinal tract in a beneficial way, especially regarding lipid accumulation and innate immunity actions.

Keywords: intestinal epithelial cells; hepatocytes; monocytes; phenolic acids; quercetin; rutin; resveratrol; apigenin; myricetin; kaempferol



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1. Introduction

Intestinal mucosa is where dietary phytochemicals are absorbed and where intestinal resident macrophages coordinate immune tolerance to dietary antigens and microbiota, while also providing surveillance and protection against infection. Hepatocytes are the main target of phytochemicals entering the liver through portal veins and are responsible for metabolizing these compounds [1].

Some dietary phytochemicals reach the small intestine in the form of glycosides, which are cleaved by the enterocyte brush border enzymes, such as lactase phlorizin hydrolase (LPH) or cytoplasmic β -glucosidase. These enzymes release biologically active aglycons [2–4]. Such compounds diffuse into the mucus layer and are absorbed through paracellular or transcellular routes. Water, ions, sugars and solutes up to 600 Da in molecular weight are destined to follow the first route, but most phytochemicals undergo the

latter, either through passive diffusion for nonpolar aglycones or carotenoids, or through facilitated transport by transmembrane carriers [5]. Usually, sodium–glucose-linked transporter 1 (SGLT-1) or mono-carboxyl transporter 1 (MCT-1) are engaged in polyphenol aglycone uptake [6,7]. Once phenolic compounds enter the intestinal epithelial cells (IECs), they undergo a broad range of activities, including anti-inflammatory actions through modulation of the signal transduction pathways or through epigenetic regulation [8,9].

After being absorbed in the intestine, phenolic compounds reach the liver through portal circulation. The liver is responsible for the metabolism of a majority of xenobiotics, including phytochemicals and polyphenols, and can also be their main target. For that reason, plant-derived compounds such as silymarin or berberine are used as hepatoprotective agents to promote liver regeneration and help in prophylaxis against chronic liver diseases, including non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) [10].

NAFLD manifests through the accumulation of lipid droplets in hepatocytes and results from an imbalance between de novo lipogenesis in liver, uptake of dietary fats and insufficient mitochondrial fatty acid catabolism and expenditure. Acute lipid accumulation in the liver leads to steatosis, hepatocyte cell death caused by lipotoxicity and inflammation [11]. Risk factors for NAFLD onset include obesity, insulin resistance, type II diabetes and high percentage of consumed calories coming from fats.

Macrophages are the most abundant immune cell population in the gut [12]. Intestinal, lamina propria resident macrophages are also a target for dietary bioactive compounds absorbed in the small intestine. Kupffer cells constitute a special population of macrophages residing in the liver that support hepatocyte functions by taking part in iron recycling, clearing apoptotic cells and tolerance to innocuous dietary antigens [13]. During infection, they are the first line of innate immune defence against pathogens that enter through the gastrointestinal route. They also promote hepatocyte proliferation and liver regeneration after injury [14]. In NAFLD, Kupffer cells respond to hepatocyte damage with enhanced synthesis of proinflammatory cytokines, such as IL-1 β and TNF α . The inflammatory environment further stimulates lipid accumulation in hepatocytes, which creates a vicious cycle of disease progression to NASH [15]. Currently, a “multiple hits” hypothesis is the prevailing explanation for the onset of NAFLD [16,17]. Initial metabolic conditions, such as prolonged excessive energy intake leading to obesity, insulin resistance and a diet rich in fat and fructose, are considered “the first hit”. These factors lead to gut dysbiosis and a prevalence of *Proteobacteria* in the microbiota community, which impair the intestinal mucosa integrity and initiate so-called “leaky gut” problem. A disrupted intestinal barrier in leaky gut does not sufficiently protect against bacterial endotoxins (including lipopolysaccharide, LPS) entering circulation and further reaching liver. The innate immune response of Kupffer cells to LPS, which manifests due to nitric oxide (NO) secretion, oxidative burst (i.e., rapid and intensive reactive oxygen species, ROS release) and pro-inflammatory cytokine release are considered “the second hit” that drives NAFLD progression to NASH [18].

Mononuclear phagocytes, including Kupffer cells, show remarkable plasticity and can acquire a spectrum of phenotypes according to current exposure to regulatory signals from the cellular environment. Lipid uptake and metabolism are intrinsically bound to the monocyte and macrophage polarization process and to the formation of either pro-inflammatory M1 or anti-inflammatory, pro-regenerative M2 phenotype. M1 macrophages, which predominate during acute infection, are also engaged in the pathogenesis of NASH and usually rely on intensive glycolysis, actively synthesize fatty acids and secrete IL-1 β and NO, a product of inducible NO synthase (iNOS). Inflammation-resolving M2 cells carry out oxidative phosphorylation and fatty acid β -oxidation and release IL-10 [19]. Lipid uptake and metabolism in monocytes and macrophages is an important factor that determines progression of cardiovascular diseases. Oxidized LDL particles (oxLDL) are recognized and avidly engulfed by monocytes via scavenger receptors, and the event

of oxLDL phagocytosis leads to activation of the inflammatory response via NF- κ B and MyD88 signalling pathways and subsequent production of IL-1 β [20].

Phenolic compounds representing several classes (flavonols, flavanols, anthocyanins, stilbenoids, etc.) derived from various plant species have been tested as protecting agents in NAFLD models [10,21,22] due to their promising bioactivities in liver cells. Sea buckthorn and Cornelian cherry are traditional utilitarian plants in Europe and Asia, used in the ethnic medicine and cuisine of many countries. Most commonly, the berries are consumed in processed form as fruit jams, jellies, liquors and syrup preserves.

Sea buckthorn (*Hippophae rhamnoides* L., *Elaeagnaceae* family) is a rich source of folate, tocopherols, carotenoids and pantothenic acid, with a very high content of ascorbic acid (over 400 mg/100 g of fresh fruits) [23,24]. Cornelian cherry (*Cornus mas* L., *Cornaceae* family) has gained considerable interest due to its high content of anthocyanins (flavonoids) and iridoids (monoterpenoids) [25], but it is also a great provider of ascorbic acid (41–101 mg/100 g fresh fruits) [26,27]. However, the natural health-promoting potential of sea buckthorn and Cornelian cherry is not their sole advantage. Both these plants have very modest soil requirements, tolerate drought and frost, are resistant to pests and diseases, do not require chemical fertilizer support and are very well suited to ecological recultivation of areas endangered due to erosion [26,28,29]. Sea buckthorn grows well on poor sand and clay soils, easily adapts to salinity and can serve as a pioneer plant in land reclaimed territories, due to its symbiosis with nitrogen-fixating actinobacteria from *Frankia* species, which reside in the root nodules [28,30]. Cornelian cherry is a slow-growing, long-lived shrub or small tree that adapts very well on rocky slopes and is recommended as a streambank protective plant in landscape bioengineering [26,29]. Therefore, both plants are well-suited for wasteland management and offer a valuable option for land reclamation and recultivation of arid areas, in addition to producing crops rich in bioactive compounds. These two advantages make these species ideal for sustainable and profitable non-timber plant production, with beneficial effects on the environment.

In this paper, we aim to expand the current knowledge on phenolic phytochemicals in reference to their impact on the physiology of cells that represent various parts of the gastrointestinal tract. We seek to answer how sea buckthorn and Cornelian cherry extracts, rich in phenolic acid and flavonoids, affect the intestinal epithelial cells, hepatocytes and monocytes. Thus, we characterised the contents of the extracts from leaves and fruits and analysed intestinal differentiation markers, lipid droplet accumulation in metabolically challenged hepatocytes and innate immune response of monocytes. Our results indicate novel and previously undescribed bioactivities of the plant extracts, including promotion of Caco-2/HT-29-MTX differentiation, a decrease in the intracellular lipid droplet size and number of HepG2 cells and the restriction of monocyte oxidative burst.

2. Materials and Methods

2.1. Chemicals

Single-component standards: *trans-p*-coumaric acid ($\geq 98.0\%$, HPLC), rutin trihydrate ($\geq 97.84\%$, reference standard), *trans*-ferulic acid ($\geq 99.4\%$, certified reference material TraceCERT[®]), *trans*-caffeic acid ($\geq 99.3\%$, certified reference material TraceCERT[®]), protocatechuic acid ($\geq 99.8\%$, primary reference standard), myricetin ($\geq 98\%$, analytical standard), resveratrol ($\geq 99.2\%$, certified reference material TraceCERT[®]), quercetin ($\geq 98\%$, HPLC), *trans*-cinnamic acid ($\geq 98\%$, analytical standard), apigenin ($\geq 96.86\%$, primary reference standard), kaempferol ($\geq 93.9\%$, primary reference standard), methanol (HPLC grade), acetonitrile (HPLC grade) and phosphoric acid (ACS grade) were purchased from Merck KGaA (Darmstadt, Germany). Double deionized water (ddH₂O) was treated ($18.2 \text{ M}\Omega \text{ cm}^{-1}$) in a Simplicity 185 purification system (Millipore SAS, Molsheim, France). Single-component standard stock solutions were prepared by dissolving $\sim 5 \text{ mg}$ of each compound in 10 mL of methanol (HPLC grade) and filtered through syringe PVDF filters ($0.45 \mu\text{m}$, 13 mm diameter) (Frisenette ApS, Knebel, Denmark) into the HPLC vials. The calibration standard

solutions were prepared by dilution of stock solutions with methanol (HPLC grade) in the concentration ranges 0.03–0.3 µg.

2.2. RP-HPLC-DAD Analysis of the Selected Polyphenol Compounds

The plant material (fruits and leaves from *Hippophae rhamnoides* L. and *Cornus mas* L.) was collected from the Botanical Garden of the Slovak University of Agriculture in Nitra, Slovak Republic. Samples were prepared by extracting 2 g of dried plant material in 20 mL 80% ethanol (*v/v*) for 4 h using a horizontal shaker Unimax 2010 (Heidolph Instruments, GmbH, Schwabach, Germany). The extracts were filtered through a syringe with PVDF filters (0.45 µm, 13 mm) (Agilent Technologies, Waldbronn, Germany). All studied polyphenol compounds were determined by HPLC-DAD Agilent 1260 (Agilent Technologies, Santa Clara, CA, USA) according to Kulig et al. (2019) [31]. All analyses were conducted on a Cortecs C18 column (4.6 mm × 150 mm × 2.7 µm; Waters, Milford, MA, USA). The mobile phases were 0.1% H₃PO₄ in ddH₂O (*v/v*) (A) and acetonitrile (B). The gradient elution program was as follows: 0–1 min, isocratic elution (90% A, 10% B); 1–5 min, linear gradient (85% A, 15% B); 5–10 min, linear gradient (80% A, 20% B), held until 12 min; 12–15 min, linear gradient (30% A, 70% B). A 3 min post-run was included. The mobile phase flow rate was 0.6 mL/min. The column was maintained at 30 °C, and samples were kept at 4 °C in the autosampler. Detection was performed at 265 nm, 320 nm and 372 nm, with spectra scanned from 210 to 600 nm. Spectral data were collected and processed using the Agilent OpenLab ChemStation software for LC 3D Systems C.01.06 (Agilent Technologies, Santa Clara, CA, USA). Compounds were identified by comparing their retention times and absorbance spectrum profiles with those of known standards.

2.3. Cell Culture Methods

Caco-2 human adenocarcinoma cells (ECACC 86010202, purchased from Sigma-Aldrich, St. Louis, MA, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with a 4.5 g/L D-glucose concentration and GlutaMAX addition (Gibco; ThermoFisher; Waltham, MA, USA) supplemented with 10% (*v/v*) fetal bovine serum (EurX, Gdańsk, Poland) and an antibiotic–antimycotic mixture (streptomycin 100 mg/mL, penicillin 100 U/mL and amphotericin B 250 ng/mL; Biowest, Lakewood Ranch, FL, USA) in standard conditions (37 °C, 5% CO₂, 98% humidity). HT-29-MTX cells were cultured in the same conditions as Caco-2 cells. For the 2D differentiation experiments, Caco-2 and HT-29-MTX cell suspensions were mixed in a 9:1 ratio and placed in the upper compartments of 12-well 0.4 µm PET Transwell inserts. The extracts were added to the upper chamber and the media was changed every second day. For spheroid culture, a mixture of Caco-2/HT-29-MTX (9:1 ratio, 40,000 cells/200 µL of medium with 1% of Matrigel® Corning, NY, USA) was placed on a Biofloat U-shaped plate (Sarstedt, Germany). The spheroids were formed after 24 h and photographed in the microscope every second or third day for 2 weeks. HepG2 human hepatocellular carcinoma cells (ATCC HB-8065, batch #70039681) were cultured in DMEM medium with a low glucose concentration (1.0 g/L, Gibco; Thermo Fisher; Waltham, MA, USA). Raw 264.7 and P388D1 murine monocytic leukemia cells were grown in RPMI 1640 medium with 10% FBS and the same supplements as for Caco-2 cells.

2.4. Cell Proliferation Assays

The cells were seeded on the 96-well plate (20,000 cells/well) and allowed to attach. Extracts were added to each well in the range of 1–50 µg/mL and the appropriate amount of solvent (80% ethanol diluted in the cell culture media) was used as the control treatment. After a 48 h incubation period, the cell number in each well was determined either by adding MTS reagent (CellTiter 96® Aqueous One, Promega, Walldorf, Germany), incubation for 1 h in 37 °C and the measuring absorbance at 490 nm or by cell fixation in 3.7% buffered formaldehyde, 0.5% crystal violet staining and measuring absorbance at 540 nm.

2.5. Evaluation of Epithelial Cell Differentiation Markers

2.5.1. TEER

After the Caco-2/HT-29-MTX monolayers on the inserts reached confluence, epithelial integrity was assessed by measuring transepithelial electric resistance (TEER) with an EVOM electrode (World Precision Instruments, Sarasota, FL, USA) on the indicated time points.

2.5.2. Actin Cytoskeleton Staining

To visualize actin filaments in the brush border formed in the 3D Caco-2/HT-29-MTX cultures, spheroids were fixed in 3.7% buffered formaldehyde for 15 min, then stained with phalloidin–Texas Red (Molecular Probes, Eugene, OR, USA) 1:200 diluted in PBS for 45 min. For the last 5 min, Hoechst 33342 (NucBlue™ reagent, Thermo Scientific, Waltham, MA, USA) was added to counterstain the nuclei. The spheroids were mounted on the microscopic slides and observed using a fluorescent microscope (ZEN AxioObserver, Zeiss, Oberkochen, Germany).

2.5.3. Intestinal Alkaline Phosphatase (ALPI) Activity and Staining

ALPI enzymatic activity assay was performed on live differentiated Caco-2/HT-29-MTX cultures on inserts, according to the previously published procedure [32] using p-nitrophenol phosphate (p-NPP) as a substrate. The released p-NP was measured spectrophotometrically at 400 nm at several time points and the initial rate (v_0) was calculated as ($\mu\text{mols of p-NP}/(\text{min} \times \text{mg of protein})$). The presence of ALPI in the microscopic 2D and 3D (spheroid) cultures were demonstrated through incubation with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT substrate, Promega, Germany) for 60 min at room temperature. Subsequently, the microscopic slides were prepared and observed in the microscope. The quantification of staining was performed with ImageJ Fiji software (<https://imagej.net/software/fiji/> (accessed on 30 June 2024)) equipped with an image custom colour deconvolution tool and measurements in the RGB scale. In-gel ALPI activity was also detected after the SDS-PAGE electrophoresis of cell lysates (20 μg protein/well) in non-denaturing conditions. After electrophoresis, the gels were incubated using a BCIP/NBT substrate solution for 30 min at 37 °C, then fixed in 70% ethanol and scanned.

2.5.4. Alcian Blue Staining

Mucins were visualised using 3.7% formaldehyde-fixed 2D cultures on glass slides or spheroids by staining with alcian blue solution. First, the slides were incubated in 3% acetic acid (pH 2.5) for 3 min. This solution was then replaced with 1% alcian blue in acetic acid (pH 2.5) for 30 min. After washing with acetic acid and distilled water the slides were mounted and observed using the microscope. The staining intensity was quantified using ImageJ Fiji software with an image custom colour deconvolution tool and measurements in the RGB scale.

2.6. Evaluation of Intracellular Lipid Accumulation

HepG2 cells were seeded on the glass coverslips in 24-well plate (100,000 cells/well) and left for attachment. The next day the media was changed to serum-free low glucose DMEM for 24 h. On the following day the lipid accumulation was induced by replacing the media with (i) high glucose serum-free DMEM (HG); (ii) HG with 100 nM of insulin (HG + I); (iii) HG with 100 μM palmitate conjugated with BSA (HG + P); (iv) HG with 100 nM insulin and 100 μM palmitate-BSA (HG + I + P). At this stage all extracts (50 $\mu\text{g}/\text{mL}$) were added and the control cells were treated with the appropriately diluted solvent (80% ethanol). After 48 h of incubation, the cells were washed 3 $\times$ in PBS with $\text{Ca}^{2+}/\text{Mg}^{2+}$ and stained with Nile Red (Merck Life Sciences, Kenilworth, NJ, USA) solution (0.5 $\mu\text{g}/\text{mL}$) in PBS with $\text{Ca}^{2+}/\text{Mg}^{2+}$ supplemented with 5 mM of glucose and BSA (0.1%) for 15 min in 37 °C. Hoechst 33342 was then added for 5 min and the cells were fixed in 3.7% formaldehyde

for 15 min at room temperature. The microscopic slides were mounted with ProLong anti-fade mountant (Thermo Scientific, USA). The fluorescent images were analysed using the ImageJ Fiji software. The Nile Red channel images were calibrated to μm , thresholded and converted to binary using the watershedding method. Automated detection of particles (i.e., lipid droplets) larger than $0.2 \mu\text{m}^2$ was performed. Statistical analysis was conducted on the data obtained from each experimental group to compare distribution parameters.

2.7. Oxidised LDL Phagocytosis

Raw 264.7 monocytes were seeded on the glass coverslips in 24-well plates (50,000 cells/well) and left for attachment. The extracts (50 $\mu\text{g}/\text{mL}$) were then added for 24 h preincubation. Next, the cells were stimulated with 100 ng/mL lipopolysaccharide (LPS from *E. coli* O26:B6, Merck Life Sciences, USA) for 30 min. A suspension of oxidised LDL from human plasma (Thermo Scientific, USA, #L34357) in the final concentration of 50 $\mu\text{g}/\text{mL}$ was then added for 90 min. The cells were washed $3 \times$ in PBS with $\text{Ca}^{2+}/\text{Mg}^{2+}$, fixed in 3.7% buffered formaldehyde, incubated in 60% isopropanol for 3 min and stained with Oil Red O solution (Merck Life Sciences, USA) for 5 min. The excess stain was washed with tap water and the microscopic slides were mounted and observed using the microscope. The images were analysed using ImageJ Fiji software equipped with an image RGB colour deconvolution tool and measurements of red channel intensity of individual cells.

2.8. Immunoblotting

Whole-cell extracts were prepared from differentiated Caco-2/HT-29-MTX cultures by cell scraping in radioimmunoprecipitation assay (RIPA) buffer (Cell Signalling Technology, Danvers, MA, USA, #9803) supplemented with phenylmethylsulfonyl fluoride (PMSF), sodium ortho-vanadate (final concentrations 1 mM) and a protease inhibitor cocktail (Cell Signalling Technology, USA, #5871). The protein concentration was determined using a QuantiPro BCA kit (Merck Life Sciences, USA) and the lysate samples (15 μg of protein) were resolved through routine SDS-PAGE electrophoresis. The immunoblotting procedure was carried out as previously described [32], using the following primary antibodies for detection: intestinal alkaline phosphatase (Novus Biologicals, Centennial, CO, USA, #NBP2 37389); villin 1 (Novus Biologicals, #NBP1 85336), ATP citrate lyase (Cell Signalling Technology, #4332), fatty acid synthase (CST, #3180), lipin 1 (CST, #14906), long chain acyl-CoA synthetase 1 (CST, #9189) and long chain acyl-CoA dehydrogenase 9 (CST, #9796), all in a 1:1000 dilution. To confirm equal loading, an anti- β -actin mouse monoclonal antibody (1:4000 dilution, Sigma-Aldrich, Steinheim, Germany, #A2228) was used. The luminescent signal was recorded using the ChemiDocTM system (Bio-Rad, Hercules, CA, USA) and the densitometric analysis of the protein bands was performed using ImageJ Fiji. The studied protein band intensities were divided by the corresponding β -actin band intensity and this ratio was set to 100% for the controls.

2.9. Detection of Nitric Oxide Metabolites and IL-1 β Secretion

P388D1 monocytes were seeded in 96-well plates (70,000 cells/well) and left to attach overnight. The next day, they were starved in serum-free RPMI medium for 24 h in the presence of the extracts. The media were then replaced with RPMI with 1% FBS and LPS (100 ng/mL) as well as the tested extracts. After 24 h the cell culture supernatants were collected and the cells were fixed and stained with crystal violet to determine the cell number in each well. The supernatant samples (50 μL) were mixed with Griess reagents (sulfanilamide and N-1-naphthylethylenediamine dihydrochloride, Promega, Germany), incubated for 10 min in the darkness and the absorbance at 540 nm was measured. A standard curve with NaNO_2 was also performed. For IL-1 β quantification in the cell supernatants, an ELISA kit (RayBiotech, Peachtree Corners, GA, USA, #ELM-IL1b-1) was used and the assay was performed according to the provided instructions.

2.10. Oxidative Burst Measurements

Raw 264.7 cells were seeded on 6-well plates (100,000 cells/well) and incubated in regular RPMI medium supplemented with the tested extracts for 48 h. On the assay day the cells were detached by incubation with PBS w/o Ca^{2+} / Mg^{2+} , centrifuged (1500 rpm, 5 min) and resuspended in Hank's balanced salt solution with 0.1% BSA and 0.1% glucose with 1 mM luminol. After a 5 min incubation at 37 °C, the reaction was started by adding phorbol 12-myristate 13-acetate ester (PMA, 100 ng/mL) and the luminescence was immediately measured with a GloMax 20/20 luminometer (Promega, Madison, WI, USA). The luminescence was measured every 4 min for the next 20 min. After the experiment the cells were centrifuged and lysed in RIPA buffer and the protein concentration was used for data normalization.

2.11. Statistical Analysis

The Shapiro–Wilk test was conducted to check data distribution normality. For multiple comparisons, either a one-way ANOVA and Tukey's post-hoc test or a non-parametric Kruskal–Wallis test were performed. For the lipid droplet size distribution data, the Kolmogorov–Smirnov test was performed to compare each of the experimental group with the control. The statistical significance of the differences was recognized for $p < 0.05$. The Statistica 13.3 PL (Tibco Software, Palo Alto, CA, USA) was used for calculations and the majority of the plots were prepared using the BioRender software (<https://www.biorender.com/> (accessed on 27 August 2024)).

3. Results

3.1. The Polyphenol Composition of the Plant Extracts and Their Impact on Cell Proliferation

The HPLC analysis of the ethanolic extracts obtained from the sea buckthorn and Cornelian cherry tissues (leaves and fruits) revealed that they are a rich source of bioactive polyphenols, but also that the extracts differ considerably in their compositions (Table 1). Sea buckthorn leaves (RL) were particularly abundant in rutin, *trans* cinnamic acid and apigenin, whereas fruits of this plant (RO) contained exceptionally high concentrations of myricetin, resveratrol and kaempferol. Cornelian cherry leaves had the most complex composition and provided 10 out of the 11 analysed polyphenols, being particularly rich in phenolic acids: protocatechuic, *trans* caffeic, *trans* ferulic, *trans* *p*-coumaric as well as quercetin. In contrast, Cornelian cherry fruit extract had the least complex composition, with rutin being present in the highest concentration (Table 1). The extracts were used in the cell culture experiments in the range of concentrations comparable to those achieved in the gastrointestinal tract after oral administration of plant-based dietary supplements or berry consumption, i.e., 1–50 µg/mL. Neither intestinal epithelial cells (Caco-2) nor hepatic-derived cells (HepG2) or immunocompetent monocytic cell lines (P388D1 and Raw 264.7) showed any signs of cytotoxicity in response to the culture in the presence of the extracts (Supplementary Figure S1a–d). In the majority of cases, the extracts did not affect the cell proliferation rate significantly, except for sea buckthorn and Cornelian cherry fruits extracts, which increased the HepG2 cell number, as compared to the control (Supplementary Figure S1b).

3.2. The Plant Extracts Increase the Selected Intestinal Epithelial Differentiation Markers

Next, we aimed to check if prolonged exposure to sea buckthorn and Cornelian cherry extracts is able to modulate the intestinal epithelial differentiation in two separate intestinal mucosa models using a mixture of Caco-2/HT-29-MTX cells at a 9:1 ratio: (i) a 3D spheroid culture in the non-adherent Biofloat U-shaped plate (Sarstedt) and (ii) a 2D confluent culture on the porous polycarbonate inserts. In both models the prolonged culture (2 weeks in the presence of the plant extracts or solvent as a control) enabled Caco-2's differentiation into absorptive enterocyte-like cells, whereas HT-29-MTX acquire goblet cell features, such as mucin secretion.

Table 1. The content of selected polyphenols in the plant tissue extracts ($\mu\text{g/g}$ DW sample; mean $\pm$ SD, $n = 3$). ND—not detected.

Phenolic Compounds	Sea Buckthorn Leaves (RL)	Sea Buckthorn Fruit (RO)	Cornelian Cherry Leaves (DL)	Cornelian Cherry Fruit (DO)
Protocatechuic acid	ND	38.63 ± 0.31^a	436.00 ± 8.44^b	ND
<i>Trans</i> caffeic acid	38.64 ± 4.38^c	12.14 ± 1.03^b	64.16 ± 0.91^d	1.65 ± 1.43^a
<i>Trans p</i> -coumaric acid	7.74 ± 0.06^a	ND	63.09 ± 2.34^b	7.41 ± 0.10^a
<i>Trans</i> ferulic acid	ND	1.09 ± 0.24^a	111.00 ± 4.51^b	2.72 ± 0.82^a
Rutin trihydrate	366.00 ± 16.53^d	86.00 ± 1.14^b	155.59 ± 1.46^c	41.41 ± 2.51^a
Myricetin	4.66 ± 0.24^a	2994.98 ± 26.54^b	3.57 ± 0.06^a	ND
Resveratrol	3.35 ± 0.06^a	27.86 ± 2.90^b	4.70 ± 0.41^a	ND
Quercetin hydrate	4.77 ± 0.03^a	ND	5.42 ± 0.05^b	ND
<i>Trans</i> cinnamic acid	84.65 ± 0.29^c	0.67 ± 0.24^a	4.12 ± 0.03^b	ND
Apigenin	3.67 ± 0.03^b	ND	1.69 ± 0.02^a	ND
Kaempferol	4.42 ± 0.02^a	36.04 ± 2.43^b	ND	ND

The same letters in superscript indicate the lack of statistically significant differences at $p < 0.05$.

The spheroids in all the experimental groups formed the brush border structures (Figure 1a, fluorescent β -actin staining panel) facing the environment (i.e., upside-out spheroid morphology). This indicated that the apical surfaces of Caco-2 cells were polarised, which is an important step in the differentiation process. The presence of the plant extracts during the culture did not significantly affect the spheroid polarisation (Figure 1a) or size (Figure 1b).

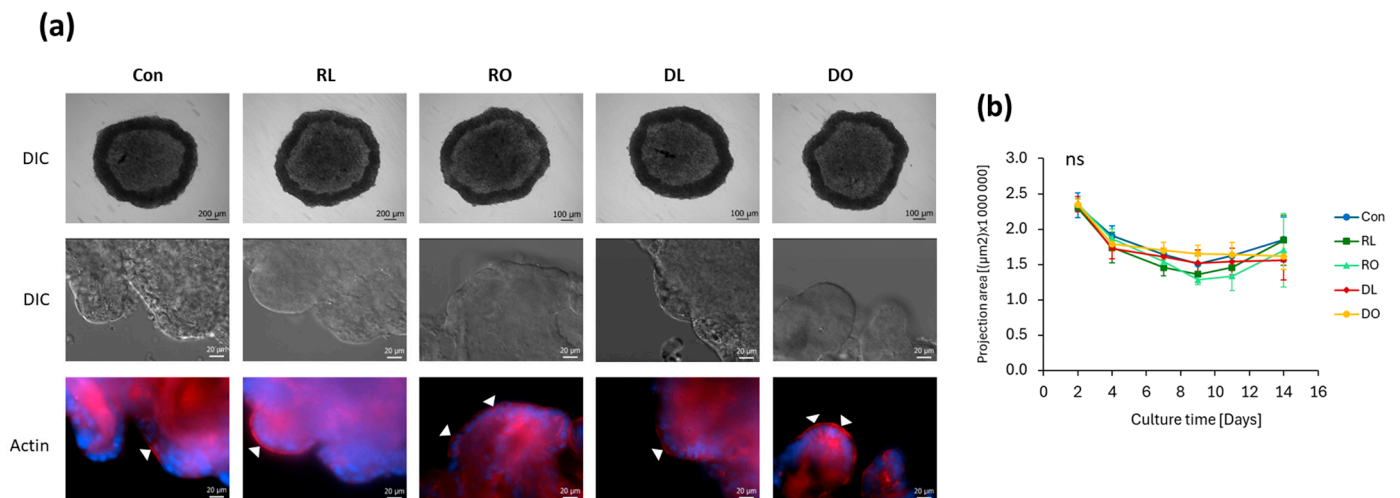


Figure 1. (a) Caco-2/HT-29-MTX spheroid growth in the presence of sea buckthorn leaf extract (RL, $50 \mu\text{g/mL}$) or fruit extract (RO, $50 \mu\text{g/mL}$) and Cornelian cherry leaf (DL, $50 \mu\text{g/mL}$) and fruit (DO, $50 \mu\text{g/mL}$) extracts. Con—cells treated with the appropriate amount of solvent. Phalloidin–Texas Red staining for actin indicates the presence of a brush border in the differentiated Caco-2 cells (arrows). Nuclei are counterstained with Hoechst 33342. Differential interference contrast images (DIC) show the spheroid structure. (b) The projection areas of the spheroids from all the experimental groups do not differ significantly during the culture (mean $\pm$ SD, $n = 5$); ns—differences not significant.

Nevertheless, all the extracts, but particularly sea buckthorn (RO) and Cornelian cherry fruit (DO), enhanced the differentiation markers of goblet cells, as indicated by the darker Alcian blue staining of the mucins (Figure 2a). Similarly, the spheroids that grew in the presence of the extracts demonstrated a stronger activity of intestinal alkaline phosphatase (ALPI), a brush border enzyme regarded as a microvilli marker (Figure 2a). In the two-dimensional cultures, Cornelian cherry leaf extract (DL) significantly promoted differentiation into both enterocyte and goblet cells grown on microscopic slides (Figure 2b–d)

and a similar trend was observed for the sea buckthorn leaf extract (RL), although the differences did not reach statistical significance (Figure 2b–d).

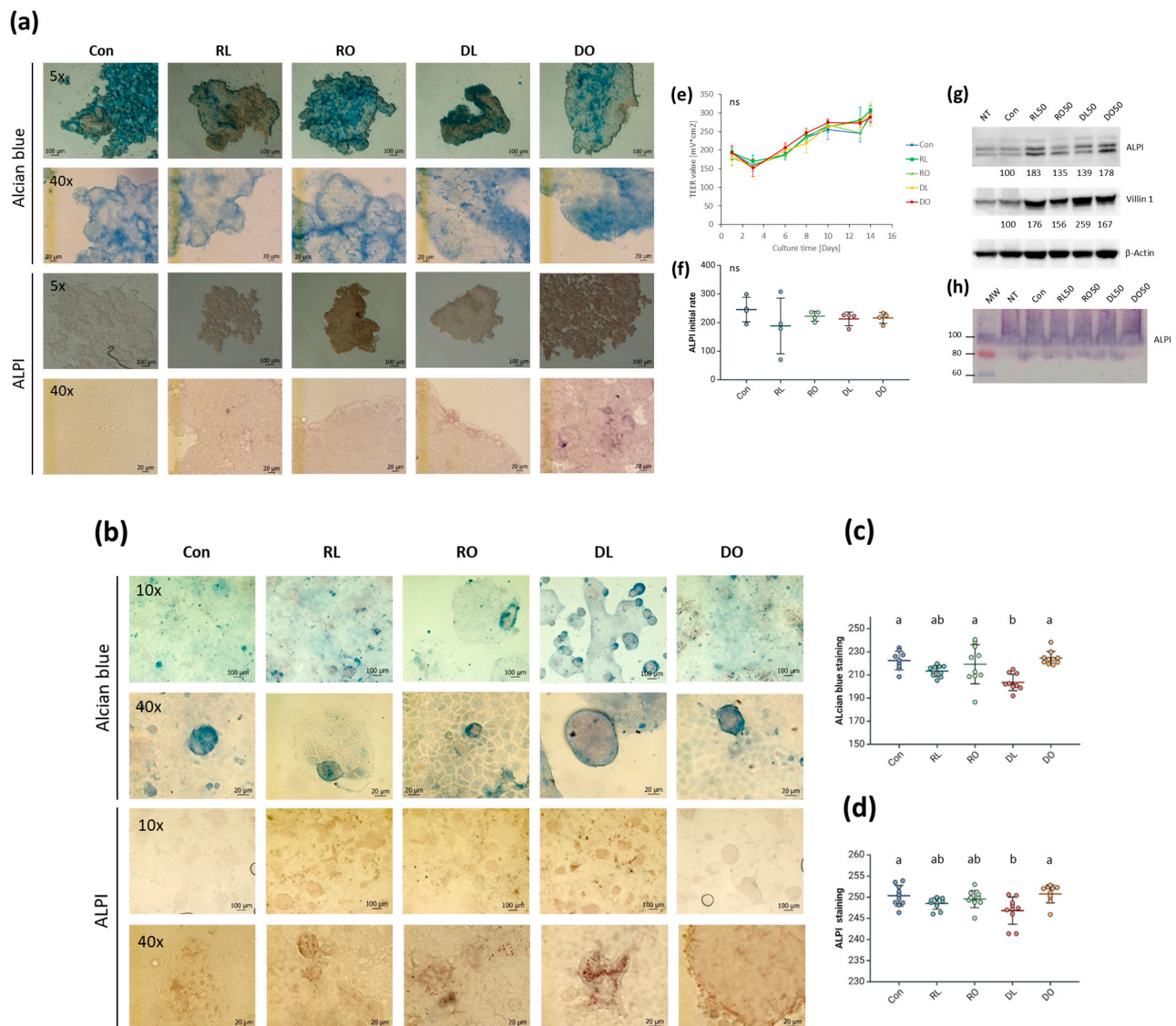


Figure 2. Mucin (alcian blue) and intestinal alkaline phosphatase (ALPI) staining of Caco-2/HT-29-MTX spheroid cultures (a) and 2D cultures on glass slides (b). Quantitative analysis of microscopic images from mucin (c) and ALPI staining (d). The plots are expressed in arbitrary values (mean ± SD) of image analysis software (ImageJ Fiji), the lower the value, the darker staining colour (the same letters represent lack of statistically significant differences at $p < 0.05$). (e) Trans-epithelial electric resistance (TEER, mean ± SD) measured in Caco-2/HT-29-MTX monolayer cultures on inserts (Transwell chambers, BD Biosciences); ns—differences not significant. (f) ALPI enzymatic activity (mean ± SD) in lysates from the differentiated Caco-2/HT-29-MTX cultures, expressed as the initial rate of p-nitrophenol phosphate (p-NPP) hydrolysis (μmol of p-NP/(min × mg protein)); ns—differences not significant. (g) Immunoblotting detection of ALPI and villin-1 in the lysates from the differentiated Caco-2/HT-29-MTX cultures. Probing for β -actin serves as a loading control. The numbers below the protein bands express the band intensity normalised to β -actin in relation to the control (Con = 100%). (h) In-gel detection of ALPI enzymatic activity using NBT/BCIP colour reaction. RL—sea buckthorn extract (50 $\mu\text{g}/\text{mL}$); RO—sea buckthorn fruit extract (50 $\mu\text{g}/\text{mL}$); DL—Cornelian cherry leaf extract (50 $\mu\text{g}/\text{mL}$); DO—Cornelian cherry fruit extract (50 $\mu\text{g}/\text{mL}$); Con—cells treated with the appropriate amount of solvent; NT—non-treated cells. Panels (c,d) created with <https://www.biorender.com/>.

Formation of the impermeant cell barrier with functional tight junctions is a hallmark of well-developed intestinal mucosa. 2D Caco-2/HT-29-MTX cultures on inserts are a widely established model for studying the intestinal epithelial barrier, and its integrity can be assessed by measuring the trans-epithelial electric resistance (TEER). In our experimental setting, the presence of the plant extracts during the 2-week culture did not affect the TEER values (Figure 2e). Nevertheless, all the tested plant extracts increased the protein levels of ALPI and villin 1, a structural protein that binds actin filament bundles, stabilising the brush border structure (Figure 2g). The differences in the ALPI activity in the lysates (Figure 2f,h) were not statistically significant.

3.3. The Plant Extracts Reduce Lipid Accumulation in the Metabolically Challenged Hepatocytes

Next, we checked if the sea buckthorn and Cornelian cherry extracts were able to alter the fatty acid metabolism and lipid droplet accumulation in HepG2 hepatocytes subjected to several conditions that mimic metabolic challenges, namely (i) hyperglycaemia (25 mM glucose in the cell culture medium); (ii) hyperglycaemia with insulin (25 mM glucose, 100 nM insulin); (iii) hyperglycaemia and palmitate load (25 mM glucose, 100 μ M palmitate); and (iv) hyperglycaemia with insulin and palmitate (25 mM glucose, 100 nM insulin, 100 μ M palmitate). All these conditions promoted intracellular lipid storage in HepG2 cells, but the combination of hyperglycaemia and palmitate was particularly effective in the induction of large droplet accumulation (Figure 3a). In order to assess the impact of the plant extracts on the extent of lipid storage, we performed a quantitative image analysis of the slides stained with Nile red, focusing on the size distribution of the intracellular lipid droplets, mean fluorescence in the red channel and average number of lipid droplets per cell in each experimental group.

Lipid droplet size (mean area) distributions in all the groups were strongly asymmetric, with higher representations of small droplets (the minimal size was set to 0.2 μm^2 , which is approximately the resolution limit in fluorescent microscopy) and fewer large droplets. This feature reflects the high values of skewness and kurtosis (Table 2). The largest lipid droplets were observed in control groups with hyperglycaemia with insulin and palmitate or hyperglycaemia with palmitate (Table 2). In the majority of cases, the plant extracts reduced the mean and median value of the lipid droplet area and a statistically significant size reduction was observed in the DL treated groups (HG, HG + Ins, HG + Ins + Palm conditions) and RO treated groups (HG, HG + Palm, HG + Ins + Palm). The RL and DO extracts reduced the lipid droplet size for HG + Palm and HG + Ins + Palm conditions (Table 2). In general, the cells grown the presence of each tested plant extract accumulated fewer lipid droplets on average (Figure 3b).

The presence of well-defined lipid droplets is a characteristic pathogenic manifestation of metabolic diseases such as NAFLD. Apart from the size of individual lipid droplets, the mean intensity of Nile red fluorescence is also an important parameter that reflects overall cytoplasmic lipid storage. Diffused staining indicates a more dynamic intracellular lipid circulation. In two conditions that promote acute lipid accumulation, the significant differences between the groups were revealed: sea buckthorn fruit extract (RL) decreases fluorescence in HG + Palm, which might indicate more efficient lipid catabolism, but the same extract increases fluorescence intensity in HG + Ins + Palm, as compared to the control (Figure 3c). Cornelian cherry leaf (DL) extract significantly decreases fluorescence in HG + Ins + Palm, but fruit extract (DO) in HG + Palm condition shows elevated fluorescence as compared to the control (Figure 3c).

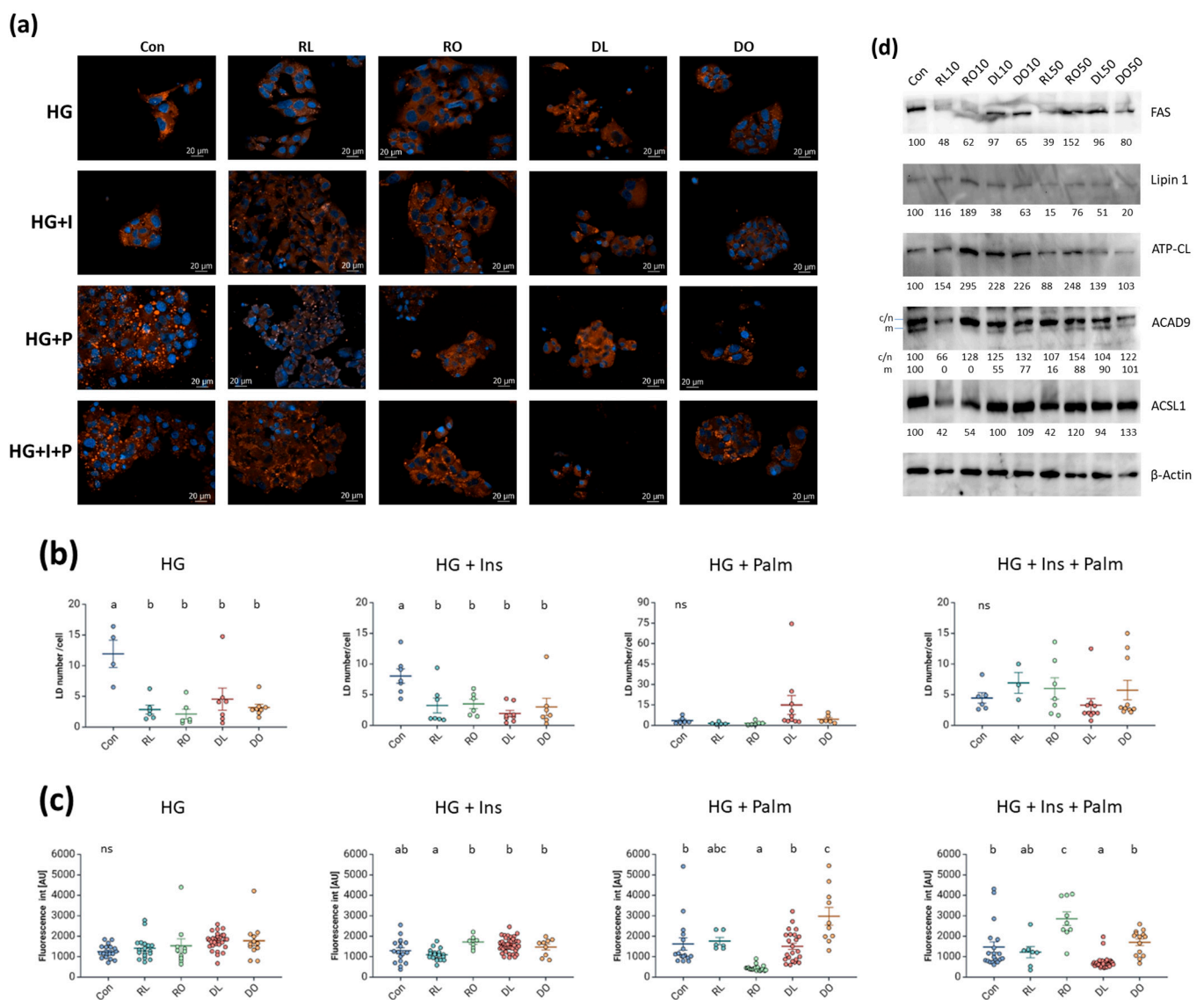


Figure 3. Nile red staining for intracellular lipid storage in HepG2 cells subjected to metabolic stress: 25 mM glucose (HG); 25 mM glucose and 100 nM insulin (HG + I); 25 mM glucose and 100 μ M palmitate (HG + P); 25 mM glucose, 100 nM insulin and 100 μ M palmitate (HG + I + P). (a) Fluorescent staining of lipids (red), nuclei are counterstained with Hoechst 33342 (blue), (b) Average number of lipid droplets per cell (mean $\pm$ SEM). (c) Mean intensity of Nile red fluorescence in arbitrary units (mean $\pm$ SD), the same letters represent lack of statistically significant differences at $p < 0.05$, ns—differences not significant. (d) Immunoblotting detection of enzymes engaged in lipid metabolism in the HepG2 lysates: FAS—fatty acid synthase; ATP-CL—ATP citrate lyase; ACAD9—long chain acyl-CoA dehydrogenase 9; m—mitochondrial, c/n—cytoplasmic/nuclear form; ACSL1—long chain acyl-CoA synthetase 1. Probing for β -actin serves as a loading control. The numbers below the protein bands express the band intensity normalised to β -actin in relation to the control (Con = 100%). RL—sea buckthorn extract (50 μ g/mL); RO—sea buckthorn fruit extract (50 μ g/mL); DL—Cornelian cherry leaf extract (50 μ g/mL); DO—Cornelian cherry fruit extract (50 μ g/mL); Con—cells treated with the appropriate amount of solvent. Panels (b,c) created with <https://www.biorender.com/>.

Table 2. Lipid droplet size distribution descriptive statistics. Lipid droplets from multiple images were counted and analysed using ImageJ Fiji software. RL—sea buckthorn extract (50 µg/mL); RO—sea buckthorn fruit extract (50 µg/mL); DL—Cornelian cherry leaf extract (50 µg/mL); DO—Cornelian cherry fruit extract (50 µg/mL); Con—cells treated with the appropriate amount of solvent.

Treatment	n	Mean Area [µm ²]	Median	Min	Max	Coefficient of Variation	Skewness	Kurtosis
HG								
Con	438	1.92	1.07	0.20	45.27	151.57	8.54	115.41
RL	460	1.61	0.98	0.20	14.46	115.78	2.91	11.67
RO	455	1.47 *	0.83	0.20	19.07	137.66	4.30	25.41
DL	466	1.51 *	0.83	0.20	21.38	141.05	4.26	25.55
DO	487	1.76	1.12	0.20	20.86	115.77	3.76	23.24
HG + Ins								
Con	353	1.75	0.99	0.20	25.31	133.36	4.49	32.75
RL	620	2.00	0.94	0.20	23.28	135.66	3.15	13.49
RO	730	1.81	0.96	0.20	25.75	142.17	4.44	27.05
DL	306	1.62 *	0.74	0.20	62.12	256.54	11.01	150.50
DO	324	1.83	0.94	0.20	30.67	7.62	150.88	48.21
HG + Palm								
Con	1116	2.63	1.33	0.20	49.64	143.52	4.29	31.00
RL	406	2.75 *	1.09	0.20	54.81	171.79	5.20	42.71
RO	116	0.58 *	0.42	0.20	4.63	102.65	3.75	19.17
DL	499	2.85	1.22	0.20	56.09	4.92	172.29	49.31
DO	716	2.34 *	1.08	0.20	27.89	142.80	3.31	14.04
HG + Ins + Palm								
Con	1005	3.05	1.51	0.20	57.96	141.95	4.23	32.54
RL	278	2.41 *	0.99	0.20	27.32	146.92	3.47	16.20
RO	658	2.62 *	1.05	0.20	36.79	148.89	3.49	17.22
DL	118	1.45 *	0.77	0.20	14.49	126.55	3.91	22.42
DO	693	2.69 *	1.12	0.20	48.75	164.89	4.41	27.80

The differences in lipid droplet area (treatment vs. control) were analysed using the Kolmogorov–Smirnov test. * indicates the statistically significant difference vs. control at $p < 0.05$.

Liver plays a central role in intermediary lipid metabolism in both fed and fasted conditions, facilitating storage or release of fats and distribution to peripheral tissues when needed. Therefore, hepatocytes abundantly synthesise enzymes involved in fatty acid anabolism, such as fatty acid synthase (FAS) and ATP-citrate lyase (ATP-CL); in mitochondrial fatty acid oxidation, such as acyl-CoA dehydrogenase 9 (ACAD9); as well as proteins that control or engage in lipid turnover in both anabolic and catabolic directions, such as lipin 1 and long-chain acyl-CoA synthetase 1 (ACSL1). Having observed that sea buckthorn and Cornelian cherry extracts affect lipid accumulation in metabolically challenged hepatocytes, we checked the levels of the mentioned proteins in HepG2 cells treated with the extracts. RL, RO and DO extracts downregulate FAS, whereas they increase ATP-CL (Figure 3d). RO, DL and DO extracts increase the level of the higher molecular weight precursor form of ACAD9, which can also be found in the cytoplasm and nucleus (ACAD9 c/n). On the other hand, they decrease the level of the mitochondrial ACAD9 (m) form (Figure 3d). All the extracts applied in higher concentrations (50 µg/mL) reduce lipin 1 level; the only extract that upregulates this protein is RO at a concentration of 10 µg/mL (Figure 3d). RL extracts in both concentrations decrease ACSL1, whereas the other extracts do not seem to alter this protein expression significantly (Figure 3d). Taken together, the sea buckthorn and Cornelian cherry extracts do have an impact on the enzymes that control lipid metabolism, but their influence is multidirectional.

3.4. The Plant Extracts Do Not Affect oxLDL Phagocytosis, but Modulate Monocyte Innate Response Activities

Bearing in mind this connection between metabolic reprogramming, lipid uptake and inflammatory phenotype, we decided to check how the sea buckthorn and Cornelian cherry extracts influence monocyte oxLDL phagocytosis, lipid metabolizing enzymes as well as IL-1 β and NO production. Preincubation with plant extracts prior to exposure to oxLDL had no influence on phagocytosis (Figure 4a,b). The extracts influenced the levels of enzymes involved in lipid metabolism in quite a diverse way: sea buckthorn leaf extracts (RL 10 and 50 $\mu\text{g}/\text{mL}$) FAS and ATP-CL were modulated in opposite directions and increased FAS was accompanied by decreased ATP-CL and vice versa (Figure 4c). This suggests the existence of a kind of compensatory mechanism. The RL extract in higher concentrations (50 $\mu\text{g}/\text{mL}$) upregulated the mitochondrial ACAD9 level (Figure 4c), which might indicate redirection to fatty acid oxidation and oxidative metabolism in general. The DL extract globally downregulated all the tested enzymes, which implies less active intracellular lipid conversion. In case of the DO extract, the concentration used (10 or 50 $\mu\text{g}/\text{mL}$) seems to determine the direction of the changes in ATP-CL and ACSL1 levels: the lower concentration upregulates ATP-CL and decreases ACSL1, the higher concentration acts in the opposite manner (Figure 4c).

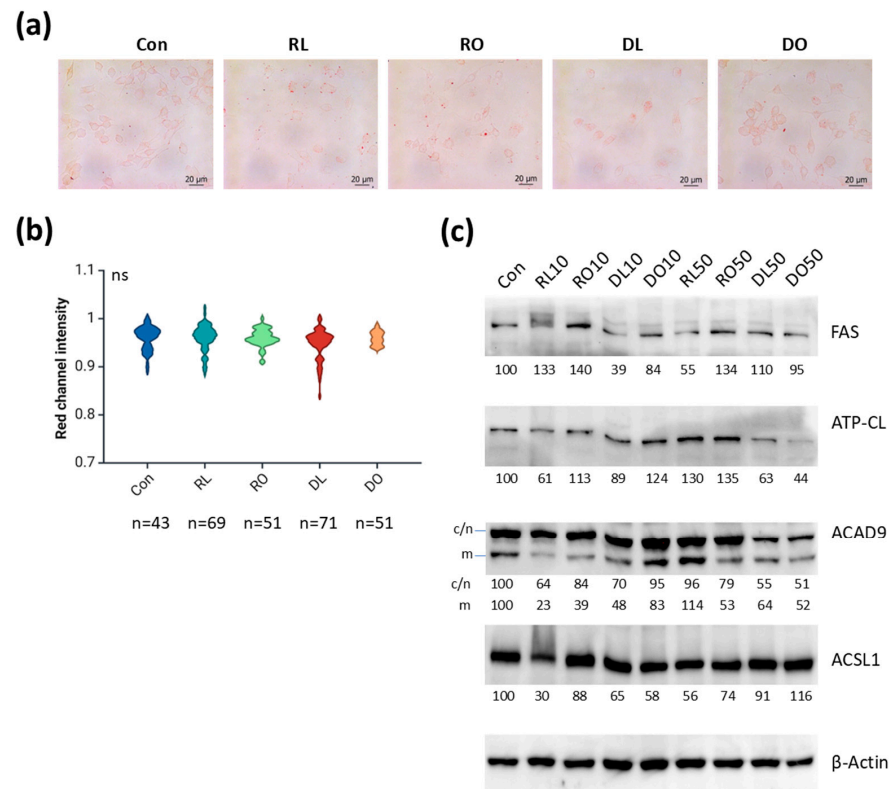


Figure 4. Phagocytosis of oxLDL particles by Raw 264.7 monocytes (oil red O staining, (a)). Quantitative analysis of microscopic images (b). The fiddle plots present the distribution of cell staining intensity using arbitrary values from the ImageJ Fiji software, the lower values are the darker staining colour; differences not significant (ns). (c) Immunoblotting detection of enzymes engaged in lipid metabolism in the Raw 264.7 lysates: FAS—fatty acid synthase; ATP-CL—ATP citrate lyase; ACAD9—long chain acyl-CoA dehydrogenase 9; ACSL1—long chain acyl-CoA synthetase 1. Probing for β -actin serves as a loading control. The numbers below the protein bands express the band intensity normalised to β -actin in relation to the control (Con = 100%). RL—sea buckthorn extract (50 $\mu\text{g}/\text{mL}$); RO—sea buckthorn fruit extract (50 $\mu\text{g}/\text{mL}$); DL—Cornelian cherry leaf extract (50 $\mu\text{g}/\text{mL}$); DO—Cornelian cherry fruit extract (50 $\mu\text{g}/\text{mL}$); Con—cells treated with the appropriate amount of solvent. Panel (b) created with <https://www.biorender.com/>.

Next, we checked if the tested plant extracts were able to alter monocyte innate immune response activities, such as reactive oxygen species production (“oxidative burst”) and NO release, as well as secretion of pro-inflammatory IL-1 β , which exhibit features characteristic of the M1 phenotype. The RL, RO and DL extracts administered at a higher dose of 50 $\mu\text{g}/\text{mL}$ effectively blocked the monocyte oxidative burst (Figure 5a–d). Surprisingly, the DO (50 $\mu\text{g}/\text{mL}$) and DL (10 $\mu\text{g}/\text{mL}$) extracts stimulated reactive oxygen species release (Figure 5c,d). The RL and DO extracts applied in 50 $\mu\text{g}/\text{mL}$ concentrations significantly decreased NO and IL-1 β secretion, respectively, as compared to the control (Figure 5e,f).

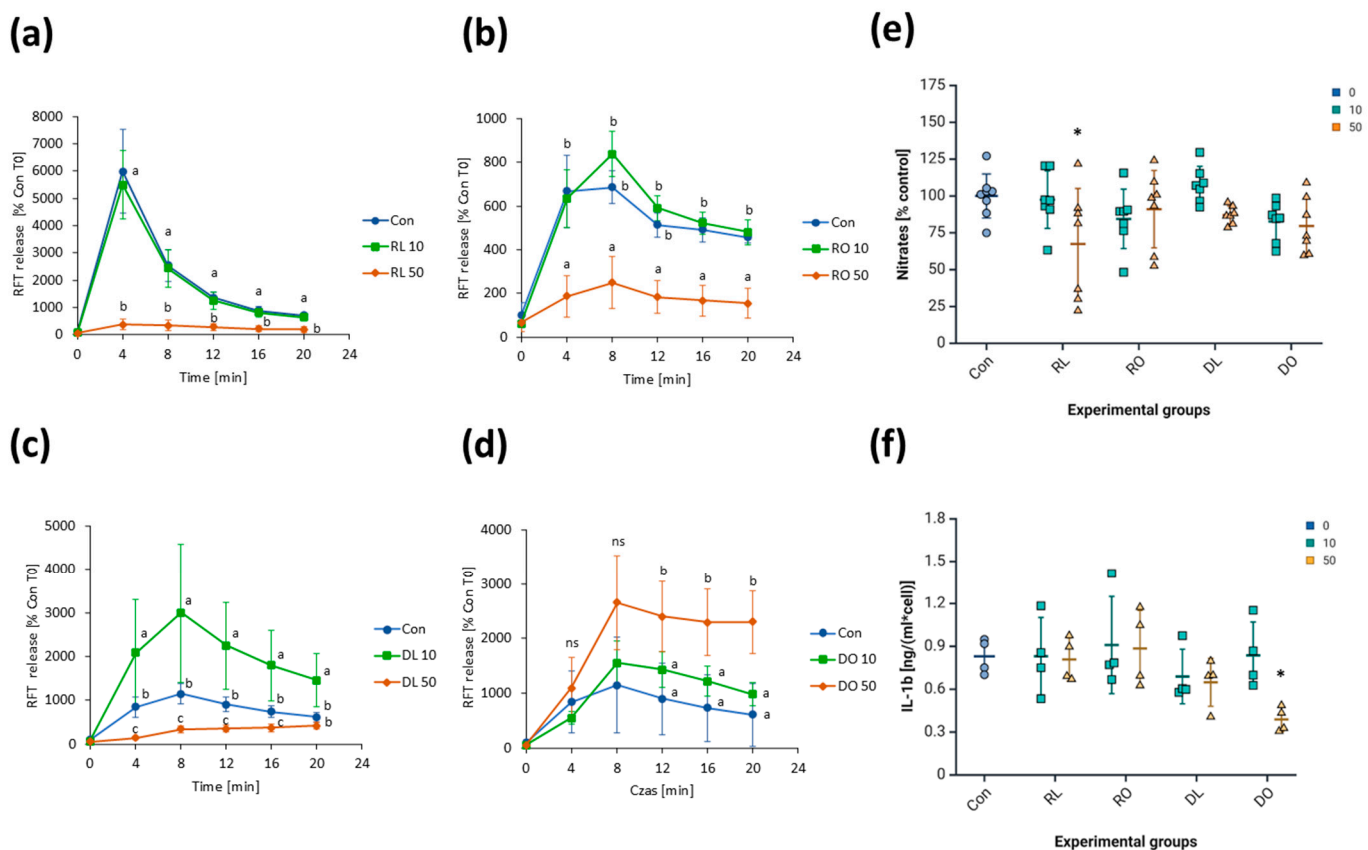


Figure 5. Reactive oxygen production (oxidative burst) in PMA-stimulated Raw 264.7 cells (a–d). The plots show luminescent signals using arbitrary units expressed as a percentage of the value of the control at the start of the reaction (time 0), the same letters represent lack of statistically significant differences at $p < 0.05$; ns—insignificant differences. (e) Concentration of NO metabolites (nitrites nmol/(ml $\times$ cell)) in the culture supernatant from P388D1 monocytes stimulated with LPS. (f) Concentration of IL-1 β in the culture supernatant from P388D1 monocytes stimulated with LPS. * $p < 0.05$ vs. Con. RL—sea buckthorn extract (10, 50 $\mu\text{g}/\text{mL}$); RO—sea buckthorn fruit extract (10, 50 $\mu\text{g}/\text{mL}$); DL—Cornelian cherry leaf extract (10, 50 $\mu\text{g}/\text{mL}$); DO—Cornelian cherry fruit extract (10, 50 $\mu\text{g}/\text{mL}$); Con—cells treated with the appropriate amount of solvent. Panels (e,f) created with <https://www.biorender.com/>.

4. Discussion

A huge body of literature presents the various beneficial and health-promoting effects of phenolic phytochemicals, administered orally either as food components or as purified supplements. The knowledge regarding the underlying mechanisms of their action is constantly expanding, but it is still far from being complete. Our results indicate that Cornelian cherry leaf extract upregulated the level of intestinal differentiation markers in Caco-2/HT-29-MTX 2D co-cultures, which manifested through enhanced ALPI and mucin staining (Figure 2b) and ALPI protein levels as compared to the control (Figure 2g),

although the differences in ALPI enzymatic activity were not significant. In spheroid co-cultures, all the tested extracts increased both ALPI's and mucin's staining intensity (Figure 2a). The observations about the positive impact of Cornelian cherry fruit extract on the intestinal epithelial differentiation markers are novel and, as far as we are aware, have not been reported yet.

ALPI plays an important role in the maintenance of gut homeostasis through bicarbonate release and the regulation of luminal pH, facilitating nutrient absorption and the detoxification of microbial pathogen molecular patterns (PAMPs) such as LPS [33]. Removing phosphate from the lipid A moiety of LPS using ALPI dramatically diminishes LPS recognition by Toll-like receptor 4, which helps to control the immune response to commensal microbiota in the gut [33]. High levels of ALPI in the intestinal mucosa provides protection from exaggerated inflammation, whereas ALPI deficiency or loss of catalytic function causes the development of inflammatory bowel disease (IBD) [34].

Mucins, being a vital part of the innate immune barrier mechanisms of the gastrointestinal tract, play a protective role against infection and the onset of IBD. Mucins create a layer that is difficult to penetrate for pathogenic bacteria and limit the risk of their direct contact with epithelial cells, serve as a carrier for antimicrobial peptides and IgA antibodies, facilitate the chyme passage and provide a friendly environment for commensal beneficial bacteria such as *Akkermansia muciniphila*. Dietary polyphenols and the phytochemical present in the tested extracts can possibly come into contact with goblet cells (in our model HT-29-MTX cells) and stimulate them to secrete mucin. Volstatova and co-authors [35] observed the increase in trans-membrane mucin Muc17 in Caco-2/HT-29-MTX models after treatment with quercetin, although the expression of Muc2, the main mucin secreted in the gut, was not altered. We did not analyse the particular types of mucins but focused on the staining of mucin glycans with alcian blue, because mucin post-translational modifications, particularly glycosylation, determines mucin's protective functions. We observed that the treatment with DL extract produced the most intensive staining, and this extract contained the highest amount of quercetin out of all four extracts, so it is possible that this flavonoid contributed to mucin upregulation. Our results are in line with the report that the Cornelian cherry iridoid–polyphenol extract prevented the loss of the mucus layer during chemically induced colitis in rats [36].

Sea buckthorn water extracts administered in the concentration of 500 µg/mL improved the Caco-2 barrier functions by inhibiting the translocation of LPS added to the apical chamber of the insert to the basal chamber [37]. Similarly, various polyphenols, including resveratrol, rutin and quercetin, were shown to protect the epithelial layer's integrity in the Caco-2 or IPEC-J2 intestinal mucosa models challenged with mycotoxins or indomethacin [38,39]. In these reports, the polyphenols restored the TEER values and barrier functions of the cell monolayer. We did not detect any significant changes in TEER values in the Caco-2/HT-29-MTX co-cultures, which reflected a good barrier integrity. However, in our experiments, no external challenge, such as a drug, chelator, toxin or LPS, was used to disrupt the epithelial barrier, so we could not evaluate any eventual protective activity of the tested extracts.

Numerous polyphenols have been tested so far in the context of their protective role against NAFLD development or alleviation of NAFLD or NASH symptoms. Tie and co-authors observed that kaempferol and kaempferide isolated from sea buckthorn alleviated oleic acid-induced lipid accumulation in HepG2 cells [40]. The fatty acid induced lipid droplet accumulation in primary hepatocytes or HepG2 cells was also decreased after the treatment with pure quercetin, resveratrol, myricetin, rutin, ferulic acid, caffeic acid, cinnamic acid and *p*-coumaric acid [21,41–49]. Our data showing that the RL (rich in rutin), RO (rich in myricetin) and DO (rich in rutin and *p*-coumaric acid) extracts decreased lipid droplet size in HepG2 cells challenged with hyperglycaemic glucose concentration and palmitate confirms these results. Additionally, we analysed other conditions that were meant to model diabetes (hyperglycaemia), insulin resistance (hyperglycaemia with insulin) and high fat diets with insulin resistance (hyperglycaemia, insulin and palmitate)

in our HepG2 experiments, as it is known that the aforementioned metabolic conditions contribute to NAFLD development. All the tested extracts decreased the lipid droplet size in at least two of these conditions (Table 2). All the extracts restricted overall intracellular lipid storage in diabetes and insulin resistance—mimicking those conditions (Figure 3b). In some cases, the extracts (e.g., RO extract in HG + Ins + Palm condition, DO extract in HG + Palm, Figure 3c) decreased the droplet size but increased their number. This may indicate stimulation of lipid droplet dynamics and their emulsification, which often leads to their easier breakdown, as the interface of lipid to cytosol is increased [50].

In terms of the protein level of the enzymes involved in lipid metabolism, it is hard to get a clear picture of generally increased or decreased levels of enzymes taking part in fatty acid synthesis or β -oxidation. This might be caused by the application of extracts that have a complex composition and the timeline of the changes induced by individual phenolic compounds interfering with each other, therefore we observe a dynamic resulting effect. It would be better to collect lysates from several time points and compare the results. Out of the numerous acyl-CoA dehydrogenases engaged in the mitochondrial β -oxidation, we chose ACAD9 because it plays a dual function, being not only responsible for providing long-chain fatty acyl-CoA for the breakdown, but it is also necessary for the proper assembly of subunits to form the functional respiratory complex I; therefore, its level reflects the cellular respiratory capacity [51–54]. ACAD9 is produced in a precursor form with a higher molecular mass (Figures 3d and 4c) and resides mainly in the cytoplasm or nucleus (c/n form). However, after processing with mitochondrial peptidases a 37-amino acid leader peptide is cleaved and a lower molecular mass, approximately 65 kDa, form is finally found in the mitochondria [55]. In HepG2 lysates from the cells treated with the extracts, we noticed a decreased abundance of lower MW mitochondrial forms (Figure 3d) but increased levels of the higher MW forms, which might suggest increased production of premature forms not yet transported to the mitochondria.

Decreased FAS protein levels and concomitantly higher ATP-CL amounts in the HepG2 cells treated with RL and RO extracts indicate a possible accumulation of malonyl-CoA (produced by ATP-CL), which is a direct substrate for FAS. Downregulation of FAS suggests that malonyl-CoA cannot be readily utilised, therefore it may act as a carnitine palmitoyltransferase CPT1 inhibitor that blocks fatty acyl-CoA entry to the mitochondria and their subsequent β -oxidation. Such a situation would not be beneficial for efficient lipid catabolism, which is necessary to reduce intracellular lipid storage. On the other hand, malonyl-CoA can be also transformed into acetyl-CoA by malonyl-CoA decarboxylase (MCD). Acetyl-CoA fuels the Krebs cycle to produce reducing equivalents NADH and FADH₂ for oxidative phosphorylation, as well as numerous other important metabolites, so this is the other catabolic route for acetyl-CoA.

Although we did not detect any significant differences in the oxLDL phagocytosis by the monocytes treated with the extracts and the control ones, we noticed a strong impact of the treatment on the intensity of the oxidative burst. The RL, RO and DL extracts in the concentration of 50 μ g/mL blocked ROS production by monocytes, whereas 10 μ g/mL of DL and 50 μ g/mL of DO enhanced this response as compared to the control (Figure 5a–d). Large quantities of superoxide anion released during the oxidative burst can react with NO secreted by monocytes and macrophages to form a highly microbicidal and cytotoxic peroxynitrite (ONOO[−]). In our experimental setting, the higher dose of RL extract significantly decreased the amount of NO metabolites in the cell culture supernatants from LPS-stimulated monocytes in comparison to the control. Other extracts showed a modest effect, not reaching statistical significance (Figure 5e). The experiments with J774.A1 monocytes treated with methanolic extracts from the *Crescentia alata* medicinal plant revealed the inhibition of NO release from the LPS-stimulated cells, as well as decreased iNOS protein levels [56]. The extracts contained rutin, kaempferol glycoside and kaempferol aglycone, but the authors identified kaempferol as the most effective inhibitor of NO production, whereas rutin and kaempferol glycoside were inactive. In our case,

the RO extract was the richest source of kaempferol, but it did not significantly affect NO secretion (Figure 5e).

Uncontrolled production of reactive oxygen and nitrogen species contributes to intestinal mucosa damage seen in chronic inflammatory conditions such as IBD [57]. The loss of barrier function in the damaged epithelium increases bacterial endotoxin leakage from the lumen, which leads to increased vascular permeability in the jejunum and colon and further aggravates inflammation [58]. RL, RO and DL extracts are particularly rich in rutin and cinnamic acid (RL), myricetin and resveratrol (RO) and phenolic acids and quercetin (DL). All these phytochemicals were reported to scavenge free radicals (such as DPPH) and ROS [59–63].

The anti-inflammatory activities of phenolic phytochemicals are well studied and there are numerous reports about the inhibition of pro-inflammatory cytokine release from various cell types treated with the individual, separately administered compounds that we detected in the analysed extracts [60,64–69]. Therefore, we expected to see the modulation of the LPS-induced inflammatory response by the application of the extracts. However, only the DO extract at a dose of 50 µg/mL significantly inhibited IL-1β secretion (Figure 5f). This supports previous observations that Cornelian cherry fruit extract decreased IL-1β production via peripheral blood neutrophils stimulated with LPS [70]. Surprisingly, the DO extract has the least complex composition and the concentrations of rutin and phenolic acids in this extract are lower than in the others (Table 1). This suggests that the application of the complex mixture of polyphenols might not give the expected results, possibly due to different kinetics or a mode of action of the individual compounds on the intracellular signalling pathways that control cytokine production and secretion. Apparently, there is no synergism among these phenolic compounds, but possibly a kind of antagonism.

5. Conclusions

The sea buckthorn and Cornelian cherry fruits and leaves are rich sources of bioactive phenolic compounds, and they differ considerably in the number of compounds that belong to different classes: phenolic acids (protocatechuic, *trans*-caffeic, *trans* *p*-coumaric, *trans*-ferulic and *trans*-cinnamic), stilbenes (resveratrol), flavones (apigenin) and flavonols (rutin, myricetin, quercetin and kaempferol). The extracts increase intestinal epithelial differentiation markers and the Cornelian cherry leaf extract was the most effective. All the tested extracts significantly reduced the intracellular lipid droplet size in hepatocytes metabolically challenged with hyperglycaemia, insulin and palmitate, which is a novel observation. Our findings also suggest a possible beneficial effect of NAFLD prophylaxis. The extracts from sea buckthorn leaves, fruit and Cornelian cherry leaves blocked monocyte oxidative bursts, while the sea buckthorn leaf and Cornelian cherry fruit extracts downregulated LPS-induced NO and IL-1β, respectively. The complexity of the extract composition is a factor that makes the final biological effect harder to predict than in cases of pure substances. This would also be true for dietary supplements of plant origins.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/app14177992/s1>, Figure S1: The proliferation of cells grown in the presence of the sea buckthorn and Cornelian cherry leaves and fruits.

Author Contributions: Conceptualization, M.G. and I.S.; methodology, M.G., M.P. and J.L.; formal analysis, M.G., I.S., M.P., J.L. and V.F.; plant material collection, I.S. and V.F.; investigation M.G., G.W., A.W., K.J., J.L. and M.P.; data curation, M.G. and J.L.; writing—original draft preparation, M.G.; writing—review and editing, M.G., M.P., J.L. and V.F.; visualization, M.G.; supervision, M.G.; project administration, M.G. and I.S.; funding acquisition, M.G. and I.S.; G.W., A.W. and K.J. equally contributed to the experimental work. All authors have read and agreed to the published version of the manuscript.

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