Immunomodulatory effect of a wild blueberry anthocyanin-rich extract in human Caco-2 intestinal cells

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Abstract

Intestinal inflammation is a natural process crucial for the maintenance of gut functioning. However, abnormal or prolonged inflammatory responses may lead to the onset of chronic degenerative diseases, typically treated by means of pharmacological interventions. Dietary strategies for prevention of inflammation are a safer alternative to pharmacotherapy. Anthocyanins and other polyphenols have been documented to display anti-inflammatory activity. In the present study, three bioactive fractions (anthocyanin, phenolic and water soluble fractions) were extracted from a wild blueberry powder. The Caco-2 intestinal model was used to test the immunomodulatory effect of the above fractions. Only the anthocyanin-rich fraction reduced in a dose-dependent manner the activation of NF-κB, induced by IL-1β in intestinal epithelial Caco-2 cells. Specifically, concentrations of 50 µg mL⁻¹ and 100 µg mL⁻¹ decreased NF-κB activation by 68.9% and 85.2%, respectively (p≤0.05). These preliminary results provide further support for the role of food bioactives as potential dietary anti-inflammatory agents.

Keywords: Wild blueberry; anthocyanins; Caco-2 cells; NF-κB; luciferase reporter system
**Introduction**

Inflammation is a non-specific immune response consisting of complex reactions as the body defends itself against injuries that are encountered daily by host cells such as the mucosal surface of the gastrointestinal tract. The orchestration of these responses guarantees both effective protection against harmful agents and maintenance of homeostasis. The nuclear factor κB (NF-κB) pathway is an immunological route through which inflammation processes are triggered and regulated. The NF-κB transcription factor exists in an inactive state in the cytosol of cells; upon its activation and translocation into the nucleus the induction of genes involved in immune and pro-inflammatory responses is induced, leading to the expression of cytokines and chemokines, adhesion molecules and inflammatory enzymes.¹

In some individuals, or in particular conditions, the above system fails to suppress the inflammatory processes once activated, leading to exacerbated responses and to the onset of pathological consequences like intestinal bowel diseases (IBD). Usually, effective therapies are mainly based on pharmacological interventions, which are often connected with adverse side effects. Therefore, the potential of utilizing dietary strategies to prevent and/or reduce the inflammatory state may represent a safer and affordable tool.

Polyphenols are a class of phytochemicals that have been shown to modulate inflammatory processes.² *In vitro* and *in vivo* studies report that pure polyphenolic molecules, but also plant extracts, can interfere with immunological pathways and the production of inflammatory mediators.³

The anti-inflammatory abilities of polyphenols have been partially attributed to a direct action on the host immune system. It is reported that these bioactives could affect different intracellular pathways in the intestinal mucosa by triggering specific immune responses.⁴ In fact, diverse phenolic compounds (e.g. kaempferol, quercitin, genistein, luteolin) have been documented to exert
their effects through the attenuation of pro-inflammatory cytokines.\textsuperscript{5,6} Consequently, it has been proposed that such compounds may decrease the risk of individuals to develop chronic inflammation.\textsuperscript{4} Specifically, previous investigations (both in \textit{in vitro} and in mouse models) documented that polyphenols can exert modulatory effects in presence of different inflammatory stimuli.\textsuperscript{2}

ACNs are pigments of the polyphenol class that confer red to blue color to several common fruits and vegetables, which have been shown to exert different health-promoting properties as documented both in \textit{in vitro} and \textit{in vivo} studies.\textsuperscript{7-12} Apart from their antioxidant activity\textsuperscript{13}, ACNs play a key role in promoting host health through diverse mechanisms: from improvement of lipid profiles, vasomotor tone and blood pressure to the modulation of detoxifying enzymes\textsuperscript{7-10} and inflammation.\textsuperscript{11,12,14}

ACNs are poorly absorbed\textsuperscript{15}, even if new evidence suggests higher bioavailability than previously reported\textsuperscript{16}. Consequently most of them reach the colon where their concentrations, as well as that of other polyphenols, can reach high concentrations, justifying a beneficial effect against IBD.\textsuperscript{17}

Thus the present study aims to investigate the effect of different bioactive fractions, obtained from an ACN-rich Wild Blueberry (WB, \textit{Vaccinium angustifolium}) powder, on immune responses in CaCo-2 cells following exposure to a pro-inflammatory stimulus.

\section*{Materials and methods}

\textit{Chemicals and Materials} - Standard of cyanidin (Cy)-, delphinidin (Dp)-, petunidin (Pt)-, peonidin (Pe)-, and malvidin (Mv)-3-O-glucoside (glc), Cy- and Pt-3-O-arabinoside (ara), Cy-3-O-galactoside (gal), Dp, Cy, and Mv were purchased from Polyphenols Laboratory (Sandes, Norway). Eagle’s minimum essential medium (EMEM), Trypsin, fetal calf serum (FCS), D-luciferin, adenosine triphosphate (ATP), phosphate buffer saline (PBS), penicillium, streptomycin, non
essential amino acids, L-glutamine, HEPES, and Tris-HCl buffer, intereukin 1β and standards of
colorgenic, caffeic, ferulic, glucose, fructose, malic, citric acid were obtained from Sigma-Aldrich.
Hydrochloric acid, methanol, acetonitrile, triethylamine, phosphoric acid, trifluoroacetic acid (TFA)
and ethyl acetate were from Merck (Darmstadt, Germany). Water was obtained from Milli-Q
apparatus (Millipore, Milford, MA). Zeocin and plasmid pNiFty2-Luc where purchased from
Invivogen (Labogen, Rho, Italy). Freeze-dried Wild Blueberry (WB) powder, standardized at 1.5%
total ACNs, was provided by Future-Ceuticals Company (Momence, IL, USA).

Separation and chemical characterization of WB fractions

Extraction of bioactives from the WB powder - The freeze-dried WB powder was used to extract
three different fractions providing the main WB bioactives: the water soluble fraction (containing
mainly sugars and organic acids - WS fraction), the ethyl acetate soluble fraction (containing
mainly chlorogenic acid - Phe fraction) and the methanol soluble fraction (containing mainly
anthocyanins - ACN fraction).

Extraction was performed following the method described by Wrolstad\textsuperscript{18} with few modifications.
Briefly, the WB powder was suspended in water, sonicated for 10 min, and centrifuged at 3000 × g
for 10 min. Fraction separation from the supernatant was obtained through solid-phase extraction
(SPE)-cartridge (Strata-X 300 mg/3 mL, Phenomenex, Torrence, CA). Three mL of supernatant was
loaded and the elution of WS, Phe and ACN fractions were carried out respectively with HCl 0.01
N (5 mL), ethyl acetate (10 mL) and methanol (5 mL) containing 0.1% HCl. The fractions were
dried under vacuum with rotavapor (RC Jouan 10, Jouan, Winchester, VA) at 20 °C for ACN, 40 °C
for Phe and up to 60 °C for the WS fraction. The residues were dissolved in methanol acidified with
HCl (0.05 mM) for the ACNs, methanol for the Phe, and water for the WS fractions. The solutions
were analyzed for the content of ACNs, Phe, sugars and organic acids, and stored at -20 °C until
use.
Analysis of ACN and Phe-rich fractions - Analysis was performed with a liquid chromatographic system, which consisted of an Alliance mod. 2695 (Water, Milford, MA) equipped with a mod. 2998 photodiode array detector (Waters). The separation was carried out through a C18 Kinetex column (150 x 4.6 mm, 2.6 µm, Phenomenex, Torrence, CA) at 45 °C and 1.7 mL min⁻¹ as flow rate. The eluents were (A) 1% H₃PO₄ and (B) acetonitrile/water (35:65, v/v). The elution gradient was linear as indicated: 0-15 min 14% B; 15-25 min from 14 to 20% B; 25-35 min from 20 to 32% B; 35-45 min from 32 to 50% B; 45-48 min from 50 to 90% B; 90% for 3 min. Chromatographic data were acquired from 200 to 700 nm and integrated at 520 (ACNs) and 320 nm (Phe). Calibration curves ranged from 2 to 50 µg mL⁻¹ were obtained for cyanidin (Cy-), delphinidin (Dp-), petunidin (Pt-), peonidin (Pe-) and malvidin (Mv-) 3-O-glc, Cy- and Pt-3-O-gal and Pt-3-O-ara and chlorogenic acid. For the ACNs, the working solution was diluted from the stock solution with methanol acidified with 0.1% TFA. Each analysis was carried out in duplicate. The identification of single ACNs was confirmed by LC coupled to ESI-MS (electrospray ionization - mass spectrometry) as already described by Del Bo’ et al. Briefly, the mass spectrometer operated in positive full-scan mode in the range 200-800 Da. The capillary voltage was set to 3.5 kV, the cone voltage to 20 V, the source temperature to 130 °C, and the desolvating temperature to 350 °C. Data were acquired by Masslinx 4.0 software (Micromass, Beverly, MA).

Analysis of sugars - Glucose and fructose were quantified in WB fractions by UPLC (Acquity, Waters) coupled with a triple quadrupole mass spectrometer mod. Quattro micro (Micromass, Beverly, MA).

The separation was carried out on BEH (Ethylene Bridged Hybrid) Amide column (150 x 2.1 mm, 1.7 µm, Waters) at 35 °C. Solvents were (A) triethylamine 0.2% and (B) triethylamine 0.2% in acetonitrile. Flow-rate was 0.40 mL min⁻¹. The calibration curve was obtained from 1 to 50 µg mL⁻¹
for both sugars. The mass spectrometer operated in ESI negative mode monitoring the ions with $m/z$ 179 (glucose, fructose). The capillary voltage was set to 3.0 kV, the cone voltage to 20 V, the source temperature to 120 °C, and the desolvating temperature to 250 °C.

**Analysis of organic acids** - Malic and citric acids were analyzed by UPLC (Acquity, Waters) coupled to a triple quadrupole mass spectrometer mod. Quattro micro (Micromass, Beverly, MA). The separation was performed on C$_{18}$ Atlantis T3 column (150 x 2.1 mm, 1.7 µm, Waters) at 45 °C and the flow rate was 0.5 mL min$^{-1}$. The eluent was formic acid 0.05%. The calibration curves were obtained over the concentration range 2 to 25 µg mL$^{-1}$ for both malic and citric acid. The capillary voltage was set to 3.5 kV, the cone voltage and the collision energy was specific for each compound. The source temperature was 120 °C, the desolvating temperature was 300 °C and argon was used at 1.3x10$^{-3}$ mbar to improve fragmentation in the collision cell. Masslynx 4.0 acquired data with Quan-Optimize option for fragmentation study. The fragmentation transitions for the multiple reaction monitoring (MRM) were ($m/z$) 133→115, 133→71 for malic and ($m/z$) 191→111, 191→87 for citric acid, with a dwell time of 0.2 s per transition.

**Immunomodulatory activity of ACN-rich fraction evaluated on Caco-2 cell line**

**In vitro cultivation** - Caco-2 cells (human epithelial colorectal adenocarcinoma cell line; ATCC HTB-37) is a well-established and validated model of the human intestinal barrier.$^{19}$ They were routinely grown in Eagle’s minimum essential medium (EMEM) supplemented with 10% (v/v) heat-inactivated (30 min at 56 °C) fetal calf serum (FCS), 100 U mL$^{-1}$ penicillin, 100 µg mL$^{-1}$ streptomycin, 0.1 mM nonessential amino acids (NEAA), and 2 mM L-glutamine and were incubated at 37 °C in a water-jacketed incubator in an atmosphere of 95% air and 5% carbon dioxide.
Study of NF-κB activation. Stable recombinant Caco-2 cell line was generated by transfecting cells with the plasmid pNiFty2-Luc as described by Guglielmetti et al.\textsuperscript{20} This plasmid contains a promoter with five NF-κB-binding sites followed by the firefly luciferase reporter gene luc. Stimuli that activate NF-κB promote its binding to the vector promoter, resulting in the expression of the luciferase gene. Briefly, Caco-2 were transfected by means of the StoS transfection kit (GeneSpin, Milan, Italy), in accordance with the manufacturer’s protocol. Afterwards, cells were resuspended in fresh EMEM, seeded in 24-well plates, and incubated for 48 h, in order to obtain the expression of the antibiotic resistance. Finally, stable recombinant clones were selected by adding into the culture medium 50 µg mL\textsuperscript{-1} of zeocin.

Stimulation protocol and NF-κB-luciferase assay - After growth in the presence of 50 µg mL\textsuperscript{-1} zeocin, differentiated cell monolayers (approximately 3×10\textsuperscript{5} cells well\textsuperscript{-1}) were carefully washed with 0.1 M Tris-HCl buffer (pH 8.0). Subsequently, fresh EMEM medium, containing 100 mM HEPES (pH 7.4) was added to Caco-2 cells. The WS, Phe and ACN-rich fractions obtained by WB powder have been tested. Concentrations used were 25, 50 and 100 µg mL\textsuperscript{-1} for the WS fraction (calculated considering the sugars and organic acid concentration), and 5, 25, 50 and 100 µg mL\textsuperscript{-1} for the Phe-rich fraction (calculated considering the chlorogenic acid concentration) and the ACN-rich fraction (calculated considering the total ACNs concentration). Only minor changes of pH were registered (pH 7.6±0.1) when supplementing cells with extract doses corresponding to the highest concentration. Recombinant Caco-2 cells were simultaneously stimulated with interleukin-1β (IL-1β, 2 ng mL\textsuperscript{-1}), used as pro-inflammatory stimulus. After incubation at 37 °C for 4 h, 24-well plates were put on ice for 15 min; recombinant Caco-2 cells were detached mechanically from the bottom of a well, samples were transferred into an eppendorf tube and subjected to sonication at maximum power for 5 s using a Bandelin SONOPLUS Ultrasonic Homogenizer (Bandelin electronic GmbH & Co., Berlin, Germany). Insoluble particles were removed by centrifugation and the supernatants
were transferred into a new tube. One hundred μL of supernatants were aliquoted in duplicate into the wells of a 96-well white microtiter plate (PerkinElmer, Monza, Italy) by means of epMotion Automated Pipetting System (Eppendorf, Milan, Italy). Then 12.5 μL of a 10 mM ATP solution (i.e. up to the final concentration of 1 mM) and 12.5 μL of 0.1 mM D-luciferin solution were added, and the emitted bioluminescence was immediately measured every 120 s with a VICTOR3 1420 Multilabel Counter (PerkinElmer). The maximum of light production curve was considered for comparison of results. All conditions were analyzed in duplicate in eight independent experiments.

Cell viability assay- The methosulfate (MTS) test is a spectrophotometric method used to evaluate vitality and number of living cells. The test was performed on cells treated with ACN-rich fraction (5, 25, 50 and 100 μg mL⁻¹), with media (Control 1), and media added with MetOH + 0.05 mM HCl (Control 2. After incubation at 37 °C for 4 h, the medium was removed from Caco-2 cells, and the cells were carefully washed with 500 μL of PBS. Trypsin (Trypsin 0.25%, 2.5 g porcine trypsin + 0.2 g EDTA) was added to lyse cells. EMEM medium, containing 100 mM HEPES (pH 7.4) was added and 100μL of supernatant was aliquoted into a 96-well microtiter plate (PerkinElmer, Monza, Italy) by means of epMotion Automated Pipetting System and 10μL of MTS was added. The plate was incubated at 37 °C for 90 min and the absorbance of MTS at 490 nm was measured by a spectrophotometer (Biorad, UK).

Statistical analysis

Statistical analysis was performed by means of STATISTICA software (Statsoft Inc., Tulsa, OK, US). Analysis of variance (ANOVA), with type of treatment as the dependent factor, was used to evaluate the immunomodulatory activity of the different WB fractions on Caco-2 cells. The same analysis was performed to ensure cell viability following the ACN-rich fraction supplementation. Post-hoc analysis of differences between treatments was assessed by the Least Significant Difference (LSD) test with p ≤ 0.05 as level of statistical significance.
Results

Characterization of Wild blueberry (WB) fractions

The three fractions obtained from the WB powder were analyzed to evaluate the content of sugars and organic acids, phenols and ACNs. The water soluble fraction (WS) contained 4.12 μg mL⁻¹ of sugars, corresponding to 2.28 ± 0.12 μg mL⁻¹ and 1.84 ± 0.13 μg mL⁻¹ of glucose and fructose, respectively. The content of malic acid and citric acid was 19.9 ± 2.10 μg mL⁻¹ and 47.2 ± 4.23 μg mL⁻¹, respectively. Conversely the content of ACNs and phenols was negligible. Sugars and organic acids were detected only in the WS fraction.

In the Phe-rich fraction, throughout chlorogenic acid was the main phenolic compound (719.2 ± 21.4 μg mL⁻¹), with traces of caffeic and ferulic acids. No ACNs, sugars or organic acids were detected.

The ACN-rich fraction composition is reported in Table 1. The total ACN content was 1563 ± 102 μg mL⁻¹. Fourteen different ACNs were detected in the fraction; the dominant compounds were: Mv glycosides (about 540 μg mL⁻¹; 35% of the total ACN amount) and Dp glycosides (about 492 μg mL⁻¹; 31%) followed by Cy glycosides (about 293 μg mL⁻¹; 19%), while Pt and Pe glycosides represented about 11% and 4.2% of the total ACNs, respectively. The rank order for conjugated sugars was glucoside>galactoside>arabinoside from the most to the least representative (Table 1).

No phenolic compounds, sugars and organic acids, were detected.

Modulation of NF-κB activation by WS, Phe and ACN-rich fractions in Caco-2 cells

To identify the class of potential bioactive molecules provided by our WB powder, we tested the effects of the three different fractions isolated from WB (i.e. WS, Phe, ACN-rich) on NF-κB pathway activation. Experiments on Caco-2 cells were performed in the presence of the pro-inflammatory stimulus IL-1β. No significant differences in light emission levels were observed
following Phe and WS exposure, and none of the Phe or the WS concentrations used (up to 100 μg mL⁻¹) resulted in a significant reduction in the NF-κB induction level compared to control. On the contrary, the ACN-rich fraction was able to reduce significantly NF-κB activation (Figure 1). In particular, the bioluminescence, and therefore NF-κB activation, was reduced by 68.9% and 85.2% at the concentration of 50 and 100 μg mL⁻¹ respectively (p≤0.05).

Cell viability

No significant difference in cell viability was observed following Caco-2 cell supplementation. Thus, the reduction of NF-κB activation observed was not ascribable to a cytotoxic effect of high concentrations of the ACN-rich fraction (Table 2).

Discussion

Prolonged inflammation mediated by NF-κB activation may contribute to the pathogenesis of chronic diseases such as inflammatory bowel disease, diabetes, neurodegenerative diseases, cancer, and cardiovascular diseases. In particular the regulation of NF-κB activation at the epithelial interfaces, such as at gut level, is of particular importance for the maintenance of mucosal integrity, barrier function and homeostasis. Excessive activation of NF-κB at the gut level has been associated with inflammatory bowel disease.

NF-κB is the main target in several anti-inflammatory therapies (e.g. non-steroidal anti-inflammatory drugs) that are not specific for NF-κB inhibition and are required in relatively high concentrations leading to problems of adverse side effects and toxicity.

The use of natural bioactive molecules, like polyphenols, which possess the ability to attenuate NF-κB activation, might represent a safer and effective strategy in repressing inflammatory processes. Several studies suggest that blueberry polyphenols display anti-inflammatory effects that are not specifically related to their antioxidant properties. ACNs, flavonols and phenolic acids could be the
phytochemicals mainly responsible for this activity. Thus, in our study, we extracted and characterized three fractions (WS, Phe and ACN-rich fraction) from the freeze dried WB powder in order to investigate the potential anti-inflammatory ability exerted by the different bioactive-rich fractions. The ACN profile of the WB used in the current research differs from that of previous studies both in qualitative and quantitative composition. Generally, Mv, Dp, Cy, Pt, Pe glycosides are the most abundant ACNs found in wild blueberry. In the ACN-rich fraction prepared for this study that originated from wild blueberry (Vaccinium angustifolium), the dominant compounds were Mv glycosides (35% of the total ACN amount), Dp glycosides (31%) and Cy glycosides (19%). On the contrary, Yi et al. using three different blueberry cultivars (Briteblue, Tifblue, and Powderblue) reported higher level of Pt and Pe glycosides in the ACN fraction, whereas Triebel et al. detected higher level of Dp glycosides and Cy glycosides from bilberry (Vaccinium myrtillus L.). These differences may be explained considering that ACN profile of blueberry can vary between different cultivars and growing conditions; finally the extraction procedures may affect molecule recovery. However, it is noteworthy that ACN absorption and their anti-proliferative effects were demonstrated to be comparable in Caco-2 cell monolayers supplemented with different ACN fractions obtained from several blueberry cultivars. Moreover, the authors reported that ACN absorption and average transport efficiency in the Caco-2 cell model was comparable to that evidenced in in vivo studies (i.e. about 3-4%).

Concerning the Phe-rich fraction, chlorogenic acid was the main compound detected. Traces of hydroxycinnamic acids, such as caffeic and ferulic acids, were also found. These results disagree with findings by Yi et al. in which gallic, caffeic, p-coumaric and ferulic acids were identified in three different cultivars. The differences may be due to the blueberry cultivar investigated as well as to the freeze-drying process which may contribute to the decrease in phenols.
Finally, the immunomodulatory effect of the WS fraction was investigated to ascertain the potential contribution of sugars (i.e. glucose and fructose) and organic acids (i.e. malic acid and citric acid), not included in the ACN and Phe-rich fractions.

The WS fraction did not show any immunomodulatory potential, even though some studies suggest an induction of the NF-kB pathway following supplementation with sugars. Similarly, no significant effect was observed following Phe-rich fraction exposure. These results are in contrast with other studies which documented an anti-inflammatory and inhibitory activity of phenolic compounds on NF-κB. In particular, Rodriguez-Ramiro et al. documented that a cocoa polyphenolic extract (10 µg mL\(^{-1}\)) decreased the nuclear levels of NF-kB and the expression of several cytokines in the colon of rats. Additionally, the authors confirmed in Caco-2 cells that the cocoa polyphenol-rich extract, downregulated the levels of inflammatory markers by inhibiting NF-κB activation. The capacity of polyphenols to downregulate the NF-κB signaling pathway was also observed by Romier et al. in a Caco-2 intestinal model upon supplementation with different polyphenols (50 µmol L\(^{-1}\)). Moreover, Kim et al. reported that the flavonoid luteolin (50 µmol L\(^{-1}\)) prevented lipopolysaccharide-induced NF-κB signaling and gene expression by blocking IκB kinase activity in intestinal epithelial cells. These conflicting results could be attributed to the different type and amount of polyphenols used, and the specific role played by each single phenolic compound.

In the present study, only the ACN–rich fraction tested revealed an immunomodulatory potential on transfected human intestinal epithelial Caco-2 cells. In particular, a significant decrease of NF-κB activation was observed at ACN-rich fraction concentrations of 50 and 100 µg mL\(^{-1}\). Our results are in agreement with others demonstrating the ability of ACNs in the modulation of NF-κB, and down-stream signalling \textit{in vitro} in different cell models. For example, supplementation with ACN-rich extract (100 µg mL\(^{-1}\)) isolated from bilberries and blackcurrants modulated \textit{in vitro} LPS-induced activation of NF-κB in monocytes.
Kim et al.\textsuperscript{42} showed that the administration of a soybean seed coat extract (providing from 1 to 100 µg mL\textsuperscript{-1} ACNs) significantly inhibited NF-κB activation in endothelial cells.

Beneficial effects were also observed \textit{in vivo} with ACN-rich foods. For example, the use of a wild-blueberry-enriched diet in Zucker rats was able to downregulate the expression of NF-κB, both in the liver and the abdominal adipose tissue, and to reduce several circulating inflammatory markers as CRP, IL-6 and TNF-α.\textsuperscript{14} Moreover, interestingly, in a parallel-design controlled clinical trial, the supplementation of healthy subjects with ACNs from blueberry (Medox, 300 mg d\textsuperscript{-1} for 3 weeks) reduced the levels of circulating NF-κB-dependent inflammatory mediators such as IL-8 and IFN-α.\textsuperscript{3}

In the present \textit{in vitro} study, the use of extracts instead of single ACNs is limited since it does not reveal the effect of single bioactive compounds on the NF-κB pathway. In this regard, the ACN-rich fraction was further characterized for the content of vitamin A and E\textsuperscript{43}, ascorbic acid\textsuperscript{44}, carotenoids\textsuperscript{43}, fatty acids\textsuperscript{45}, fiber\textsuperscript{46} and minerals eventually recovered during the extract preparation. None of the compounds were detectable apart from traces of fatty acids (palmitic, stearic, oleic and linoleic acids). Concerning minerals, only traces of magnesium, phosphorus and manganese were present, while calcium, sodium and zinc were the main compounds found. However, Caco-2 cell supplementation with the ACN-rich fraction provided less than 1.1 µg mL\textsuperscript{-1} of the above minerals. In addition we cannot exclude that other bioactive compounds not identified in the ACN-rich fraction could have contributed to the anti-inflammatory effect observed. In fact, some of them might interact synergistically or additively with ACNs or other bioactives. In this regard, the contribution of ACN metabolites such as protocatechuic acid, whose concentration \textit{in vivo} is reported to be much higher than the parent compound\textsuperscript{47,48} cannot be ruled out. The extensive activity of the gut microbiota in the colon may also be another contributory factor.\textsuperscript{49}
Additionally, it is known from cell culture studies, that under simulated physiological conditions (pH and temperature) a significant rate of degradation of ACNs occurs with formation of isoforms (e.g. chalcone, hemiketal) and phenolic acids that can contribute to the effects observed. Finally a possible limitation of cell culture studies where food extracts are used, is the omission of the step simulating gastrointestinal digestive processes as it happens in vivo.

In conclusion, our preliminary results show that the ACN-rich fraction extracted from the WB powder displayed anti-inflammatory properties by decreasing the activation of NF-κB in presence of the pro-inflammatory stimulus IL-1β. This ability might be particularly useful in the prevention/control of several inflammatory disorders, like IBD, as already observed in mouse model. Further investigations are necessary to identify the individual components and/or metabolites of the ACN-rich fraction responsible of this immunomodulatory ability. Future research should target the identification of potential functional components that could aid in the development of innovative strategies preventing chronic inflammatory diseases without the side effects of pharmacotherapy.
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References


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FIGURE CAPTIONS

Figure 1

Modulation of light emission expressed by Caco-2 cells transfected with a NF-κB/luciferase reporter vector and incubated in presence of interleukin-1β with the ACN-rich fraction. Data are reported as percent variation of light emission, referred to the control. Control: Caco-2 cell layers incubated only with media supplemented with MetOH + 0.05 mM HCl. ACNs: anthocyanin-rich fraction. ACN concentrations (5-25-50-100) are referred to µg mL⁻¹. The values are the means (+ standard deviations) for eight independent experiments conducted in duplicate. Asterisks indicate statistically significant differences compared to results for the control (*, P<0.05).
Table 1: Characterization of the ACN-rich Fraction

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Data are reported as mean±standard deviation.

a=glc, glucoside; b=gal, galactoside; c=ara, arabinoside
Table 2: MTS Viability Test. Data Are Reported as a Measure of Absorbance at 490 nm. Caco-2 Cell Layers Incubated with ACN-rich Fraction (5, 25, 50, 100 µg mL⁻¹) only with Media (Control 1) and Media with MetOH + 0.05 mM HCl (Control 2). ACNs: Anthocyanin-rich Fraction. The Values Are Expressed as Percentage Mean (± Standard Deviations) for two Independent Experiments.

<table>
<thead>
<tr>
<th>Cell viability</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>100</td>
</tr>
<tr>
<td>Control 2</td>
<td>74.5 ± 18.3</td>
</tr>
<tr>
<td>ACNs 5 µg mL⁻¹</td>
<td>77.4 ± 14.9</td>
</tr>
<tr>
<td>ACNs 10 µg mL⁻¹</td>
<td>73.3 ± 18.9</td>
</tr>
<tr>
<td>ACNs 50 µg mL⁻¹</td>
<td>65.7 ± 25.1</td>
</tr>
<tr>
<td>ACNs 100 µg mL⁻¹</td>
<td>90.1 ± 10.2</td>
</tr>
</tbody>
</table>
Figure 1

% change biofilm score

ACNs 5 µg/mL  ACNs 25 µg/mL  ACNs 50 µg/mL  ACNs 100 µg/mL