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Modulatory effect of Andean blackberry polyphenols on genes related to antioxidant and inflammatory responses, the NLRP3 inflammasome and autophagy

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30 **Abstract**

31 BACKGROUND: The Andean blackberry (*Rubus glaucus* Benth) is one of Ecuador's iconic
32 berries that grows in its Andean region, of which a high anthocyanin content has been described.
33 OBJECTIVE: The aim of the present study was to determine the chemical composition and anti-
34 inflammatory potential of the Andean blackberry from Ecuador, with emphasis on its effects on
35 NLRP3 inflammasome activation and autophagy processes. RESULTS: Andean blackberry
36 extracts were rich in hydroxycinnamates (coumaric acid and derivates), in addition to quercetin
37 and kaempferol as main flavonols. Cyanidin and its glycosides were identified as the main
38 anthocyanins present. Andean blackberry extracts efficiently reduced oxidative stress markers in
39 the lipopolysaccharide-stimulated RAW 264.7 cells. The extract also caused a moderate decrease
40 in the expression of the pro-inflammatory and antioxidant genes NFκB1, TNF, IL-1, IL-6 and
41 NOS2 expression, while it significantly increased the mRNA levels of both SOD1 and NFE2L2
42 genes. Andean blackberry extracts significantly decreased the activation of the NLRP3
43 inflammasome complex, as well as p62 levels and the LC3I/LC3II ratio increased, suggesting a
44 direct action of Andean blackberry compounds on the inflammatory response and restoration of
45 the autophagy process. CONCLUSION: These results suggest the potential anti-inflammatory
46 effect of Andean blackberry, through their ability to regulate genes related to the inflammatory
47 and antioxidant response, as well as modulate the activation of the NLRP3 inflammasome complex
48 and autophagy processes.

49

50 **Keywords:** Anthocyanins, Autophagy, Blackberries, Inflammation, NRLP3 inflammasome

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55 ## 1. Introduction

56 Current epidemiological evidence increasingly highlights the relationship between nutritional
57 patterns based on frequent consumption of fruits and vegetables with a low incidence of
58 noncommunicable diseases, such as diabetes, cardiovascular and neurodegenerative diseases and
59 cancer [1]. All these pathologies have a common pathophysiological feature in their development
60 and progression based on high production of pro-inflammatory mediators and are characterized by
61 excessive production of reactive oxygen species (ROS) with the consequent oxidative damage on
62 macromolecules and cellular structures [2]. Thus, the inhibition of the excessive inflammatory
63 response and oxidative damage has been proposed as an alternative to minimize their damage. It
64 is in this context that a diet rich in fruits and vegetables, with its important contribution of
65 antioxidants and anti-inflammatory compounds, plays a fundamental role based mainly on the
66 ability of these compounds not only to block the excessive circulating free radicals but also on
67 their biological effects that go beyond the simple antioxidant character [3]. Indeed, in the last
68 decades an increasing body of articles have described the anti-inflammatory and
69 immunoregulatory properties that polyphenols have on different pathways related to the
70 antioxidant response and, therefore, their contribution to protection against oxidative damage
71 [4,5]. Within the polyphenols described with anti-inflammatory activity, anthocyanins (ACNs)
72 play a prominent role. Anthocyanins are natural phenolic pigments with broad and outstanding
73 biological activity. They have potent antioxidant and anti-inflammatory activity, properties that
74 have been closely related to other of their biological effects such as their antidiabetic and
75 anticancer activity, and their role in cardiovascular and neuroprotective prevention [6,7].
76 Anthocyanins are widespread in the plant world, providing the characteristic red/blue colour to
77 various fruits, vegetables, flowers and leaves, and their content is influenced by various factors,
78 such as species, cultivars, climatic conditions and level of maturity [8], and others [9]. Among its
79 natural sources, berries stand out (strawberries, blueberries, blackberries, blackcurrant, redcurrant,

80 and raspberries) for being a rich source of anthocyanins, with levels ranging from about 100 to
81 about 700 mg/100 g of fresh fruits [9].

82 The Andean blackberry (*Rubus glaucus* Benth) is one of Ecuador's iconic berries that grows
83 wild or cultivated, isolated, dispersed or in groups with other plants in the Andean highlands of
84 Ecuador. They are traditionally collected and commercialized by the indigenous people of these
85 regions [10] and they have become a popular fruit among the Ecuadorian population in general
86 consumed in the form of jams, wine, and boiled drinks such as the famous "*Colada morada*"
87 (purple strained) that is consumed in the day of the dead celebration [11]. Although several studies
88 have highlighted the chemical composition and biological effects of blackberries from other
89 geographical regions [12–15], few studies have been developed in the case of the Andean
90 blackberry. Only a few investigations have described its chemical composition, evidencing its high
91 anthocyanin content [16–18], as well as its biological properties, such as its ability to protect
92 against oxidative damage [19]. Thus, more studies are necessary to define its chemical
93 composition, as well as the biological effects and mechanisms that it uses to generate them, to
94 gradually determine its true health potential and therefore justifying its consumption. With this
95 background, in the present study we firstly determined the polyphenolic composition of the
96 Andean blackberry fruit. In order to assess its anti-inflammatory effects, a murine macrophage
97 RAW 264.7 cells were used as an *in vitro* validation model both in the presence or absence of the
98 endotoxin lipopolysaccharide (LPS). The levels of the principal biomarkers related to oxidative
99 damage and inflammatory markers were estimated together with the activity of the main
100 antioxidant enzymes. Finally, the expression of different genes related to antioxidant and
101 inflammatory response was also determined with the objective of discovering the possible
102 molecular pathways involved in the anti-inflammatory action of Andean blackberry. Finally, given
103 the importance of the NLRP3 inflammasome complex as an important mediator of inflammatory
104 processes, we studied the effects of Andean blackberry compounds on its activation by using an
105 *in vitro* model of THP-1 human monocytes cells. At the same time, given the repairing effect of

106 the autophagy process and therefore its importance in cellular hemostasis, we also studied the
107 effect of the polyphenols of this fruit on the activation of this process using this cellular model.

108 2. Material and methods

109 *2.1. Chemical and reagents*

110 All chemicals and solvents were of analytical grade. Solvents, lipopolysaccharides (LPS) from
111 *Escherichia coli* O55:B5, RPMI and 1640 Medium, fetal bovine serum, penicillin-streptomycin,
112 RIPA Buffer, hydrogen peroxide solution (H_2O_2), reduced glutathione (GSH), β-Nicotinamide
113 adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH), glutathione
114 peroxidase from bovine erythrocytes (GPx), L-glutathione reduced (GSH), glutathione S-
115 transferase from equine liver (GST), 2-Thiobarbituric acid, malondialdehyde tetrabutylammonium
116 salt (MDA), 2,4-Dinitrophenylhydrazine (DNPH), guanidine hydrochloride, streptomycin, Tris-
117 HCl, Triton™ X-100, phenylmethylsulfonyl fluoride, leupeptin and Bradford reagent were
118 purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), while the 2′-7′-dichlorofluorescin
119 diacetate (DCFH) kit was purchased from Merck, Darmstadt, Germany. TRIzol™ reagent,
120 Turbo™ DNase and SuperScript III Platinum SYBR Green One-Step RT-qPCR kit were
121 purchased from Invitrogen Carlsbad, CA, USA) while specific monoclonal antibodies were
122 obtained from Santa Cruz Biotechnology (Dallas, TX, USA). A cocktail of protease inhibitors
123 (cOmplete Protease Inhibitor Cocktail) was purchased from Boehringer Mannheim (Indianapolis,
124 IN). The Immun-Star horseradish peroxidase (HRP) substrate kit was obtained from Bio-Rad
125 Laboratories Inc. (Hercules, CA, USA).

126 *2.2. Plant materials and sample preparation*

127 Ripe Andean blackberries (*Rubus glaucus* Benth) fruits were harvested during the berries crop
128 season (October – December, 2018) from plants growing in the highlands of the province of
129 Tungurahua, located in the inter-Andean region of Ecuador at an altitude between 3000 and 3200
130 meters above sea level. Fruits were collected on two different occasions at random from different
131 plants based on a similar degree of ripeness and without blemishes or any signs of damage or

132 decay. Two batches of samples (~1kg) were collected on each occasion and the specimens were
133 identified using the reference vouchers for specimens deposited in the herbarium of the Jardín
134 Botánico de Quito, Ecuador. During collection time, the fruits were kept at 4°C protected from
135 light and transported to the laboratory the same day they were harvested. Afterwards, samples
136 were cleaned, removing remains of leaves, stems and other residues, washed with distilled water
137 and allowed to dry on absorbent paper at room temperature and protected from light. Finally, fruits
138 were frozen at -20 °C overnight and then freeze-dried, ground to a fine powder, and stored at
139 -20°C until analysis.
140 For chemical composition determination and *in vitro* assays, a polyphenol-rich extract was
141 obtained as previously reported [20]. Briefly, the fine powder of fruits (10g) was added to 100 mL
142 of the extraction solution consisting of methanol acidified with 0.1% HCl and Milli-Q water (80%,
143 *v/v*) and stirred overnight at 3–5°C. Then, the solution was centrifuged for 15 minutes at 4000*g* at
144 4°C and filtered through a 2.5 μm pore size Whatman® cellulose filter paper (Sigma-Aldrich Co.,
145 St. Louis, MO, USA). The solid residues were washed with the extraction solution as many times
146 as necessary until no red colour was observed in the extracted solution. Finally, the resulting
147 extraction solutions were combined, water was added, and the final solution was concentrated in
148 a vacuum rotary evaporator (~30°C) until obtaining a dry crude extract.

149 ### *2.3. HPLC-DAD/ESI-MS^n characterization of phenolic acids, flavonols and anthocyanins*

150 The Andean blackberry crude extract (ABCext) was dissolved in 0.5 mL of 0.1% trifluoroacetic
151 acid/acetonitrile (70:30, *v/v*), filtered through a 0.22 μm disposable LC filter disk for analysis by
152 HPLC and analyzed using double online detection by diode array spectrophotometry and mass
153 spectrometry (MS). The HPLC system consisted in a Hewlett-Packard 1200 chromatograph
154 (Hewlett-Packard 1200, Agilent Technologies, Santa Clara, CA, USA) equipped with a binary
155 pump and a diode array detector (DAD) coupled with an HP ChemStation (*rev*. A.05.04) data
156 processing station coupled with an MS detector API 3200 Qtrap (Applied Biosystems, Darmstadt,
157 Germany) that was controlled by the Analyst 5.1 software. As stationary phase, an AQUA®

158 (Phenomenex, Torrance, CA, USA) reverse phase C18 column (5 µm, 150 mm × 4.6 mm)
159 thermostated at 35°C was used. The solvents were: (A) 0.1% trifluoroacetic acid and (B)
160 acetonitrile. The elution gradient established was: isocratic 10% B for 3 min, 10–15% B in 12 min,
161 isocratic 15% B for 5 min, 15–18% B over 5 min, 18–30% B over 20 min, 30–35% B over 5 min,
162 and re-equilibration of the column to initial solvent conditions. The flow rate used was 0.5 mL/min.
163 For detection, the DAD was set using 520, 330 and 280 nm as preferred wavelengths, while the
164 MS operated in the positive ion mode for the analysis of anthocyanins and in the negative mode
165 for the rest of the compounds, the spectra were recorded between *m/z* 100-1000. Zero grade air
166 served as the nebulizer gas (30 psi) and as turbo gas (400°C) for solvent drying (40 psi). Nitrogen
167 served as the curtain (20 psi) and collision gas (medium). Both quadrupoles were set at unit
168 resolution and EMS and EPI analyses were also performed. The EMS parameters were set as
169 follows: ion spray voltage −4500V, DP -50V, EP -6V, CE -10V and cell exit potential (CXP) −3V,
170 whereas EPI settings were: DP -50V, EP -6V, CE -30V and CES 10V. The individual phenolic
171 compounds were tentatively identified based on their UV and mass spectra, and comparison with
172 data reported in the literature, as well as with internal standards when available.

173 ***2.4. Evaluation of anti-inflammatory properties***

174 ***2.4.1. Cell culture and treatments***

175 Murine RAW 264.7 macrophage cell line was purchased from ATCC (ATCC-TIB71)
176 (Manassas, VA, USA) and maintained in RPMI 1640 medium supplemented with 10% fetal
177 bovine serum and 1% penicillin-streptomycin antibiotics (100 IU/mL penicillin and 100 mg/mL
178 streptomycin) in a humidified atmosphere at 37°C with 5% CO_2. For all the assays cells were used
179 between the 4th and the 6th passage. The Andean blackberry crude extract (ABCext) was
180 resuspended in RPMI 1640 medium to achieve the final concentration of 80 µg/mL. RAW
181 macrophages were treated with: (*i*) RPMI 1640 medium only (Ctrl group), (*ii*) lipopolysaccharides
182 (LPS) (*Escherichia coli* serotype 055:B5) at 1 µg/mL for 24h (LPS group), (*iii*) ABCext (80
183 µg/mL) for 24h (ABCext group), or (*iv*) LPS (1 µg/mL) for 24h and then ABCext (80 µg/mL) for

184 another 24h (LPS/ABCext group), (*v*) ABCext (80 μg/mL) for 24h and then LPS (1 μg/mL) for
185 another 24h (ABCext/LPS group) and (*vi*) ABCext (80 μg/mL) + LPS (1 μg/mL) for 24h
186 (ABCext+LPS group). The appropriate dose/time combination of ABCext and LPS treatments was
187 selected according to our previously reported studies using the same type of extract [19] as well
188 as the same cell model according to the culture conditions in our laboratory [21].

189 ***2.4.2. Measurement of intracellular ROS level***

190 Intracellular reactive oxygen species (ROS) levels were determined using the 2′-7′-
191 dichlorofluorescin diacetate (DCFH) kit according to the manufacturer's instructions. RAW cells
192 previously treated under the same/above-mentioned conditions were incubated with 5 μmol/L of
193 DCFH at 37°C for 30 min in the dark at room temperature. Next, the fluorescence intensity was
194 read using a microplate reader (Thermo Scientific Microplate Reader, Multiskan®, Winooski, VT,
195 USA) at 485/530 nm excitation/emission wavelength. Total protein levels were determined by the
196 Bradford method [22] and results were expressed as arbitrary units of fluorescent intensity/μg cell
197 proteins.

198 ***2.4.3. Determination of antioxidant enzyme activities, biomarkers of oxidative damage in lipid***
199 ***and proteins and nitrite production***

200 After treatment, murine RAW cells were washed with PBS, incubated with RIPA buffer on ice
201 for 5 min and the obtained lysate was stored at −80°C until analysis. For the analysis of antioxidant
202 enzymes, superoxide dismutase (SOD) was analyzed based on the inhibition of the formation of
203 NADHphenazine methosulphate-nitroblue tetrazolium (NBT) formazon by SOD [23]. Catalase
204 (CAT) was analyzed following the decomposition of hydrogen peroxide [24]. Glutathione
205 peroxidase (GPx) activity was determined according to the ability of GPx to remove H_2O_2 by
206 coupling its reduction to H_2O with oxidation of reduced glutathione in the presence of NADPH
207 [25]. Glutathione reductase (GR) activity was determined based on the capacity of GR to reduce
208 oxidized glutathione back to reduced glutathione (GSH), in presence of NADPH [26], while
209 glutathione transferase (GST) activity was determined by measuring the content of dinitrophenyl

210 thioether produced by the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced
211 glutathione [27]. SOD and CAT activity were expressed as U/mg prot/min, GPx and GR activity
212 were expressed as nmole NADPH oxidized/mg prot/min, while GST activity was expressed as
213 nmol 1-chloro-2,4-dinitro benzene (CDNB)-reduced glutathione (GSH) conjugate/mg
214 protein/min. Protein carbonyl content [28] and thiobarbituric acid-reactive substance (TBARS)
215 [29] were used as markers of protein and lipid oxidative damage, respectively and the results were
216 expressed as nmol/mg protein for total protein carbonyl content, and nmol/100 mg protein for
217 TBARS levels. Nitrite oxide (NO) accumulation in cell culture media was determined by the
218 Griess reagent system following the manufacturer's protocol and NO content was expressed as μM
219 of $NaNO_2/10^5$ cells.

220 ***2.4.4. RT-qPCR analysis of related genes to antioxidant and inflammatory responses***

221 RNA from RAW cells was extracted using the TRIzol™ reagent followed by DNase I treatment
222 to eliminate genomic DNA interference (Turbo™ DNase kit) according to the manufacturer's
223 instructions. RNA concentrations and purity were determined using a NanoDrop 2000
224 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Reverse transcription and
225 subsequent quantitative real-time PCR were performed on a CFX96 Real-Time PCR Systems
226 (Bio-Rad Laboratories Inc., Hercules, CA, USA) using the SuperScript III Platinum SYBR Green
227 One-Step RT-qPCR kit and 100 ng per RNA sample. cDNA synthesis was carried out for 3 minutes
228 at 50°C followed by an initial denaturation step at 95°C for 5 minutes. The cycling conditions
229 comprised an initial denaturation step at 95°C for 5 min, followed by 40 cycles of: denaturation at
230 95°C for 30 s, primer annealing at 65°C for 30s and extension at 72°C for 30s, followed by a final
231 elongation step at 40ºC for 1 min. For gene expression analysis primers for 10 different genes
232 associated with pro-inflammatory, anti-inflammatory, and oxidative stress responses designed
233 online with the NCBI Primer-BLAST tool were used (Table 1). Beta actin (ACTB) was used as a
234 housekeeping gene. Finally, a melting curve analysis, whereby the qPCR products were run from

235 65°C to 95°C and an increment of 0.5°C for 0.05s per step, was used to verify specific product
236 formation. Relative gene expression analysis was calculated using the ΔΔCt method [30].

237 ***2.5. Assessment of inflammasome and autophagy activation***

238 ***2.5.1. Cell culture and treatments***

239 THP-1 cells, a monocyte isolated from peripheral blood from an acute monocytic leukemia
240 patient, were purchased from ATCC (ATCC® TIB-202™) (Manassas, VA, USA) and cultured in
241 RPMI media with 10% fetal serum and penicillin (100 U/ml)/streptomycin (100 µg/ml) at 37°C
242 under an atmosphere of 5% CO_2. To determine the effects of the Andean blackberry crude extract
243 (ABCext) on the LPS + ATP-induced inflammasome, the cells were divided into 3 groups: (*i*)
244 RPMI 1640 medium only (Ctrl group), (*ii*) LPS (*Escherichia coli* serotype 055:B5) at 500 ng/mL
245 LPS for 4h followed by stimulation with ATP (5 mM) for 6h (LPS+ATP group) and LPS (500
246 ng/mL) for 4h followed by ATP (5 mM) and ABCext (80 µg/mL) for 6h (LPS+ATP+ABCext
247 group). The appropriate dose/time combination of LPS and ATP treatments for inflammasome
248 activation was previously established by researchers at the Research Laboratory, Oral Medicine
249 Department, University of Seville, Seville, Spain [31]. Moreover, ABCext was established
250 according to preliminary dosage assays for protein expression induction (data not shown).

251 ***2.5.2. Western blotting***

252 Whole cellular lysate from THP-1 cells was prepared by gentle shaking with a buffer containing
253 0.9% NaCl, 20 mM Tris-HCl, pH 7.6, 0.1% triton X-100, 1 mM phenylmethylsulfonylfluoride and
254 0.01% leupeptin. The protein content was determined by the Bradford method [22].
255 Electrophoresis was carried out in a 10-15 % acrylamide SDS/PAGE and proteins were transferred
256 to Immobilon membranes (Amersham Pharmacia, Piscataway, NJ, USA). Next, membranes were
257 washed with PBS, blocked over night at 4°C and incubated with the primary antibody solution
258 (1:1000) specific for the detection of the following proteins or multiproteic complexes: NLRP3
259 inflammasome, caspase 1, caspase 1 p20 unit (p20), interleukin 1 beta (IL-1β), mature form of IL-
260 1β (p17), the autophagy markers microtubule-associated protein light chain 3 (LC3I and LC3III)

261 and p62. Membranes were then probed with their respective secondary antibody (1:2500).
262 Immunolabeled proteins were detected by chemiluminescence method (Immun Star HRP substrate
263 kit, Bio-Rad Laboratories Inc., Hercules, CA, USA). Western blot images were quantified using
264 ImageJ software (see: http://rsb.info.nih.gov/ij/download.html).

265 ***2.6. Statistical analysis***

266 Statistical analysis was carried out with SPSS v.24 software and the Jamovi built of R statistical
267 language [32–34]. Median ± standard deviation (SD) of three independent experiments are
268 presented. Correlations between the different stimulation groups were processed using one-way
269 ANOVA analysis and the Tukey post hoc test with a $p<0.05$ was considered statistically
270 significant, with a $p<0.01$ being considered as highly significant.

271 **3. Results and Discussion**

272 **3.1. Phenolic composition of Andean blackberries fruit**

273 In this study, a hydroalcoholic extract obtained from Andean blackberries fruit was analyzed
274 for its polyphenol composition by HPLC-DAD-ESI/MS^n. Figure 1A shows representative HPLC
275 chromatograms of the polyphenol profile obtained at different λ (330 and 360 nm for phenolic acid
276 derivatives and flavonoids and 520 nm for anthocyanins), while Fig. 1B shows the specific profile
277 for anthocyanins and Fig. 1C, the specific profile of phenolic acids and flavonoids. On the other
278 hand, Table 2 compiles the tentative identity of the peaks, assigned based on their UV-Vis spectra,
279 mass spectral data of their (pseudo)molecular ions and MS^2 fragmentation patterns. The detected
280 phenolic acids corresponded mainly to hydroxycinnamoyl derivatives, whereas the majority of
281 flavonoid peaks corresponded to the group of flavonols and anthocyanins. Hydroxycinnamates
282 here identified (coumaric acid and ferulic acid derivatives) have been previously reported in berries
283 [4,13], similarly to quercetin and kaempferol which have been reported as the main flavonols
284 present in this fruit [13,18,35]. Among the anthocyanins reported in berries, cyanidin and its
285 glycosides have been identified as the main form of this group of pigments [13,35] in fact, in our
286 study cyanidin was identified predominantly in its 3-form glycosidic and only two minor peaks of

pelargonidin derivatives were detected. In this context, the chemical composition reported here result crucial to justify part of the biological effects reported here below, as well as those previously reported [35]. Hydroxycinnamates, for example, are among the most widely distributed phenylpropanoids present in the insoluble-bound, conjugated-soluble and free forms in plants and render relevant antioxidant activity by their capacity to scavenge different free radicals (FR) such as hydroxyl radical ($HO^•$), superoxide radical anion ($O_2^{-•}$), peroxyl radical ($ROO^•$), peroxynitrite (NO_3^-), singlet oxygen ($^1O_2$) and several organic radicals [36]. Coumaric acids have been reported as an important antioxidant component in different berries [37], while in addition to its antioxidant capacity, it has also been associated with antibacterial, anticancer, antihistaminic effects and an enhancement of ATP production [38]. Quercetin and kaempferol were also associated with significant antioxidant, anti-inflammatory and antitumor effects [7,39], similar to anthocyanins that have been widely studied for their powerful antioxidant and anti-inflammatory effects [7,35,40], antitumoral capacity [7,41], and protective effects against cardiovascular diseases [5,7,42].

3.2 Effects of Andean blackberry extract on radical levels, antioxidant enzyme and oxidative biomarkers in LPS-stimulated cells

During inflammation, different macrophage subsets have a dual behavior: on one side, macrophages displaying an M1 phenotype secrete pro-inflammatory cytokines and produce elevated amounts of reactive oxygen species (ROS) and nitric oxide (NO), enhancing the inflammatory status of the immune response. On the other hand, alternatively activated M2 macrophages are involved in the inflammation resolution and tissue reconstruction, being responsible for apoptotic and necrotic cell phagocytosis and secretion of anti-inflammatory, proangiogenic and profibrotic cytokines, such as interleukin 10 (IL-10), vascular endothelial growth factors (VEGF) and transforming growth factor β (TGF-β), respectively. The excessive production of ROS and NO has been correlated with a chronic inflammation typical of some diseases, including, for example, asthma, ulcerative colitis and rheumatoid arthritis [43].

Therefore, it is very important to investigate the role that these mediators have on these processes, together with the effects of natural extracts in improving inflammation. In line with the above, this work analyzed the effect of ABCext on the intracellular production of ROS and NO in RAW 264.7 macrophages, as well as on a group of oxidative stress markers related to oxidative stress derived from the inflammatory response (Table 3).

Regarding ROS, LPS significantly ($p<0.05$) increased the intracellular % of ROS up to 2.8 times compared to control cells, while the treatment with ABCext led to a slight decrease compared to the control; in the combined treatments, we found that ABCext significantly ($p<0.05$) decreased the intracellular ROS levels, in LPS/ABCext group, but especially in the ABCext/LPS group, compared to LPS group, highlighting the protective effects of blackberry extract before the LPS damage (Table 3). The similar trend was found for NO production (Table 3), with the highest values ($p<0.05$) found in LPS group, the lowest ($p<0.05$) in ABCext group and with the pretreatment with ABCext the most effective ($p<0.05$) compared to the other combined treatments. Our results agree with previous data, which highlighted the ability of the main blackberry compounds, such as anthocyanins, to decrease basal intracellular production of inflammatory-based ROS and NO as a predisposing factor for several noncommunicable chronic degenerative diseases [6,44–46]. In fact, through different pathways, anthocyanins possess the ability to act as reducing agents in the electron-transfer reaction pathway, donating electrons to the free radicals with unpaired electrons [47]. However, recent studies have shown that the biological activities of anthocyanin also extend to other implicated pathways such as improvement of mitochondrial function [48], immunomodulatory properties [49], regulation of lipid metabolism [50], as well as mitochondrial biogenesis and functionality, modulation of intracellular ROS production and regulation of antioxidant enzymes production involved in intracellular antioxidant defense [51].

Regarding the activities of the main antioxidant enzymes, in LPS treated cells, a significant ($p<0.05$) decrease in the activities of GPx, GR, GST, SOD and CAT was observed compared to the control group (Table 3), while in ABCext treated cells similar values with the control group

339 were measured. Interestingly, the pretreatment with ABCext (ABCext/LPS group) exerted a more
340 protective effect against LPS damage, compared to the LPS/ABCext and ABCext+LPS. The
341 effects of ABCext on ROS and NO levels, as well as on antioxidant enzyme activities, followed a
342 similar behavior to protein and lipid damage (Table 3). The highest values ($p<0.05$) of the carbonyl
343 group and TBARS were found in LPS group, while the lowest ($p<0.05$) were measured in ABCext
344 group and in the ABCext/LPS group, confirming the efficacy of the ACNs-rich ABCext treatment
345 in counteracting oxidative damage, as previously reported by our group in ANCs-rich extracts
346 from different red fruits [21,40].

347 **3.2. Effects of Andean blackberry extract on the expression of proinflammatory and**
348 **antioxidant genes in LPS-stimulated RAW 264.7 cells**

349 Given the direct effect of various natural compounds from, the same families as those reported
350 here on gene regulation beyond their antioxidant power [35,49,50], here we investigate the effect
351 of the ABCext on the expression levels of genes that codify for key proinflammatory and
352 antioxidant mediators in LPS-stimulated murine macrophages. Among the genes analyzed, we
353 observed some interesting changes in the expression profile of 7 of these important mediators (Fig.
354 2). More specifically, although the nuclear factor kappa B (NFκB) expression was relatively low,
355 not allowing for statistically significant differences between the different stimulation groups to be
356 revealed, we observed that treating RAW cells with ABCext alone or with LPS and ABCext
357 simultaneously shows a tendency to lower NFκB expression compared with LPS-stimulated cells
358 (Fig. 2A). In the same line, pretreating murine macrophages with ABCext before LPS stimulation
359 appeared to confer a protective effect though lower NFκB expression did not reach statistical
360 significance compared to LPS-stimulated cells.

361 On the other hand, the analysis for tumor necrosis factor (TNF) expression showed statistically
362 significant differences between the different stimulation groups ($p<0.05$) (Fig. 2B). Additionally,
363 we observed that mean TNF expression in both RAW cells treated first with LPS and then ABCext
364 and cells treated first with ABCext and then LPS was almost half of the LPS-treated cells, although

365 these values did not reach statistical significance, while stimulating RAW cells simultaneously
366 with LPS and ABCext did not change *TNF* expression compared with LPS stimulation alone.

367 Interestingly, similar expression patterns were found between the other two proinflammatory
368 cytokines, namely IL-1β and IL-6 (Fig.2C and D, respectively). Indeed, for both genes codifying
369 for these cytokines, treating cells with ABCext alone lowered expression levels compared with
370 LPS-stimulated macrophages (p<0.001) for each gene, respectively) as well as with constitutive
371 expression levels. However, treating cells simultaneously with LPS and ABCext, first with LPS
372 and then with ABCext or first ABCext and then LPS did not manage to significantly lower the
373 expression levels of either of these genes compared with the levels induced by LPS alone
374 stimulation (Fig. 2).

375 Nitric oxide synthase 2 (NOS2*)* expression exhibited a similar pattern whereby ABCext-
376 stimulated cells were found to significantly reduce NOS2 mRNA levels against LPS-treated cells
377 (p<0.05) and even reach lower expression levels than in unstimulated cells (Fig. 2E). However,
378 LPS appears to be a strong promotor of NOS2 activation as either of the treatments that involved
379 LPS and ABCext were shown to induce similar NOS2 expression levels as the LPS alone
380 condition.

381 Finally, NFE2L2 and SOD1 expression patterns were largely similar (Fig. 2F and G,
382 respectively). Treating RAW cells with LPS was shown to lower expression levels of both genes,
383 although the differences with basal expression levels did not reach statistical significance.
384 However, treating cells with ABCext after LPS stimulation incremented the mRNA levels of both
385 genes reaching statistical significance for both superoxide dismutase (SOD1) and nuclear factor
386 erythroid 2-related factor 2 (NFE2L2) (p<0.05). Toll-like receptor (TLR) ligands like LPS and
387 cytokines like IFNG drive M1 polarization. The outcome of this stimulation is a significant
388 increment in the expression of proinflammatory cytokines, such as IL-1β, IL-12, TNF, IL-6,
389 chemokines like IL-8 (CXCL8), MHC-II molecules and production of nitrous oxide and ROS. The
390 principal promoter of the aforementioned mediators is NFκB, a pivotal transcription factor that

391 bridges TLR signalling and the expression of the whole armamentarium of molecules employed
392 by classically activated macrophages [52] Indeed, LPS, a master activator of TLR4, induced
393 overexpression of NFκB, TNF, IL-1β, IL-6, and NOS2, while slightly downregulating the
394 expression of NFE2L2 that codifies for the NRF2 protein, a known regulator of anti-inflammatory,
395 detoxifying and antioxidant responses [53].

396 Treatment with ABCext, whether before, after, or concomitantly with LPS, largely reversed the
397 LPS-induced upregulation of NFκB suggesting an important protective role for ABCext in
398 excessive inflammation, although generally low LPS-induced NFκB expression did not allow for
399 statistical significance to be reached. Moreover, treating murine macrophages with ABCext before
400 or after LPS stimulation seemed to partially mitigate TNF, but not IL-1β or IL-6 upregulation (Fig.
401 2C and D, respectively). This finding implies that the expression of these proinflammatory
402 cytokines is either not exclusively NFκB mediated in RAW cells, as LPS-induced NFκB
403 expression levels suggest, or that these cells are so sensitive to LPS stimulation that even lower
404 concentrations of this ligand are needed to allow the full anti-inflammatory effect of ABCext to
405 be revealed. Additionally, the induction of NFE2L2 and SOD mRNA expression by ABCext in
406 RAW cells, especially if added after LPS stimulation, revealed a possible antioxidant role of this
407 extract in an endeavour to minimize oxidative stress adverse effects.

408 Overall, gene expression analysis in stimulated RAW 264.7 cells strongly suggest an important
409 anti-inflammatory and antioxidant role of ABCext as previously has been demonstrated in ANCs-
410 rich extracts from different fruits [15,48–50,54]. Nonetheless, the anti-inflammatory and
411 antioxidant effects of ABCext warrant further investigation in *in vivo* models and the exact
412 components exerting these effects need to be identified.

413

414 **3.3 Effects of Andean blackberry extract on NLRP3 inflammasome and autophagy 415 process**

416 Given the direct effect that molecular regulation has on the inflammatory response, and
417 therefore on the deleterious effects that it can produce, considering the results presented here, it is
418 imperative to know how the polyphenolic constituents of Andean blackberry can influence the
419 regulation of the response. With this aim, we set out to determine whether ABCext administration
420 can alleviate the inflammatory response by mediating regulation of the NLRP3 inflammasome
421 complex. For this, we used an *in vitro* model of THP-1 cells. Since NLRP3 activation requires two
422 signals, the priming, and the activation signal, we treated the cells with LPS and ATP, respectively
423 [55]. The NLRP3 inflammasome is the best characterized NOD-type receptors (NLR) which plays
424 an essential role in the inflammation response. It is a multiprotein complex, also known as caspase-
425 1 activation platform, which once triggered by pathogens, stress, or danger-signals, leads to the
426 activation of caspase-1(CASP1) and thus to the maturation and secretion of interleukin 1 beta (IL-
427 1β) and interleukin 18 (IL-18) which initiate the inflammatory signalling [56,57]. NLRP3
428 inflammasome plays a crucial role in the inflammation response maintaining the homeostasis by
429 repairing tissues, eliminating pathogens, and sensing and adapting stress levels. However,
430 dysregulation of its activation leads to a wide range of diseases such as metabolic, inflammatory,
431 cardiovascular, neurological and cancer. In addition, the ageing process is commonly accompanied
432 by low grade sterile chronic inflammation, being NLRP3 inflammasome activated and thus leading
433 to the accumulation of pro-inflammatory mediators. This process is already known as inflaming
434 [58,59]. For this reason, it is increasingly necessary to identify compounds, such as those present
435 in food, that target the inflammasome to improve our general health and well-being.

436 The treatment with LPS+ATP in THP-1 cells (LPS+ATP group) significantly increased
437 ($p<0.01$) the expression levels of NLRP3 proteins (1.35 times), activated CASP1 (p20) (1.24
438 times) and activated IL-1β (p17) (0.65 times) compared to the control group (Ctrl group) (Fig.
439 3A), indicating that the NLRP3 inflammasome was triggered and therefore the inflammatory
440 response was on. However, after treatment with ABCext (LPS+ATP+ABCext group), the levels
441 of these proteins showed a significant decrease ($p<0.01$) (0.89 times for NLRP3 protein; 0.73 times

for activated caspase-1 (p20) and 0.95 times for activated IL-1β (p17)~~)~~ compared to the group treated with LPS and ATP (LPS+ATP group). These values are close to the basal levels, where, in the case of activated IL-1β (p17), the levels decreased below the basal values of the control group (Ctrl group) (Fig. 3A). In addition, our results showed that there is a downward trend in NFκB levels after treating the cells with ABCext (Section 3.2 of the Results and Discussion), which correlated with the observed decrease in NLRP3 inflammasome and IL-1β levels after the treatment with ABCext, since NFκB mediates their expression after the priming signal which in our case is the LPS addition. These results are promising since they suggest a possible modulatory effect of Andean blackberry polyphenols, more probably the anthocyanins, on the NLRP3 inflammasome complex, due to its ability to decrease its activation, as well as activated CASP1 and IL-1β protein levels, evidencing its potential anti-inflammatory effects. In fact, several works have already reported the modulating effect of polyphenols, as anthocyanins on the NLRP3 complex [60]. It was recently reported that anthocyanin extract from Purple Sweet Potato was able to restrict NLRP3 inflammasome activation in alveolar macrophages infected with *Klebsiella pneumoniae* [61]. On the other hand, the anthocyanins extracted from the petals of *Hibiscus syriacus* L. were able to inhibit NLRP3 Inflammasome in BV2 microglia cells by alleviating NFκB and endoplasmic reticulum stress induced Ca^{2+} accumulation and mitochondrial ROS production [62]. In general, the anti-inflammatory molecular mechanisms of anthocyanins have been associated with some mechanisms, among which it is worth noting, and which are associated with the results presented here: (*i*) their ability to suppress the activation of NFκB [63], and (*ii*) suppress LRR, NACHT and PYD domains-containing protein 3 (NLRP3) inflammasomes by activation of *NRF2* and the thioredoxin-1/thioredoxin-interacting protein (Trx1/TXNIP) inhibitory complex [64].

After NLRP3 activation, we observed a significant increase (p<0.01) in the levels of p62 and the LC3I/LC3II ratio in the LPS+ATP group, proteins that play a fundamental role in the autophagy process (Fig. 3B). It consists in a guided degradation of unnecessary or dysfunctional

468 cellular components such as misfolded proteins and dysfunctional organelles by a double
469 membrane vesicle, or autophagosome, and then delivered to the lysosomes for their degradation
470 [65]. High levels of p62 together with high levels of LC3I/LC3II ratio suggest an impairment in
471 the autophagy with the accumulation of non-degraded substrates. However, after treatment with
472 ABCext (LPS+ATP+ABCext group), the p62 levels showed a significant decrease (p<0.01) and
473 the LC3I/LC3II ratio increase ($p < 0.05$), compared to the LPS+ATP group, indicating a restoration
474 of the autophagy process (Fig. 3B). Autophagic dysfunction, as well as NLRP3 dysregulation, are
475 associated with ageing and age-related diseases. By treating the cells with ABCext after triggering
476 the inflammatory response, we have observed a reduction in NRLP3 protein level which leads,
477 according to other studies [66–68], to a decrease in p62 and an increase in the LC3I/LC3II ratio,
478 restoring the autophagy flux. In light of these results the consumption of Andean blackberry
479 polyphenols, more probably the anthocyanins seem certainly promising to treat inflammatory-
480 based and age-related diseases. The results presented here agree with several studies that have
481 demonstrated that anthocyanins from different natural sources induce autophagy [69,70]. More
482 recently it was demonstrated that anthocyanins from *Sambucus canadensis* could significantly
483 reduce cell senescence and lens ageing by increasing autophagic and mitophagy flux and
484 enhancing mitochondria and cell renewal through the inhibition of the PI3K/AKT/mTOR
485 signaling pathway, which leads to attenuation of ageing [71].

486 **4. Conclusions**

487 According to the results presented here, it was possible to demonstrate a modulating effect of
488 inflammation by the polyphenolic components of the Andean blackberry. This effect was not only
489 due to its antioxidant capacity, evidenced in the improvement of markers of oxidative damage to
490 cellular macromolecules, but also through its ability to regulate certain genes related to the
491 antioxidant and inflammatory response. The components of the Andean blackberry showed a
492 modulatory effect of the NLRP3 inflammasome complex, decreasing its activation, in addition to
493 restoring the autophagy process, which suggests a direct action of Andean blackberry compounds

494 on the inflammatory response and restoration of the autophagy process. Our findings require
495 further investigation, and more efforts in *in vivo* studies and intervention models evaluating the
496 impact of Andean blackberry components on modulating inflammatory and antioxidant responses
497 and the direct involvement of the NLRP3 complex in these effects should be done.

498 **Conflicts of interest**

499 The authors declare that they have no known competing financial interests or personal
500 relationships that could have appeared to influence the work reported in this paper.

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712

713 **FIGURE CAPTION**

714 **Figure. 1.** HPLC chromatograms of phenolic acid derivatives and flavonoid profile in the crude
715 extract of Andean blackberry (ABCext). A, shows representative HPLC chromatograms of the
716 polyphenol profile for phenolic acid derivatives and flavonoids recorded at 330 and 360 nm and
717 for anthocyanins at 520 nm. B, shows the specific profile of anthocyanins (at 520 nm) and C, the
718 specific profile of phenolic acids and flavonoids (at 330 nm).

719

720 **Figure. 2.** Expression of genes related to the inflammatory response and antioxidant response in

721 RAW 264.7 murine macrophages treated with different combinations of ABCext and/or LPS. Data

722 are expressed as mean values ± SD. $p<0.05$, $p<0.01$, significant differences between the

723 experimental groups.

724

725 **Figure. 3.** Expression levels of the proteins studied. Data are expressed as mean values ± SD.

726 $^*p<0.05$, $^{**}p<0.01$, significant differences between the experimental goups.