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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 ($p \leq 0.05$). 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

18 # Introduction

19 Inflammation is a non-specific immune response consisting of complex reactions as the body
20 defends itself against injuries that are encountered daily by host cells such as the mucosal surface of
21 the gastrointestinal tract. The orchestration of these responses guarantees both effective protection
22 against harmful agents and maintenance of homeostasis. The nuclear factor κB (NF-κB) pathway is
23 an immunological route through which inflammation processes are triggered and regulated. The
24 NF-κB transcription factor exists in an inactive state in the cytosol of cells; upon its activation and
25 translocation into the nucleus the induction of genes involved in immune and pro-inflammatory
26 responses is induced, leading to the expression of cytokines and chemokines, adhesion molecules
27 and inflammatory enzymes.[1]
28 In some individuals, or in particular conditions, the above system fails to suppress the inflammatory
29 processes once activated, leading to exacerbated responses and to the onset of pathological
30 consequences like intestinal bowel diseases (IBD). Usually, effective therapies are mainly based on
31 pharmacological interventions, which are often connected with adverse side effects. Therefore, the
32 potential of utilizing dietary strategies to prevent and/or reduce the inflammatory state may
33 represent a safer and affordable tool.
34 Polyphenols are a class of phytochemicals that have been shown to modulate inflammatory
35 processes.[2]*In vitro* and *in vivo* studies report that pure polyphenolic molecules, but also plant
36 extracts, can interfere with immunological pathways and the production of inflammatory
37 mediators.[3]
38 The anti-inflammatory abilities of polyphenols have been partially attributed to a direct action on
39 the host immune system. It is reported that these bioactives could affect different intracellular
40 pathways in the intestinal mucosa by triggering specific immune responses.[4] In fact, diverse
41 phenolic compounds (e.g. kaempferol, quercitin, genistein, luteolin) have been documented to exert

42 their effects through the attenuation of pro-inflammatory cytokines.[5,6] Consequently, it has been
43 proposed that such compounds may decrease the risk of individuals to develop chronic
44 inflammation.[4] Specifically, previous investigations (both in *in vitro* and in mouse models)
45 documented that polyphenols can exert modulatory effects in presence of different inflammatory
46 stimuli.[2]

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

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

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

59

60 Materials and methods

61 *Chemicals and Materials* - Standard of cyanidin (Cy)-, delphinidin (Dp)-, petunidin (Pt)-, peonidin
62 (Pe)-, and malvidin (Mv)-3-*O*-glucoside (glc), Cy- and Pt-3-*O*-arabinoside (ara), Cy-3-*O*-
63 galactoside (gal), Dp, Cy, and Mv were purchased from Polyphenols Laboratory (Sandes, Norway).
64 Eagle's minimum essential medium (EMEM), Trypsin, fetal calf serum (FCS), D-luciferin,
65 adenosine triphosphate (ATP), phosphate buffer saline (PBS), penicillium, streptomycin, non

66 essential amino acids, L-glutamine, HEPES, and Tris-HCl buffer, intereukin 1β and standards of
67 chlorogenic, caffeic, ferulic, glucose, fructose, malic, citric acid were obtained from Sigma-Aldrich.
68 Hydrochloric acid, methanol, acetonitrile, triethylamine, phosphoric acid, trifluoroacetic acid (TFA)
69 and ethyl acetate were from Merck (Darmstadt, Germany). Water was obtained from Milli-Q
70 apparatus (Millipore, Milford, MA). Zeocin and plasmid pNiFty2-Luc where purchased from
71 Invivogen (Labogen, Rho, Italy). Freeze-dried Wild Blueberry (WB) powder, standardized at 1.5%
72 total ACNs, was provided by Future-Ceuticals Company (Momence, IL, USA).
73

74 **Separation and chemical characterization of WB fractions**

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

80 Extraction was performed following the method described by Wrolstad[18] with few modifications.
81 Briefly, the WB powder was suspended in water, sonicated for 10 min, and centrifuged at 3000 × *g*
82 for 10 min. Fraction separation from the supernatant was obtained through solid-phase extraction
83 (SPE)-cartridge (Strata-X 300 mg/3 mL, Phenomenex, Torrence, CA). Three mL of supernatant was
84 loaded and the elution of WS, Phe and ACN fractions were carried out respectively with HCl 0.01
85 N (5 mL), ethyl acetate (10 mL) and methanol (5 mL) containing 0.1% HCl. The fractions were
86 dried under vacuum with rotavapor (RC Jouan 10, Jouan, Winchester, VA) at 20 °C for ACN, 40 °C
87 for Phe and up to 60 °C for the WS fraction. The residues were dissolved in methanol acidified with
88 HCl (0.05 mM) for the ACNs, methanol for the Phe, and water for the WS fractions. The solutions
89 were analyzed for the content of ACNs, Phe, sugars and organic acids, and stored at -20 °C until
90 use.

91

92 *Analysis of ACN and Phe-rich fractions* -Analysis was performed with a liquid chromatographic
93 system, which consisted of an Alliance mod. 2695 (Water, Milford, MA) equipped with a mod.
94 2998 photodiode array detector (Waters). The separation was carried out through a C_{18} Kinetex
95 column (150 x 4.6 mm, 2.6 μm, Phenomenex, Torrence, CA) at 45 °C and 1.7 mL min^{-1} as flow
96 rate. The eluents were (A) 1% H_3PO_4 and (B) acetonitrile/water (35:65, v/v). The elution gradient
97 was linear as indicated: 0-15 min 14% B; 15-25 min from 14 to 20% B; 25-35 min from 20 to 32%
98 B; 35-45 min from 32 to 50% B; 45-48 min from 50 to 90% B; 90% for 3 min. Chromatographic
99 data were acquired from 200 to 700 nm and integrated at 520 (ACNs) and 320 nm (Phe).
100 Calibration curves ranged from 2 to 50 µg mL^{-1} were obtained for cyanidin (Cy-), delphinidin (Dp-
101), petunidin (Pt-), peonidin (Pe-) and malvidin (Mv-) 3-*O*-glc, Cy- and Pt-3-*O*-gal and Pt-3-*O*-ara
102 and chlorogenic acid. For the ACNs, the working solution was diluted from the stock solution with
103 methanol acidified with 0.1% TFA. Each analysis was carried out in duplicate. The identification of
104 single ACNs was confirmed by LC coupled to ESI-MS (electro spray ionization - mass
105 spectrometry) as already described by Del Bo'et al.[9] Briefly, the mass spectrometer operated in
106 positive full-scan mode in the range 200-800 Da. The capillary voltage was set to 3.5 kV, the cone
107 voltage to 20 V, the source temperature to 130 °C, and the desolvating temperature to 350 °C. Data
108 were acquired by Masslinx 4.0 software (Micromass, Beverly, MA).

109

110 *Analysis of sugars* - Glucose and fructose were quantified in WB fractions by UPLC (Acquity,
111 Waters) coupled with a triple quadrupole mass spectrometer mod. Quattro micro (Micromass,
112 Beverly, MA).
113 The separation was carried out on BEH (Ethylene Bridged Hybrid) Amide column (150 x 2.1 mm,
114 1.7 μm, Waters) at 35 °C. Solvents were (A) triethylamine 0.2% and (B) triethylamine 0.2% in
115 acetonitrile. Flow-rate was 0.40 mL min^{-1}. The calibration curve was obtained from 1 to 50 µg mL^{-1}

116 for both sugars. The mass spectrometer operated in ESI negative mode monitoring the ions with *m/z*
117 179 (glucose, fructose). The capillary voltage was set to 3.0 kV, the cone voltage to 20 V, the
118 source temperature to 120 °C, and the desolvating temperature to 250 °C.
119 *Analysis of organic acids* - Malic and citric acids were analyzed by UPLC (Acquity, Waters)
120 coupled to a triple quadrupole mass spectrometer mod. Quattro micro (Micromass, Beverly, MA).
121 The separation was performed on C_{18} Atlantis T3 column (150 x 2.1 mm, 1.7 µm, Waters) at 45 °C
122 and the flow rate was 0.5 mL min^{-1}. The eluent was formic acid 0.05%. The calibration curves were
123 obtained over the concentration range 2 to 25 µg mL^{-1} for both malic and citric acid. The capillary
124 voltage was set to 3.5 kV, the cone voltage and the collision energy was specific for each
125 compound. The source temperature was 120 °C, the desolvating temperature was 300 °C and argon
126 was used at $1.3x10^{-3}$ mbar to improve fragmentation in the collision cell. Masslinx 4.0 acquired data
127 with Quan-Optimize option for fragmentation study. The fragmentation transitions for the multiple
128 reaction monitoring (MRM) were $(m/z)^-$ 133→115, 133→71 for malic and $(m/z)^-$ 191→111,
129 191→87 for citric acid, with a dwell time of 0.2 s per transition.
130

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

132 *In vitro cultivation* - Caco-2 cells (human epithelial colorectal adenocarcinoma cell line; ATCC
133 HTB-37) is a well-established and validated model of the human intestinal barrier.[19] They were
134 routinely grown in Eagle's minimum essential medium (EMEM) supplemented with 10% (v/v)
135 heat-inactivated (30 min at 56 °C) fetal calf serum (FCS), 100 U mL^{-1} penicillin, 100 µg mL^{-1}
136 streptomycin, 0.1 mM nonessential amino acids (NEAA), and 2 mM L-glutamine and were
137 incubated at 37 °C in a water-jacketed incubator in an atmosphere of 95% air and 5% carbon
138 dioxide.

139 *Study of NF-κB activation*. Stable recombinant Caco-2 cell line was generated by transfecting cells
140 with the plasmid pNiFty2-Luc as described by Guglielmettiet al.[20]. This plasmid contains a
141 promoter with five NF-κB-binding sites followed by the firefly luciferase reporter gene *luc*. Stimuli
142 that activate NF-κB promote its binding to the vector promoter, resulting in the expression of the
143 luciferase gene. Briefly, Caco-2 were transfected by means of the StoS transfection kit (GeneSpin,
144 Milan, Italy), in accordance with the manufacturer's protocol. Afterwards, cells were resuspended
145 in fresh EMEM, seeded in 24-well plates, and incubated for 48 h, in order to obtain the expression
146 of the antibiotic resistance. Finally, stable recombinant clones were selected by adding into the
147 culture medium 50 μg mL^{-1} of zeocin.

148 *Stimulation protocol and NF-κB-luciferase assay* -After growth in the presence of 50 μg mL^{-1}
149 zeocin, differentiated cell monolayers (approximately 3×10^5 cells $well^{-1}$) were carefully washed
150 with 0.1 M Tris-HCl buffer (pH 8.0). Subsequently, fresh EMEM medium, containing 100 mM
151 HEPES (pH 7.4) was added to Caco-2 cells. The WS, Phe and ACN-rich fractions obtained by WB
152 powder have been tested. Concentrations used were 25, 50 and 100 μg mL^{-1} for the WS fraction
153 (calculated considering the sugars and organic acid concentration), and 5, 25, 50 and 100 μg mL^{-1}
154 for the Phe-rich fraction (calculated considering the chlorogenic acid concentration) and the ACN-
155 rich fraction (calculated considering the total ACNs concentration). Only minor changes of pH were
156 registered (pH 7.6± 0.1) when supplementing cells with extract doses corresponding to the highest
157 concentration. Recombinant Caco-2 cells were simultaneously stimulated with interleukin-1β (IL-
158 1β, 2 ng mL^{-1}), used as pro-inflammatory stimulus. After incubation at 37 °C for 4 h, 24-well plates
159 were put on ice for 15 min; recombinant Caco-2 cells were detached mechanically from the bottom
160 of a well, samples were transferred into an *eppendorf* tube and subjected to sonication at maximum
161 power for 5 s using a Bandelin SONOPLUS Ultrasonic Homogenizer (Bandelin electronic GmbH
162 & Co., Berlin, Germany). Insoluble particles were removed by centrifugation and the supernatants

163 were transferred into a new tube. One hundred µL of supernatants were aliquoted in duplicate into
164 the wells of a 96-well white microtiter plate (PerkinElmer, Monza, Italy) by means of epMotion
165 Automated Pipetting System (Eppendorf, Milan, Italy). Then 12.5 µL of a 10 mM ATP solution
166 (i.e. up to the final concentration of 1 mM) and 12.5 µL of 0.1 mM D-luciferin solution were added,
167 and the emitted bioluminescence was immediately measured every 120 s with a VICTOR3 1420
168 Multilabel Counter (PerkinElmer). The maximum of light production curve was considered for
169 comparison of results. All conditions were analyzed in duplicate in eight independent experiments.

170 *Cell viability assay-* The methosulfate (MTS) test is a spectrophotometric method used to evaluate
171 vitality and number of living cells.[21] The test was performed on cells treated with ACN-rich fraction
172 (5, 25, 50 and 100 µg mL^{-1}), with media (Control 1), and media added with MetOH + 0.05 mM HCl
173 (Control 2. After incubation at 37 °C for 4 h, the medium was removed from Caco-2 cells, and the
174 cells were carefully washed with 500 µL of PBS. Trypsin (Trypsin 0.25%, 2.5 g porcine trypsin +
175 0.2 g EDTA) was added to lyse cells. EMEM medium, containing 100 mM HEPES (pH 7.4) was
176 added and 100µL of supernatant was aliquoted into a 96-well microtiter plate (PerkinElmer, Monza,
177 Italy) by means of epMotion Automated Pipetting System and 10µL of MTS was added. The plate
178 was incubated at 37 °C for 90 min and the absorbance of MTS at 490 nm was measured by a
179 spectrophotometer (Biorad, UK).

180 Statistical analysis

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

187 **Results**

188 **Characterization of Wild blueberry (WB) fractions**

189 The three fractions obtained from the WB powder were analyzed to evaluate the content of sugars
190 and organic acids, phenols and ACNs. The water soluble fraction (WS) contained 4.12 μg mL^{-1} of
191 sugars, corresponding to 2.28 ± 0.12 μg mL^{-1} and 1.84 ± 0.13 μg mL^{-1} of glucose and fructose,
192 respectively. The content of malic acid and citric acid was 19.9 ± 2.10 μg mL^{-1} and 47.2 ± 4.23 μg
193 mL^{-1},respectively. Conversely the content of ACNs and phenols was negligible. Sugars and organic
194 acids were detected only in the WS fraction.
195 In the Phe-rich fraction, throughout chlorogenic acid was the main phenolic compound (719.2 ±
196 21.4 μg mL^{-1}), with traces of caffeic and ferulic acids. No ACNs, sugars or organic acids were
197 detected.
198 The ACN-rich fraction composition is reported in **Table 1**. The total ACN content was 1563 ± 102
199 μg mL^{-1}. Fourteen different ACNs were detected in the fraction; the dominant compounds were: Mv
200 glycosides (about 540 μg mL^{-1}; 35% of the total ACN amount) and Dp glycosides (about 492 μg
201 mL^{-1}; 31%) followed by Cy glycosides (about 293 μg mL^{-1}; 19%), while Pt and Pe glycosides
202 represented about 11% and 4.2% of the total ACNs, respectively. The rank order for conjugated
203 sugars was glucoside>galactoside>arabinoside from the most to the least representative (**Table 1**).
204 No phenolic compounds, sugars and organic acids, were detected.
205

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

207 To identify the class of potential bioactive molecules provided by our WB powder, we tested the
208 effects of the three different fractions isolated from WB (i.e. WS, Phe, ACN-rich) on NF-κB
209 pathway activation. Experiments on Caco-2 cells were performed in the presence of the pro-
210 inflammatory stimulus IL-1β. No significant differences in light emission levels were observed

211 following Phe and WS exposure, and none of the Phe or the WS concentrations used (up to 100 μg
212 mL^{-1}) resulted in a significant reduction in the NF-κB induction level compared to control. On the
213 contrary, the ACN-rich fraction was able to reduce significantly NF-κB activation (**Figure 1**). In
214 particular, the bioluminescence, and therefore NF-κB activation, was reduced by 68.9% and 85.2%
215 at the concentration of 50 and 100 μg mL^{-1} respectively ($p \leq 0.05$).
216
217 **Cell viability**
218 No significant difference in cell viability was observed following Caco-2 cell supplementation.
219 Thus, the reduction of NF-κB activation observed was not ascribable to a cytotoxic effect of high
220 concentrations of the ACN-rich fraction (**Table 2).**
221
222 **Discussion**
223 Prolonged inflammation mediated by NF-κB activation may contribute to the pathogenesis of
224 chronic diseases such as inflammatory bowel disease, diabetes, neurodegenerative diseases, cancer,
225 and cardiovascular diseases.[3,22,23] In particular the regulation of NF-κB activation at the epithelial
226 interfaces, such as at gut level, is of particular importance for the maintenance of mucosal integrity,
227 barrier function and homeostasis. Excessive activation of NF-κB at the gut level has been associated
228 with inflammatory bowel disease.[24]
229 NF-κB is the main target in several anti-inflammatory therapies (e.g. non-steroidal anti-
230 inflammatory drugs)[25,26] that are not specific for NF-κB inhibition and are required in relatively
231 high concentrations leading to problems of adverse side effects and toxicity.[26]
232 The use of natural bioactive molecules, like polyphenols, which possess the ability to attenuate NF-
233 κB activation, might represent a safer and effective strategy in repressing inflammatory processes.
234 Several studies suggest that blueberry polyphenols display anti-inflammatory effects that are not
235 specifically related to their antioxidant properties.[27]ACNs, flavonols and phenolic acids could be the

236 phytochemicals mainly responsible for this activity. Thus, in our study, we extracted and
237 characterized three fractions (WS, Phe and ACN-rich fraction) from the freeze dried WB powder in
238 order to investigate the potential anti-inflammatory ability exerted by the different bioactive-rich
239 fractions. The ACN profile of the WB used in the current research differs from that of previous
240 studies both in qualitative and quantitative composition.[28,29] Generally, Mv, Dp, Cy, Pt, Pe
241 glycosides are the most abundant ACNs found in wild blueberry.[9,30,31] In the ACN-rich fraction
242 prepared for this study that originated from wild blueberry (*Vaccinium angustifolium*), the dominant
243 compounds were Mv glycosides (35% of the total ACN amount), Dp glycosides (31%) and Cy
244 glycosides (19%). On the contrary, Yi et al.[28] using three different blueberry cultivars (Briteblue,
245 Tifblue, and Powderblue) reported higher level of Pt and Pe glycosides in the ACN fraction,
246 whereas Triebel et al.[29] detected higher level of Dp glycosides and Cy glycosides from bilberry
247 (*Vaccinium myrtillus L.*). These differences may be explained considering that ACN profile of
248 blueberry can vary between different cultivars and growing conditions[32]; finally the extraction
249 procedures may affect molecule recovery. However, it is noteworthy that ACN absorption and their
250 anti-proliferative effects were demonstrated to be comparable in Caco-2 cell monolayers
251 supplemented with different ACN fractions obtained from several blueberry cultivars.[31]Moreover,
252 the authors reported that ACN absorption and average transport efficiency in the Caco-2 cell model
253 was comparable to that evidenced in *in vivo* studies (i.e. about 3-4%).[33]
254 Concerning the Phe-rich fraction, chlorogenic acid was the main compound detected. Traces of
255 hydroxycinnamic acids, such as caffeic and ferulic acids, were also found. These results disagree
256 with findings by Yi et al.[28] in which gallic, caffeic, *p*-coumaric and ferulic acids were identified in
257 three different cultivars. The differences may be due to the blueberry cultivar investigated as well as
258 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[34]. 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.[5,6,35] In particular, Rodriguez-Ramiro et al.[36] 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.[37] in a Caco-2 intestinal model upon supplementation with different polyphenols (50 μmol L^{-1}). Moreover, Kim et al.[38] 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 *in vitro* in different cell models.[3,39-41] For example, supplementation with ACN-rich extract (100 μg mL^{-1}) isolated from bilberries and blackcurrants modulated *in vitro* LPS-induced activation of NF-κB in monocytes.[3]

284 Kim et al.[42] showed that the administration of a soybean seed coat extract (providing from 1 to 100
285 μg mL^{-1} ACNs) significantly inhibited NF-κB activation in endothelial cells.

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

293 In the present *in vitro* study, the use of extracts instead of single ACNs is limited since it does not
294 reveal the effect of single bioactive compounds on the NF-κB pathway. In this regard, the ACN-
295 rich fraction was further characterized for the content of vitamin A and E[43], ascorbic acid[44],
296 carotenoids[43], fatty acids[45], fiber[46] and minerals eventually recovered during the extract preparation.
297 None of the compounds were detectable apart from traces of fatty acids (palmitic, stearic, oleic and
298 linoleic acids). Concerning minerals, only traces of magnesium, phosphorus and manganese were
299 present, while calcium, sodium and zinc were the main compounds found. However, Caco-2 cell
300 supplementation with the ACN-rich fraction provided less than 1.1 μg mL^{-1} of the above minerals.
301 In addition we cannot exclude that other bioactive compounds not identified in the ACN-rich
302 fraction could have contributed to the anti-inflammatory effect observed. In fact, some of them
303 might interact synergistically or additively with ACNs or other bioactives. In this regard, the
304 contribution of ACN metabolites such as protocatechuic acid, whose concentration *in vivo* is
305 reported to be much higher than the parent compound[47,48] cannot be ruled out. The extensive
306 activity of the gut microbiota in the colon may also be another contributory factor.[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.[50,51] 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.[49]

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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486

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488

489 **FIGURE CAPTIONS**

490 **Figure 1**

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

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Table 1: Characterization of the ACN-rich Fraction

Compounds	Extracted	
	μg mL^{-1}	%
malvidin 3-glc[a]	251 ± 6.3	16.4
malvidin 3-gal[b]	224 ± 8.1	14.3
malvidin 3-ara[c]	65.1 ± 4.1	4.2
delphinidin 3-glc[a]	224 ± 17.2	14.3
delphinidin 3-gal[b]	166 ± 13.1	10.6
delphinidin 3-ara[c]	102 ± 9.8	6.5
cyanidin 3-glc[a]	99.1 ± 8.6	6.3
cyanidin 3-gal[b]	69.1 ± 3.4	4.4
cyanidin 3-ara[c]	124 ± 9.9	7.8
petunidin 3-glc[a]	43.2 ± 2.3	2.8
petunidin 3-gal[b]	107 ± 11.1	6.8
petunidin 3-ara[c]	22.1 ± 3.7	1.4
peonidin 3-glc[a]	28.2 ± 2.2	1.8
peonidin 3-gal[b]	38.2 ± 2.1	2.4
Total	1563 ± 102	100

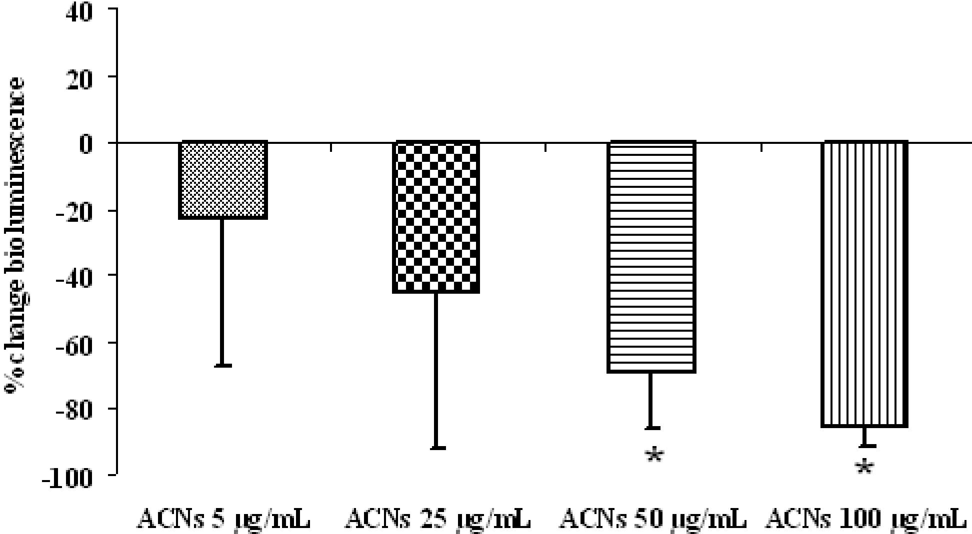
Data are reported as mean±standard deviation.

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.

Cell viability	%
Control 1	100
Control 2	74.5 ± 18.3
ACNs 5 µg mL^{-1}	77.4 ± 14.9
ACNs 10 µg mL^{-1}	73.3 ± 18.9
ACNs 50 µg mL^{-1}	65.7 ± 25.1
ACNs 100 µg mL^{-1}	90.1 ± 10.2

Figure 1



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