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

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1 **Modulatory effect of Andean blackberry polyphenols on genes related to antioxidant and**

2 **inflammatory responses, the NLRP3 inflammasome and autophagy**

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Abstract

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

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

1. Introduction

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

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

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

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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

decay. Two batches of samples (~1kg) were collected on each occasion and the specimens were identified using the reference vouchers for specimens deposited in the herbarium of the Jardín Botánico de Quito, Ecuador. During collection time, the fruits were kept at 4°C protected from light and transported to the laboratory the same day they were harvested. Afterwards, samples were cleaned, removing remains of leaves, stems and other residues, washed with distilled water and allowed to dry on absorbent paper at room temperature and protected from light. Finally, fruits were frozen at -20 °C overnight and then freeze-dried, ground to a fine powder, and stored at -20°C until analysis.

For chemical composition determination and *in vitro* assays, a polyphenol-rich extract was obtained as previously reported [20]. Briefly, the fine powder of fruits (10g) was added to 100 mL of the extraction solution consisting of methanol acidified with 0.1% HCl and Milli-Q water (80%, *v/v*) and stirred overnight at 3–5°C. Then, the solution was centrifuged for 15 minutes at 4000*g* at 4°C and filtered through a 2.5 µm pore size Whatman® cellulose filter paper (Sigma-Aldrich Co., St. Louis, MO, USA). The solid residues were washed with the extraction solution as many times as necessary until no red colour was observed in the extracted solution. Finally, the resulting extraction solutions were combined, water was added, and the final solution was concentrated in a vacuum rotary evaporator (~30°C) until obtaining a dry crude extract.

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

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

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

2.4. Evaluation of anti-inflammatory properties

2.4.1. Cell culture and treatments

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

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.

2.4.3. Determination of antioxidant enzyme activities, biomarkers of oxidative damage in lipid 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

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

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

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

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.

3. Results and Discussion

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$^\bullet$), superoxide radical anion (O$_2^{-\bullet}$), peroxyl radical (ROO$^\bullet$), peroxynitrite (NO$_3^-$), singlet oxygen (^{1}O$_2$) 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].

313 Therefore, it is very important to investigate the role that these mediators have on these processes,

314 together with the effects of natural extracts in improving inflammation. In line with the above, this

315 work analyzed the effect of ABCext on the intracellular production of ROS and NO in RAW 264.7

316 macrophages, as well as on a group of oxidative stress markers related to oxidative stress derived

317 from the inflammatory response (Table 3).

318 Regarding ROS, LPS significantly ($p<0.05$) increased the intracellular % of ROS up to 2.8

319 times compared to control cells, while the treatment with ABCext led to a slight decrease compared

320 to the control; in the combined treatments, we found that ABCext significantly ($p<0.05$) decreased

321 the intracellular ROS levels, in LPS/ABCext group, but especially in the ABCext/LPS group,

322 compared to LPS group, highlighting the protective effects of blackberry extract before the LPS

323 damage (Table 3). The similar trend was found for NO production (Table 3), with the highest

324 values ($p<0.05$) found in LPS group, the lowest ($p<0.05$) in ABCext group and with the

325 pretreatment with ABCext the most effective ($p<0.05$) compared to the other combined treatments.

326 Our results agree with previous data, which highlighted the ability of the main blackberry

327 compounds, such as anthocyanins, to decrease basal intracellular production of inflammatory-

328 based ROS and NO as a predisposing factor for several noncommunicable chronic degenerative

329 diseases [6,44–46]. In fact, through different pathways, anthocyanins possess the ability to act as

330 reducing agents in the electron-transfer reaction pathway, donating electrons to the free radicals

331 with unpaired electrons [47]. However, recent studies have shown that the biological activities of

332 anthocyanin also extend to other implicated pathways such as improvement of mitochondrial

333 function [48], immunomodulatory properties [49], regulation of lipid metabolism [50], as well as

334 mitochondrial biogenesis and functionality, modulation of intracellular ROS production and

335 regulation of antioxidant enzymes production involved in intracellular antioxidant defense [51].

336 Regarding the activities of the main antioxidant enzymes, in LPS treated cells, a significant

337 ($p<0.05$) decrease in the activities of GPx, GR, GST, SOD and CAT was observed compared to

338 the control group (Table 3), while in ABCext treated cells similar values with the control group

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

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

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

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

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

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

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

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

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

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

442   for activated caspase-1 (p20) and 0.95 times for activated IL-1β (p17)) compared to the group

443   treated with LPS and ATP (LPS+ATP group). These values are close to the basal levels, where, in

444   the case of activated IL-1β (p17), the levels decreased below the basal values of the control group

445   (Ctrl group) (Fig. 3A). In addition, our results showed that there is a downward trend in NFκB

446   levels after treating the cells with ABCext (Section 3.2 of the Results and Discussion), which

447   correlated with the observed decrease in NLRP3 inflammasome and IL-1β levels after the

448   treatment with ABCext, since NFκB mediates their expression after the priming signal which in

449   our case is the LPS addition. These results are promising since they suggest a possible modulatory

450   effect of Andean blackberry polyphenols, more probably the anthocyanins, on the NLRP3

451   inflammasome complex, due to its ability to decrease its activation, as well as activated CASP1

452   and IL-1β protein levels, evidencing its potential anti-inflammatory effects. In fact, several works

453   have already reported the modulating effect of polyphenols, as anthocyanins on the NLRP3

454   complex [60]. It was recently reported that anthocyanin extract from Purple Sweet Potato was able

455   to restrict NLRP3 inflammasome activation in alveolar macrophages infected with *Klebsiella*

456   *pneumoniae* [61]. On the other hand, the anthocyanins extracted from the petals of *Hibiscus*

457   *syriacus* L. were able to inhibit NLRP3 Inflammasome in BV2 microglia cells by alleviating NFκB

458   and endoplasmic reticulum stress induced Ca^{2+} accumulation and mitochondrial ROS production

459   [62]. In general, the anti-inflammatory molecular mechanisms of anthocyanins have been

460   associated with some mechanisms, among which it is worth noting, and which are associated with

461   the results presented here: (*i*) their ability to suppress the activation of NFκB [63], and (*ii*) suppress

462   LRR, NACHT and PYD domains-containing protein 3 (NLRP3) inflammasomes by activation of

463   *NRF2* and the thioredoxin-1/thioredoxin-interacting protein (Trx1/TXNIP) inhibitory complex

464   [64].

465   After NLRP3 activation, we observed a significant increase ($p < 0.01$) in the levels of p62 and

466   the LC3I/LC3II ratio in the LPS+ATP group, proteins that play a fundamental role in the

467   autophagy process (Fig. 3B). It consists in a guided degradation of unnecessary or dysfunctional

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

4. Conclusions

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

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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

Figure. 2. Expression of genes related to the inflammatory response and antioxidant response in RAW 264.7 murine macrophages treated with different combinations of ABCext and/or LPS. Data are expressed as mean values ± SD. *p<0.05*, *p<0.01*, significant differences between the experimental groups.

724

Figure. 3. Expression levels of the proteins studied. Data are expressed as mean values ± SD. *p<0.05*, **p<0.01*, significant differences between the experimental goups.