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Characterization of Andean Blueberry in Bioactive Compounds, Evaluation of Biological Properties and *in vitro* Bioaccessibility

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Abstract: Andean blueberries are wild berries grown and consumed in Ecuador which contain high values of bioactive compounds, mainly anthocyanins, with powerful antioxidant activity. The aim of this study was to evaluate the profile and contents of (poly)phenols and carotenoids in Andean blueberry by HPLC-DAD-MSⁿ and determine a wide range of its biological activities. The antioxidant capacity of this fruit was evaluated *in vitro* by three different methods and *in vivo* using the zebrafish animal model, also the toxicity effect was determined by the zebrafish embryogenesis test. Besides, the antimicrobial activity and the capacity of Andean blueberry to produce hemagglutination in blood cells were evaluated. Finally, the bioaccessibility of (poly)phenols and related antioxidant capacity were determined in the different phases of an *in vitro* digestion. The global results indicated no toxicity of Andean blueberry, weakly bacteriostatic activity, and high contents of anthocyanins and antioxidant capacity, which were partially bioaccessible *in vitro* (~ 50 % at the final intestinal step), contributing to the knowledge of its health benefits for consumers and its potential use in the food and pharmaceutical industry as functional ingredient.

Keywords: mortiño; *Vaccinium floribundum*; HPLC-MS/MS; anthocyanins; antioxidant; antimicrobial; toxicity; Zebrafish.

1. Introduction

The consumption of berries has been related with health-promoting effects, such as reductions in the incidence of degenerative and chronic diseases (cardiovascular diseases, type 2 diabetes and certain types of cancer, among others), mainly due to the presence of bioactive compounds (phenolic compounds, vitamins and carotenoids), associated to radical scavenging capacity and epigenetic mechanisms [1]. Clinical intervention studies have also shown that phenolic compounds from berries, particularly anthocyanins, were able to improve the profile of inflammatory markers and the total antioxidant status, being these effects more evident with chronic dietary interventions [2].



Among berry fruits, Andean blueberry (*Vaccinium floribundum* Kunth), also known as mortiño, is a promising wild berry of the family *Ericaceae* that grows spontaneously in the Andean regions of Ecuador. The demand of these small (~8 mm diameter), black and round fruits has been increased due to their antioxidant characteristic, similar to other *Vaccinium* species, such as cranberry, blueberry or bilberry, mostly related to the high content in (poly)phenolic compounds.

The phytochemical evaluation of these fruits is essential to assess their potential health-promoting effects before an intervention study, establishing their characteristics to be used for the food, nutraceutical and pharmaceutical industries. Unlike many other Ibero-American fruits and vegetables, the carotenoid profile of Andean blueberry is basically unknown [3]. The study of carotenoids is very important as they are very versatile compounds with many applications in agro-food and nutricosmetics [4,5]. As far as we know, only few works have published the profile and content of phenolic compounds in *V. floribundum* Kunth evaluated by HPLC-MS/MS [6,7]. These results are varied and influenced by many factors, including differences among varieties, maturity of the fruit, environmental parameters and pre/postharvest handling [8]. Aside of the phytochemical evaluation, *in vitro* antioxidant capacities [7,9] and antimicrobial activities [10] have been reported in Andean blueberry. However, further experiments are required before taking a step ahead through *in vivo* assays and clinical trials. In this sense, the aim of this study was to evaluate the phytochemical profile of *V. floribundum* Kunth from the local market in Machachi (Ecuador) by HPLC-DAD-MS/MS, assess its antioxidant capacity *in vitro* (by ABTS, DPPH and ORAC methods) and the antimicrobial activity against *S. aureus* and *E. coli*, identifying substantial differences with previous reports. In addition, the *in vivo* toxicity effect by the zebrafish embryogenesis test, the *in vivo* antioxidant capacity using the zebrafish animal model (TBARS test) and the capacity of Andean blueberry to produce hemagglutination in blood cells, were investigated for the first time. Finally, the bioaccessibility of phenolic compounds was studied after an *in vitro* gastrointestinal digestion, evaluating also the antioxidant activity in the different phases of digestion, which may ultimate the physiological effect and role of Andean blueberry within the organism. These results make advances of the knowledge about the health benefits linked to Andean blueberry consumption, related to bioactivity, bioaccessibility and safety, being essential before carry out further *in vivo* assays and clinical trials.

2. Materials and Methods

2.1. Fruit samples

Andean blueberry (*Vaccinium floribundum* Kunth) was purchased at local market in Machachi, Ecuador. These fruits were selected, cleaned, washed with tap water, disinfected using 100 ppm of chlorine and freeze dried. Then, samples were packed and stored at -20°C until analysis.

2.2. Standards, chemicals and solvents

The commercially available standards 5-*O*-caffeoyl-quinic acid and rutin (quercetin-3-rutinoside) were acquired from Sigma-Aldrich Chemie GmbH (Germany). Cyanidin 3-*O*-glucoside was purchased from Polyphenols (Norway). Lutein was obtained according to Meléndez-Martínez et al. [11]. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was obtained from Fluka Chemika (Neu-Ulm, Switzerland). The reagents 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS⁺), monobasic and dibasic sodium phosphate, Folin Ciocalteu's reagent and fluorescein (free acid) were purchased from Sigma-Aldrich Chemie GmbH (Germany). Finally, formic acid and solvents (ethanol, methanol, hexane, acetone, dichloromethane and acetonitrile) were all of analytical grade and were obtained from Merck (Darmstadt, Germany).

2.3. Physicochemical analysis

Weight, length, and diameter were determined according to the methods described by NTE INEN 2427 [12]. The pH of the fruits was analyzed using a pH meter Seven Compact pH/Ion S220 (Mettler Toledo, Greifensee, Switzerland), following the method described by Association of Official

Analytical Chemists (AOAC method 981.12) [13]. The method used to determine the moisture of the fruits included drying to constant weight in a vacuum oven at 70 °C and 100 mm Hg pressure until to reach a high level of water evaporation (max. 6 h) (AOAC method 950.27) [13]. The results were expressed in percentage (g H₂O 100 g⁻¹ of sample). Soluble solids were assayed using a Portable Brix Refractometer VBR90S (Boeco, Germany) (AOAC method 931.12) [13]. The results were expressed as °Brix. The titratable acidity was determined by the method suggested by AOAC (method 942.15) [13], performing titration with NaOH 0.1 N. The results were expressed in % of citric acid. Every assay was conducted in triplicate.

2.4. Identification and quantification of phenolic compounds by HPLC–DAD–ESI/MSⁿ

Phenolic compounds were extracted and analyzed following the protocol and method of Gironés-Vilaplana et al. [14]. Briefly, samples were extracted with MeOH 70 % using the ultrasound technology and kept at 4 °C overnight. Then, samples were filtered and the identification of phenolic compounds was carried out following their MS² fragmentations by an HPLC–DAD–ESI/MSⁿ, constituted by an Agilent series model HPLC 1100 with a photodiode array detector and a mass spectrometer detector in series (model G2445A) equipped with an electrospray ionization interface (Agilent Technologies, Waldbronn, Germany). The ionization conditions were selected according to those described in the method, covering an m/z range from 100 to 1200. The acquisition of the mass spectrometry data (MSⁿ) was performed in the negative ionization mode for flavonols and phenolic acids, except for anthocyanins, where the positive ionization mode was used. The quantification of phenolic compounds was performed in an Agilent 1220-Infinity HPLC-DAD system equipped with a Luna C18 column (25 cm × 0.46 cm, 5 µm particle size; Phenomenex, Macclesfield, UK) using the acquisition conditions described before. Flavan-3-ols were quantified using the external standard (+)-catechin at 280 nm, flavonols at 360 nm using the standard rutin (quercetin-3-rutinoside) and the anthocyanins by using cyanidin 3-O-glucoside at 520 nm [14]. Samples were extracted and analyzed in triplicate. Results were expressed as µg g⁻¹ dry weight (DW).

2.5. Identification and quantification of isoprenoids (carotenoids and α-tocopherol) by RRLC

The extraction and analyses of carotenoids were carried out according the method described by Stinco et al [15]. Samples (200 mg) were extracted with 1 mL of hexane/acetone (1:1 v/v) using a vortex and an ultrasonic bath for 2 min. Then, samples were centrifuged at 18,000 × g for 5 min and the colored fractions were recovered. The extraction was performed twice more until color extinction. Finally, the carotenoids extract were concentrated to dryness in a rotary evaporator at temperature below 30 °C. To obtain saponified carotenoids, the extracts were treated with 1000 µL of dichloromethane and 1000 µL of methanolic KOH (30 % w/v) for 1 h under dim light and at room temperature, after which they were washed with water to remove any trace of base. The extracts obtained were concentrated to dryness in a rotary evaporator and re-dissolved in ethyl acetate prior to their injection in the RRLC system. Samples were extracted and analyzed in triplicate. The RRLC acquisitions were made by using an Agilent 1260 system equipped with a diode-array detector, which was set to scan from 200 to 770 nm, and a Poroshell 120 C18 column (2.7 µm, 5 cm × 4.6 mm) (Agilent, Palo Alto, CA) kept at 28 °C. Chromatograms were monitored at 450 nm. The identification and quantification of isoprenoids were performed by comparison of their chromatographic UV–vis spectroscopic characteristics with the standards, as well as by comparison with the external calibration line calculated [15]. Results were expressed as µg/ g DW.

2.6. Zebrafish larvae collection and toxicity test

Adult male and female wild-type zebrafish (*Danio rerio*) were obtained from a commercial fish farm and 10 fish were kept in a 10-L glass tank under the following conditions: 28.5 °C, with a 16/8 h light/dark cycle (Westerfield, 2000). Fish were fed three times a day, 6 days a week with Tetramin flake food supplemented with live brine shrimps (*Artemia salina*). Zebrafish embryo toxicity test were performed using the method of Murphey and Zon [16], with some modifications. Embryos were

obtained by photo-induced spawning over green plants and then, cultured at 28 °C in a fishbowl. Five-hour post fecundation larvae (30 larvae per well) were incubated in 24-well plates with 1 mL of water. Freeze-dried Andean blueberry was extracted in water under constant agitation for 30 min, then, samples were centrifuged (10,000 × g, 5 min) and the supernatant was filtered. Andean blueberry was added at different concentrations (10.0, 5.0, 2.0, 1.0, 0.5, 0.2 and 0.1 mg/mL) in 200 µL of water. The effect of extract on larvae was measured after 48 h. At the end of incubation time, larvae mortality and morphologic changes were observed under microscope, determining the percentage of dead larvae. In the control wells, there should be less than 10 % of dead eggs (coagulation of fertilized eggs, lack of somite formation, lack of tail detachment from the yolk sac and lack of heartbeat).

2.7. Antioxidant capacity

2.7.1. Antioxidant capacity *in vitro*

The antioxidant capacity was evaluated using the methods DPPH, ABTS and ORAC adapted to a microscale and performed using 96-well micro plates (Nunc, Roskilde, Denmark), which were measured using an Infinite® M200 microplate reader (Tecan, Grödig, Austria). The power of scavenge DPPH and ABTS radicals was determined according to Mena et al. [17], briefly, 2 µL of the corresponding diluted sample (previously extracted for phenolic compounds analysis) was added to the wells containing the stock solution (250 µL) with absorbance ~1. Then, the plate was shaken and left for 50 min at 37 °C, thus, the variation in absorbance was measured at 515 nm or 414 nm, for DPPH or ABTS methods, respectively. Regarding the ORAC method [18], 25 µL of the properly diluted sample was added to 150 µL of fluorescein (1 µM) and, after 30 min of incubation, 25 µL of the radical AAPH (2,2'-azobis(2-methyl-propionamidine)-dihydrochloride) (250 mM) was added to the wells. Results were studied by measuring the variation in fluorescence each 2 min during 120 min of reaction with the radical. Trolox was used as standard in all the methods, following the same procedure than with the samples. According to the solubilizing agents used in the methods, samples were dissolved in MeOH in the DPPH method to evaluate less polar compounds, while in ABTS and ORAC methods, samples were dissolved in water to evaluate more polar compounds. Results were expressed as µmol Trolox equivalents (TE) g⁻¹ DW. Assays were carried out by triplicate.

2.7.2. Antioxidant capacity *in vivo*: Thiobarbituric Acid Reactive Substances (TBARS) in Zebrafish Larvae Model

The TBARS method in zebrafish larvae was used as described by Carrillo et al. [19]. Briefly, freeze-dried Andean blueberry was extracted in water under constant agitation for 30 min, then, samples were centrifuged (10,000 × g, 5 min) and the supernatant was filtered. Five days post fecundation, larvae were incubated in 24-well plates (30 larvae/well in triplicate) with different concentrations of Andean blueberry (1.0, 0.5, 0.2 and 0.1 mg/mL). Groups of 30 larvae/well in aquarium water were used as negative control. Lipid peroxidation was initiated by adding 1 mL of 500 µM H₂O₂ and incubated for 8 h at 28 °C. Then, H₂O₂ was removed and 500 µL of Tween 0.1 % were added. Larvae were mixed and homogenized with a homogenizer (T25 basic Ultra Turrax IKA, Thermo Fisher Scientific, Germany). After that, 1 mL of TBA 1 % was added and the solution was heated at 95 °C for 1 h, and then cooled down for 15 min. Absorbance of the final solution was measured at 532 nm using a spectrophotometer (Thermo Scientific Evolution 200, Germany). The synthetic antioxidant butylated hydroxytoluene (BHT) was used as positive control (0.1 mg/mL). The values of antioxidant capacity were expressed as the percentage of inhibition of lipid peroxidation in larvae homogenate as follows: % Inhibition of lipid peroxidation = [(Ab - As)/Ab] × 100, where Ab is the absorbance of blank and As is the absorbance of the sample.

2.8. Determination of anti-nutritional factor: lectins

The lectin content was determined through a hemagglutination assay, following the protocol of Boeri et al [20]. Briefly, two-fold serial dilutions of Andean blueberry (2.5, 1.25, 0.625, 0.312, 0.156 mg mL⁻¹) in 10 mM phosphate buffered saline (PBS, pH 7.4, 50 µl) were mixed with 50 µL of 4 % human

erythrocyte (group 0, Rh+) suspension in 96-well microtiter plates at 30 °C. The results of agglutination were visible macroscopically in the plate-wells, observed after 1 h of incubation.

2.9. Antimicrobial activity

Bacterial strains *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used for the screening of antibacterial activity. The antibacterial assays were performed using the method of García-Ruiz et al. [21], with some slight modifications. Inhibition of microbial growth by Andean blueberry was determined by the microtiter dilution method, using serial double dilutions of the antimicrobial agent (from 10 to 0.078 mg mL⁻¹) and initial inoculum of 5 × 10⁵ CFU mL⁻¹. Bacterial growth was determined by reading the absorbance at 620 nm. Growth inhibitory activity was expressed as a mean percentage of growth inhibition with respect to a control without antimicrobial sample as follow: % Inhibition of bacterial growth = [(Ab - As)/Ab] × 100, where Ab is the absorbance of blank and As is the absorbance of the sample.

2.10. In vitro gastrointestinal digestion

An *in vitro* gastrointestinal digestion procedure mimicking the physiological situation in the oro-gastro-intestinal transit was used according to the method of Villacis-Chiriboga et al. [22], with modifications. Briefly, 0.5 g of free-dried Andean blueberry were dissolved in 11 mL of ethanol–water (6:94), was adjusted to pH 7 with 3 mL of artificial saliva (0.22 g/L CaCl₂, 6.2 g/L NaCl, 2.2 g/L KCl, 1.2 g/L NaHCO₃) and shaking (150 rpm) for 2 min at 37 °C (oral phase). Then, samples were adjusted to pH 2.0 with HCl 5 M, and subjected to incubation in a water bath (Precision Scientific model 25, Chicago, USA) at 37 °C for 2 h under constant stirring of 150 rpm in the presence of 0.7 mL of stomach solution (20 mg of porcine pepsin and 0.7 mL of 0.1 M HCl) (gastric phase). The gastric digests were maintained in ice for 10 min to stop pepsin digestion. After this, samples were adjusted to pH 6.0 with NaHCO₃ (20 g 100 mL⁻¹) and 2.25 mL of intestinal solution (18 mg of pancreatin with 112.5 mg of bile salts dissolved in 4.5 mL of NaHCO₃ 1N) (intestinal initial phase). To stop intestinal digestion, the sample was kept for 10 min in an ice bath. Then, the sample was adjusted to pH 7.5 with NaHCO₃ 1 N at 37 °C for 2 h under constant stirring of 150 rpm (intestinal final phase). Finally, the sample was adjusted to pH 7.2 with NaOH 0.5 M and centrifuged at 5000 rpm for 20 min at 4 °C (digestion final phase). Aliquots of 2 mL of digested sample in each phase were transferred to 2.0 mL eppendorf tubes and centrifuged at 5000 rpm for 20 min at 4 °C in a Centrifuge 5415D (Eppendorf, Hamburg, Germany). Supernatants obtained were used to determine the antioxidant capacity by the ABTS method (described in section 2.7.1.), expressed as μmol TE g⁻¹ DW, and phenolic contents (bioaccessible fraction), expressed as mg of gallic acid equivalents (GAE) per gram DW. Bioaccessibility (%) was calculated as the percentage of total phenolic compounds content remaining in the bioaccessible fraction related to the original nondigested sample (Bioaccessibility % = 100 × (bioaccessible phenol content/TPC)).

2.11. Total phenolic contents

Total phenolic contents (TPC) of Andean blueberry samples were determined colorimetrically using the Folin-Ciocalteu reagent as described by Slinkard and Singleton [23]. Briefly, 500 μL of the extracts, blank or standards were placed in a 15 mL tube, where 2.5 mL of the Folin–Ciocalteu reagent was added, allowing to react for 2 min while shaking. Then, 2 mL of a solution of sodium carbonate (75 g/L) was added and properly mixed. The solution was thus incubated 15 min at 50 °C. After that, the absorbance was measured at 750 nm in a spectrophotometer (Shimadzu UV-160A, Kyoto, Japan). Gallic acid was used as standard (10–90 mg/L) and the results were expressed as mg GAE g⁻¹ DW. This assay was carried out in triplicate.

2.12. Statistical analysis

All analysis were conducted in triplicate, mean values (n = 3) ± standard deviation (SD). The data were processed using the Graph Pad Prism 6 (La Jolla, CA, US). A multifactorial analysis of variance

(ANOVA) and Tukey's multiple-range test were carried out to determine significant differences at p values < 0.05 . A nonlinear regression "Dose-response-inhibition" was used to determine the IC_{50} in zebrafish experiments.

3. Results and Discussion

3.1. Physicochemical Characterization

Andean blueberry fruits (*Vaccinium floribundum* Kunth) had high water content (~ 89 %) and appropriate size (weight, length and diameter) within the quality standards for blueberries (Table 1), nevertheless, these parameters are very varied among species and varieties [24,25]. Sugar concentration and the pH are important parameters for evaluating blueberry quality, this fruit had low pH (2.6), titratable acidity (TTA) of 1.6 %, and high amount of soluble sugars (11.2 °Brix), according to the expected range of pH 2.7 – 3.8, TTA values between 0.3 and 1.3 %, and > 11 °Brix, reported for other blueberry cultivars, being these values also influenced by environmental and growing conditions [26–28].

Table 1. Physico-chemical characterization of Andean blueberry (*Vaccinium floribundum* Kunth).

Parameters	Content
Weight (g unit ⁻¹)	3.5 ± 0.05
Length (cm unit ⁻¹)	1.75 ± 0.04
Diameter (mm unit ⁻¹)	8.5 ± 0.75
pH	2.61 ± 0.05
Moisture (%)	88.69 ± 0.08
° Brix	11.17 ± 0.03
Titratable acidity (% citric acid)	1.62 ± 0.00

¹ Mean values of three determinations ± standard deviation (SD). Fresh weight basis.

In this sense, Andean blueberry is a sweet fruit with pleasant acid flavor that could be consumed not only fresh but also as derived products, such as juice, jam, jelly, wine, or could be used as food ingredient with potential technological applications, such as antioxidant and dyeing properties [26,29].

3.2. Identification and quantification of bioactive compounds

In Andean blueberry fruits, mainly phenolic compounds were detected and also one carotenoid was found. The characterization of phenolic compounds of these fruits was performed by the identification of individual compounds by HPLC–DAD–ESI/MSⁿ (Table 2) and the subsequent quantification using HPLC–DAD (Table 3), revealing a wide range of different (poly)phenols. A total of 16 phenolic compounds were identified following their main ion [M-H]⁻ (m/z) and MSⁿ fragmentation ions.

Table 2. Phenolic compounds detected and characterized tentatively in Andean blueberry samples (n=3) by HPLC–DAD–ESI/MSⁿ. Compounds were numbered by their elution order.

Peak number	Rt (min)	DAD λ (nm)	[M-H] ⁻	Fragment ions (MS ⁿ)	Phenolic Compounds ¹
1	6.0	330	353	191, 179, 707	3-O-Caffeoylquinic acid*
2	10.8	330	353	191	5-O-Caffeoylquinic acid
3	16.7	280, 520	465	303	Delphinidin-3-O-glucoside I
4	18.5	280, 520	465	303	Delphinidin-3-O-glucoside II
5	19.6	280, 520	449	287	Cyanidin -3-O-glucoside I

6	20.8	280, 520	435	303	Delphinidin-3-O-pentose
7	21.8	280, 520	449	287	Cyanidin-3-O-glucoside II
8	23.9	280, 520	419	287	Cyanidin-3-O-pentose
9	26.6	320	335	179, 161, 131	Caffeoylshikimic acid
10	28.7	360	433	323, 179, 161	Caffeic acid derivative
11	33.6	360	463	301	Quercetin-3-O-glucoside I
12	35.2	360	463	301	Quercetin-3-O-glucoside II
13	37.5	360	433	301	Quercetin-3-O-pentose I
14	39.6	360	433	301	Quercetin-3-O-pentose II
15	41.2	360	433	301	Quercetin-3-O-pentose III
16	42.8	360	447	301	Quercetin-3-O-rhamnoside

¹ Identification of phenolic compounds based on the ion $[M-H]^-$ (m/z) in the negative mode, fragment ion (MS^n) data, and retention time compared with standards and reference sources. *Dimeric adduct. Rt: retention time.

Four hydroxycinnamic acids were found, being all of them caffeoyl acid derivatives. Compound **1** was found as an adduct of 3-O-Caffeoylquinic acid, with its $[2M - H]^-$ adduct ion at m/z 707 and $[M - H]^-$ ion at m/z 353, which produced MS^2 ions at m/z 191 and m/z 179, which evidenced its tentative identification [30]. The 5-O-caffeoylquinic acid (**2**) also showed $[M - H]^-$ ion at m/z 353 and the daughter ion at m/z 191. Compound **9**, caffeoylshikimic acid, gave its characteristic $[M - H]^-$ ion at m/z 335 with MS^2 fragmentation peaks at m/z 179, 161 and 131 [6,7,31]. Finally, compound **10** exhibited $[M - H]^-$ ion at m/z 433 and gave MS^2 fragmentation peaks at m/z 323, 179, 161, being characteristic of caffeoyl quinic acid derivatives [32]. This information, along with its characteristic spectrum with absorption at 320 nm, lead us to the tentative identification as caffeic acid derivative, according to previous works analyzing *V. floribundum* [6] and *V. meridionale* [33].

The compounds **3** – **8** were detected as glycosylated anthocyanins derivatives of delphinidin and cyanidin, with typical molecular ion at m/z 303 and 287, respectively, bound to a glucose or pentose, with a loss of 162 or 132 mass units, respectively. This anthocyanin profile agrees with previous works analyzing Andean blueberry [6,7].

The compounds **11** – **16** belonged to the flavonol family, being all of them derivatives of quercetin, with the typical MS^2 fragment of m/z 301, and a loss of 162 mass units in case of glucose, 132 due to pentose and 146 because of the deoxyhexoside rhamnose. Other authors also found quercetin-3-glycosides as the predominant flavonols in this fruit [3], additionally, small amounts of two different myricetin derivatives were identified in mortiño berries [4].

The quantification of phenolic compounds (Table 3) showed anthocyanins as the main group present in the samples (~60 % of the total phenolic compounds). Among them, cyanidin-3-O-pentose and cyanidin-3-O-glucoside I were the predominant anthocyanins (~80 % of the total), followed by delphinidin glycosides, accounting for the 19 %. These results agree with the distribution of anthocyanins described in *V. floribundum* before [6,7,34]. This accumulation of delphinidin and cyanidin-type anthocyanins have been related to the deep purple-black color of berries [35].

Table 3. Phenolic compounds and carotenoids quantified in Andean blueberry (n=3).

Phenolic compounds	Concentration
Compounds	($\mu\text{g/g DW}$)
<i>Hydroxycinnamic acids</i>	
3-O-Caffeoylquinic acid	236.1 \pm 37.7 ¹
5-O-Caffeoylquinic acid	845.5 \pm 1.25
Caffeoylshikimic acid	35.8 \pm 1.58

Caffeic acid derivative	273.0	± 40.0
Total	1390.3	± 78.9
<i>Anthocyanins</i>		
Delphinidin-3-O-glucoside I	395.7	± 58.5
Delphinidin-3-O-glucoside II	274.0	± 50.0
Cyanidin-3-O-glucoside I	1963.9	± 140
Delphinidin-3-O-pentose	392.1	± 29.5
Cyanidin-3-O-glucoside II	71.1	± 22.3
Cyanidin-3-O-pentose	2289.8	± 327
Total	5386.4	± 567
<i>Flavonols</i>		
Quercetin-3-O-glucoside I	849.7	± 25.9
Quercetin-3-O-glucoside II	70.0	± 13.9
Quercetin-3-O-pentose I	186.0	± 23.1
Quercetin-3-O-pentose II	45.4	± 2.47
Quercetin-3-O-pentose III	683.5	± 23.5
Quercetin-3-O-rhamnoside	219.0	± 25.9
Total	2095.5	± 184
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Total phenolic compounds	8875.3	± 787
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Carotenoids		
Lutein	5.94	± 1.34

¹ Mean values ± standard deviation (n=3).

Regarding flavonols, these compounds accounted for the 24 % of total phenolic compounds, being all of them quercetin glycosides. Finally, hydroxycinnamic acids constituted the 15.7 % of the total, mainly represented by caffeoylquinic acids (Table 3). Similar contents of phenolic acids and flavonols were described before by HPLC [6,7]. Nevertheless, several factors may affect the concentration of total phenolic compounds in blueberries, such as agronomic factors, cultivars and varieties, geographic region, storage conditions, ripeness, climate and others, being reported in literature varied contents of total phenolic compounds in *Vaccinium* sp. (0.5 – 7 mg g⁻¹ FW; ~ 5 – 40 mg g⁻¹ DW) [6,33,36].

On the other hand, the content in carotenoids was studied using a rapid resolution liquid chromatography (RRLC) by comparing the chromatographic UV–vis spectroscopic characteristics with the standards. Results showed lutein (5.94 µg g⁻¹ DW = 0.67 µg g⁻¹ FW) as the only carotenoid found in Andean blueberry (Table 3). Recently, other authors showed lutein as the main carotenoid in higher concentrations (8.7 µg g⁻¹ FW), but also β-carotene in lower amounts (0.7 µg g⁻¹ FW) [9]. On the other hand, only β-carotene (0.4 µg g⁻¹ FW) was found in Andean blueberry by Vasco et al. [7]. These differences among Andean blueberry fruits affirm that similar varieties may contain diverse individual and total bioactive compounds depending on factors of different nature, including stage of maturity, variety, harvesting season or production, post-harvest processing and storage conditions, among others [37].

3.3. Embryo toxicity test with zebrafish

Testing toxicity in preclinical studies comprises the evaluation of physiological, biological or molecular alterations by *in vitro* or *in vivo* models. In this work, the toxic effect of Andean blueberry was evaluated by the zebrafish embryo toxicity test, determining the percentage of egg viability after 48 h of incubation with seven different concentrations of Andean blueberry extract (0.1, 0.2, 0.5, 1, 2,

5 and 10 mg mL⁻¹). Results showed a 100 % of egg viability up to a concentration of 1 mg mL⁻¹, with no morphological abnormalities found in the growth of the body, being the toxicity of this aqueous extract strongly increased with higher concentrations (Figure 1).

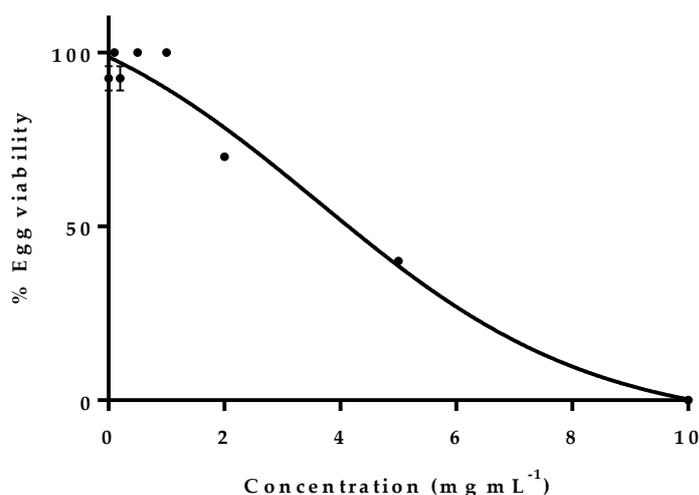


Figure 1. The embryotoxic effect expressed as % egg viability of various concentrations of Andean blueberry in the range 0–10 mg mL⁻¹. Aquarium water was used for 100 % egg viability. Results are mean values (n=3) ± standard deviation.

Furthermore, we determined the LC₅₀ dose of Andean blueberry which was able to cause the 50% percent death of test animals in 48 h, LC₅₀ = 3.63 mg mL⁻¹. According to the distinct toxicity categories of aqueous substances against zebrafish, Andean blueberry extract could be included in the safe category, as harmful concentrations are considered up to 100 mg L⁻¹ [38,39]. Based on the results, the maximum concentration causing no mortality (1 mg mL⁻¹) was selected to evaluate the antioxidant capacity *in vivo* in a further experiment with zebrafish (section 3.5).

3.4. Antioxidant capacity *in vitro*

Since there are multiple mechanisms involved in the oxidative stress in the human body, there is no universal method by which antioxidant capacity can be assessed accurately and quantitatively [40]. For *in vitro* analysis, it is recommended to use at least two methods to provide reliable results regarding antioxidant capacity [41]. Thus, in this work, DPPH and ABTS methods were selected to assess the relative antioxidant capacity for scavenging radicals, being DPPH radicals dissolved in MeOH while ABTS cations were dissolved in water, examining both hydrophilic and lipophilic antioxidants in Andean blueberry. Besides, the oxygen radical absorbance capacity (ORAC) method was used to evaluate the capacity for scavenging free radicals by competition with the reference free radical scavenger (fluorescein) for the peroxy radical (AAPH) in hydrophilic medium, which reflect physiological relevant perturbations [42]. Results showed higher antioxidant capacity values with methods ABTS and ORAC (278 and 402 μmol TE g⁻¹ DW, respectively) compared to DPPH assay, which reported the lowest value (85 μmol TE g⁻¹ DW) (Table 4). This fact can be explained by the high polarity of anthocyanins, the main phenolic group found in berries, which better contribute to the antioxidant activity in hydrophilic media. On the other hand, differences among these antioxidant capacity methods have been reported before, showing that DPPH is more selective than ABTS and ORAC methods in the reaction with hydrogen-atom donors, not reacting for example with OH-groups from aromatic compounds [43].

Table 4. Antioxidant capacity of Andean blueberry (n=3).

	Antioxidant capacity ($\mu\text{mol Trolox g}^{-1} \text{DW}$)		
	ABTS	DPPH	ORAC
Andean blueberry	278.2 ± 59	85.1 ± 27	402.2 ± 17

Mean values \pm standard deviation (n=3).

Previous works have reported ORAC values of blueberries ranged 350 – 1000 $\mu\text{mol TE g}^{-1} \text{DW}$ [44,45], ABTS results from 100 to 300 $\mu\text{mol TE g}^{-1} \text{DW}$ [10,26]; and DPPH values of 70 – 350 $\mu\text{mol TE g}^{-1} \text{DW}$ [46], reflecting great differences among genotypes, varieties, development and ripening stage of the fruit, and different contents of (poly)phenolic compounds. Thus, Andean blueberry can provide a good source of antioxidants in the diet, with potential benefits to human health.

3.5. Antioxidant capacity in vivo

The *in vivo* antioxidant activity was studied by the thiobarbituric acid reactive substances (TBARS) test using zebrafish larvae. In this experiment, the potential capacity of freeze-dried Andean blueberry to inhibit lipid peroxidation was assessed. In this work, doses from 1 to 0.1 mg mL^{-1} of Andean blueberry extracts were used to determine the antioxidant capacity *in vivo*, using as the maximum concentration the one previously selected in the toxicity test causing no mortality (1 mg mL^{-1}). The concentration of Andean blueberry that was able to inhibit the 50 % of the lipid peroxidation was 0.437 mg mL^{-1} (IC_{50}) (Figure 2). The positive effect of Andean blueberry in this assay was compared to the synthetic antioxidant butylated hydroxytoluene (BHT) at 0.1 mg mL^{-1} (control), showing the dose of 1 mg mL^{-1} a similar % of inhibition of the lipid peroxidation than the control. Thus, results suggest the potential use of Andean blueberry extracts as natural antioxidant for improving the shelf-life of food products.

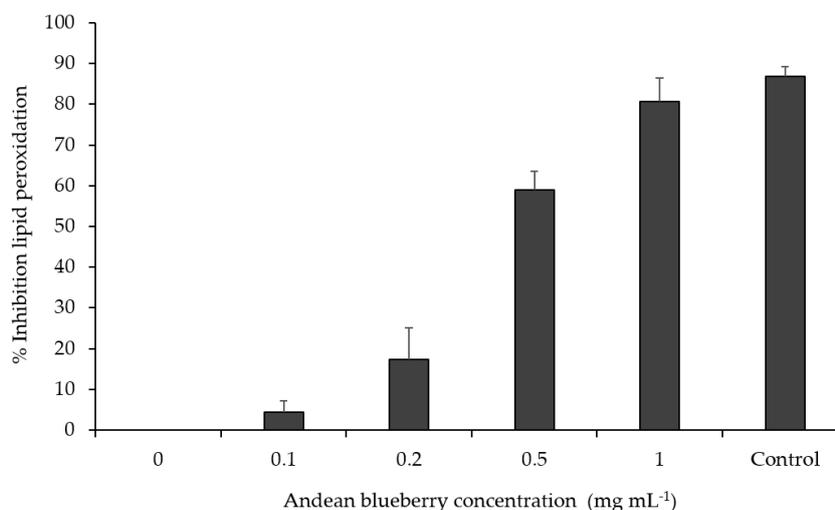


Figure 2. Inhibition of the lipid peroxidation *in vivo* using the zebrafish model. The zebrafish embryos were treated with concentrations of Andean blueberry in the range 0.1–1 mg mL^{-1} . Butylated hydroxytoluene (BHT) was used as positive control (0.1 mg/mL). Results are mean values (n=3) \pm standard deviation.

On the other hand, lipid peroxidation is a major contributor to the loss of cell function involved in many human pathological consequences, such as hepatotoxicity and hepatocarcinogenesis [19] and the zebrafish embryo model has been described to possess homologous oxidative pathways to humans [47]. There is also evidence of the absorption and metabolism of phenolic compounds by the chorion membrane of zebrafish larvae, evidencing this model as valuable for the assessment of healthy biological effects of bioactive compounds [48].

3.6. Determination of anti-nutritional lectins

The most known plant components with agglutination properties are the varied lectin proteins, which are able to reversibly bind sugar structures in the blood cells [48]. These proteins are few of the well-known antinutrients in plants and can be found in legumes, seeds extracts, fungi, and some fruits, however, their presence in berries has been little studied. Lectins may exert different responses in the human body, from allergies and gastrointestinal problems to bioactive effects related to their selectivity to bind carbohydrate residues of glycoproteins as markers in cancer research [49]. Thus, with this assay, the hemagglutination effect of Andean blueberry extract due to the presence of lectins and other compounds was evaluated using five different concentrations of the extract (2.5, 1.25, 0.625, 0.312, 0.156 mg mL⁻¹). Results showed no agglutination effect of the extracts (data not shown), revealing the possible lack of the lectin, and therefore the absence of an antinutritional effect, in Andean blueberry fruit.

3.7. Antimicrobial activity

Vaccinium spp., such as cranberry, blueberry and bilberry, have shown bactericidal activity against *S. aureus* and *E. coli*, especially in the prevention of urinary tract infections [49]. This activity was related with the presence of (poly)phenols, mainly flavonol glycosides, anthocyanins, proanthocyanidins, and flavan-3-ols. In this work, only the highest concentration of Andean blueberry aqueous extract tested in this experiment (10 mg mL⁻¹) exhibited significant antimicrobial effects toward *S. aureus* and *E. coli*, being the percentages of bacterial growth inhibition 30 % and 43 %, respectively (data not shown). These results agree with previous works showing antibacterial activities in the range of 25 – 100 mg mL⁻¹ of blueberry, being the inhibitory effect higher for gram-positive bacteria than gram-negative bacteria [50]. The concentration of 10 mg mL⁻¹ of Andean blueberry extract used in the experiment suggests a weak antibacterial effect of this fruit.

3.8. In vitro gastrointestinal digestion

The assessment of total phenolic compounds (TPC) and antioxidant capacity during *in vitro* gastrointestinal digestion of Andean blueberry allowed us to determine how the digestion process affected the stability, and therefore, bioaccessibility, of the dietary (poly)phenols present in this fruit. This experiment resembles the antioxidant role of these fruits in our gastrointestinal tract, where may exert important beneficial effects against different prooxidants (such as diet components) that have been observed to increase oxidative stress before they are absorbed. Our results showed the presence of phenolic contents in all phases of the *in vitro* digestion (Table 5), representing the availability of these compounds for absorption in the intestinal epithelium and metabolism. No significant changes in TPC were found during the oral and gastric phase, obtaining a bioaccessibility around 85 – 90 %. Afterwards, TPC were recovered in lower contents (51-56 % bioaccessibility), may be due to degradation processes of these compounds with the intestinal juice treatment, being converted to aglycones and glucuronides in the colon. These results agree with other authors who found similar losses of (poly)phenols in the intestinal steps [51,52]. Among phenolic compounds, anthocyanins have been found to experience higher losses during gastrointestinal digestion than flavonols and caffeic acid derivatives, being all of them affected by enzymes, pH levels and secretions in the digestive tract in real physiological conditions [53]. Once the release of phenolic compounds from its matrix into the intestinal lumen has been studied, the further step would be the study of their transport through the epithelium into the body and their availability to be metabolized and absorbed after reaching the colon.

Table 5. Total phenolic compounds (TPC), bioaccessibility and antioxidant capacity determined in the initial, oral, gastric, intestinal and final phase during *in vitro* digestion.

Gastrointestinal (GI) phase	Total phenolic content (mg GAE g ⁻¹)	% Loss	% Bioaccessibility	Antioxidant capacity (μmol Trolox g ⁻¹)

Initial	11.27 ¹ ± 0.20 a			40.53 ± 2.40 b
Oral	10.10 ± 0.94 a	10	90	41.67 ± 1.73 b
Gastric	9.54 ± 1.09 a	15	85	25.94 ± 2.14 c
Intestinal	6.36 ± 0.36 b	43	56	68.67 ± 2.98 a
Final	5.74 ± 0.62 b	49	51	63.97 ± 4.79 a

¹ Mean values (n=3). Different lower-case letters indicate statistically significant differences among gastrointestinal phases.

Regarding the antioxidant capacity evaluated by the ABTS method, it showed a high decrease in the gastric phase (Table 5), may be due to the lower chemical reaction of bioactive compounds with pH acid. Afterwards, the antioxidant capacity found during the intestinal and final steps of the gastrointestinal digestion (64–69 $\mu\text{mol TE g}^{-1}$) was significantly higher compared to the initial and oral steps (41 – 42 mmol TE g^{-1}). This fact could be explained by changes in the structural form of (poly)phenols in the intestine, affected by neutral pH and enzymatic activities, which promotes multiple forms of metabolites in the intestinal lumen, such as phenolic acids, resulting in a higher ability to scavenge free radicals [53,54]. Finding phenolic compounds after intestinal digestion showed their availability to be metabolized and absorbed after reaching the colon. Apart of showing antioxidant capacity, (poly)phenols may act as digestive enzymes inhibitors, affecting the activity of α -glucosidase, α -amylase and lipase, which may contribute to the control of diabetes type II and obesity, delivering other health benefits attributed to the ingestion of berries in the diet [55,56].

5. Conclusions

Andean blueberry is a relevant source of phenolic compounds, mainly anthocyanins, which may be responsible for its high antioxidant capacity. In addition, the freeze-dried extract of Andean blueberry did not show toxicity and could be included in the safe category as natural ingredient. These characteristics make Andean blueberry suitable to be used as functional ingredient with potential technological applications in the food industry, such as natural antioxidant or dye, or in the pharmaceutical industry, for the development of nutraceuticals. Due to the substantial differences in the phytochemical profile among *Vaccinium* spp. and varieties reported in literature, the identification and quantification of bioactive compounds of Andean blueberry performed in this work is part of the study of this berry as an interesting candidate to the further evaluation of its health benefits through *in vivo* assays and clinical trials. In this work, the *in vitro* simulated digestion showed a gradual release of phenolic compounds but sustained antioxidant activity, increasing reliability of antioxidant data described for berries. It should be noted that further *in vivo* and clinical studies with Andean blueberry should highlight the real effect of these bioactive compounds in the body, as the absorption and bioavailability could be affected by different interindividual factors.

Author Contributions: N.B. carried out the assays, collecting data, and drafted the manuscript. J.R., D.A.M. and A.J.M.-M. participated in the experimental design and helped to draft the manuscript. D.B. carried out the assays, collecting data, and helped to draft the manuscript. C.M.S. participated in carotenoid analyses. G.M.C. sample preparation and carried out the assays. A.G.R. participated in designing and in analyzing data, carried out the analysis and helped to draft the manuscript.

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