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Quantitative comparison of phytochemical profile, antioxidant, and anti-inflammatory properties of blackberry fruits adapted to Argentina

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ABSTRACT

The phytochemical profile by HPLC-TOF-MS of three blackberry cultivars ('Jumbo', 'Black Satin' and 'Dirksen'), adapted to the central-east of Argentina, was determined. The antioxidant capacity by DPPH and FRAP assays, and the effect of the blackberry extracts on lipopolysaccharide (LPS)-induced nitric oxide (NO) production, reactive oxygen species (ROS) production, and biomarkers of inflammation were also evaluated. 'Dirksen' fruits exhibited the highest vitamin C content (24 and 14% higher than values found in 'Black Satin' and 'Jumbo', respectively). However, 'Jumbo' and 'Black Satin' fruits presented higher total phenolic contents (more than 15%) than 'Dirksen'. Cyanidin-3-*O*-glucoside was the main polyphenolic compound quantified in all samples. 'Jumbo' and 'Black Satin' cultivars exhibited higher antioxidant capacity, and significantly reduced the release of ROS. The mRNA expression levels of cyclooxygenase-2 (Cox-2) and interleukin-6 (IL-6) were reduced consistently (more than 30%) by extracts of both cultivars, and showed slight suppressions on NO production. However, effective inhibition in the gene expression of interleukin-1β (IL-1β) and nitric oxide synthase (iNOS) was not observed by any extract. These results suggest the potential of blackberries cultivars 'Black Satin' and 'Jumbo' to contribute to a healthy diet, based on their higher polyphenol content, providing higher antioxidant and anti-inflammatory properties.

1. Introduction

Rubus fruticosus L. (*Rosaceae*) is a shrub famous for its edible blackberry fruits, which are traded globally, due to its delicious taste, pleasant flavor and nutritional profile. Blackberries are consumed fresh or processed to make food products, such as jam, wine, tea, ice cream, desserts, seedless jellies and bakery products. Furthermore, extracted pigments from fruits are used as a natural colorant in many foods (Zia-Ul-Haq et al., 2014). Blackberries are recognized as a rich source of polyphenolics, with human health benefits due to their antioxidant and anti-inflammatory properties (Koca and Karadeniz, 2009; Zia-Ul-Haq et al., 2014). Research on blackberries' composition has increased because they contain a unique and intense complement of polyphenolic compounds (Cuevas-Rodriguez et al., 2010a). The major polyphenolics in blackberries are anthocyanins, hydrolysable tannins (gallo- and ellagitannins), flavonols, and flavan-3-ols, including proanthocyanidins (Mertz et al., 2007). Blackberry anthocyanins have been well characterized, and apparently are only cyanidin-based anthocyanins at approximately 170 mg/100 g of fresh weight (FW) (Fan-Chiang and Wrolstad, 2005; Stintzing et al., 2002; Zia-Ul-Haq et al., 2014). Several studies have evaluated the ellagitannin content in blackberries with values ranging from 8 to >70 mg/100 g FW (Hager et al., 2010). Meanwhile, proanthocyanidins (PAC), also known as condensed tannins, are mixtures of oligomers and polymers composed of flavan-3-ol units, and are present in blackberries in a concentration of about 27 mg/100 g FW (Gu et al., 2003).

Argentina is the largest berry producer in South America, after Brazil and Chile. In the central-east of Argentina, the main producing regions are Coronda (Santa Fe) and surrounding areas.

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Approximately 60% of the harvested fruit from Coronda (primarily strawberries) is sold domestically for fresh fruit direct sale, and the remaining 40% is processed and exported. Export markets are in Canada, the United States of America, and European Union countries (Molina et al., 2007), especially during the off-season for berries in these countries. Recently, growers have begun to diversify the berry produce in Coronda and adapted commercial American blackberry (*R. fruticosus*) cultivars as 'Black Satin', 'Jumbo' and 'Dirksen', and have achieved good harvest indexes and market demand. However, the phytochemical content and health-relevant bioactivities of these adapted blackberries were not yet investigated. Although blackberry is a crop that can be easily adapted to different ecological conditions, it is known that growing environment could influence both phenolic concentrations and profiles, and indeed, the health-relevant potency of berries (Grace et al., 2014; Lila, 2006). Moreover, accumulating evidence suggests that genotype may have a profound influence on the content of bioactive compounds and on the antioxidant and anti-inflammatory properties of berries (Anttonen and Karjalainen, 2005; Cuevas-Rodriguez et al., 2010b). The knowledge of health benefits of blackberries may represent an actual opportunity for growers of the central-east of Argentina, first to select the cultivars in relation to their higher phytochemical content and bioactivity, and then, to increase their production of these selected cultivars, and to export blackberries that have been characterized.

The aim of this work was to rigorously establish and cross-compare the phytochemical profiles, antioxidant capacities, and anti-inflammatory properties of three blackberry cultivars ('Jumbo', 'Black Satin' and 'Dirksen') recently adapted to the central-east zone of Argentina.

2. Materials and methods

2.1. Chemicals and reagents

Reference compounds procyanidin-B2 (PAC-B2), cyanidin-3-*O*-glucoside, ellagic acid, and quercetin were purchased from Chromadex (Irvine, CA). L-(+) Ascorbic acid, sodium nitrite, DL-dithiothreitol, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), and Griess reagent were from Sigma–Aldrich Inc, (St. Louis, MO). Metaphosphoric acid was purchased from Merck KGaA (Darmstadt, Germany). Sodium acetate trihydrate, sodium hydroxide, potassium phosphate dibasic, iron(III) chloride hexahydrate, and iron(II) sulfate heptahydrate were acquired from Cicarelli (Reagents S.A., Santa Fe, Argentina). All organic solvents were HPLC grade and obtained from VWR International (Suwanee, GA).

2.2. Plant material

Cultivated blackberries (*R. fruticosus* L.) cultivars 'Dirksen', 'Black Satin', and 'Jumbo' were collected from one planting at Coronda (31°58′00″S 60°55′00″W), Santa Fe, (Argentina) in January 2014. Fruits were harvested by hand at full ripe stage and were selected for uniformity of size and colour and absence of defects. Fruits (3 kg per variety) were transported 50 km directly from the field to the laboratory of the Instituto de Tecnología de Alimentos, FIQ, UNL, (Argentina). Samples of each blackberry variety were separated for pH and soluble solids analysis and the rest was frozen at −80 °C until lyophilization in a Flexy-dry freeze dryer (SP Scientific, Stone Ridge, NY). The freeze-dried material was weighed and the dry matter content was estimated by difference in weight. Phytochemical extractions were performed on the freeze-dried material, and results were calculated on a fresh weight basis by considering the water content in each blackberry variety.

2.3. Soluble solids and pH analysis

The pH values were obtained with a pH meter (Horiba B-213 Twin pH meter, Horiba Ltd., Kyoto, Japan) in triplicate. The soluble solids content was determined in triplicate in the homogenized samples using a hand-held digital refractometer model Pal-alpha (Atago Co Ltd., WA) and results were expressed as °Brix.

2.4. Phytochemical determination

2.4.1. Extract preparation

For phenolic compound analysis and measurement of antioxidant capacities, 0.5 g of freeze–dried blackberries were placed into 15-mL centrifuge tubes. Eight milliliters of 80% methanol: 20% water (0.5% acetic acid) were added and the mixture was sonicated (ultrasonic cleaner; Testlab, Buenos Aires, Argentina) for 10 min. The mixture was then centrifuged at 5000 rpm (RC-6 plus; Sorvall, Asheville, NC) for 10 min, and the resulting supernatant was collected into a 25-mL volumetric flask. The extraction of the pellet was repeated two more times and the combined extracts were brought to a final volume of 25 mL with the extraction solvent. For anti-inflammatory activity, aliquots were concentrated four times under reduced pressure using a Sorvall Legend RT centrifuge (Thermo Fisher Scientific, Langenselbold, Germany) and freeze–dried before use. For vitamin C content, 0.5 g samples of freeze–dried blackberry were weighed into 15-mL centrifuge tubes and 5 mL extraction solvent (3% metaphosphoric acid and 8% acetic acid) were added. The mixtures were homogenized for 1 min, sonicated for 15 min, and then centrifuged at 12,000 × *g* (Neofuge 18R; Healforce, Shanghai, China) for 20 min at 4 °C. The supernatants were separated and used for analysis. All extractions were made in triplicate.

2.4.2. Phenolic determination by HPLC

HPLC analysis of phenolic compounds was conducted using an Agilent 1200HPLC (Agilent Technologies, Santa Clara, CA) with a photodiode array detector and an autosampler with Chemstation software as a controller and for data processing. Anthocyanin separation was performed according to our protocol (Grace et al., 2013) using a reversed-phase Supelcosil-LC-18 column, 25 mm × 4.6 mm, with 5 μm particle size (Supelco, Bellefonte, PA) at 30 °C. Quantification of anthocyanins was performed with reference to the external standard method calibration curve obtained with cyanindin-3-*O*-glucoside (0.125–0.375 mg/mL), and results were expressed as mg/00 g FW.

HPLC analysis for phenolic compounds was performed according to our protocol (Grace et al., 2014), using a Synergi 4 μm Hydro-RP 80A column (250 mm × 4.6 mm, with 5 μm particle size, Phenomenex, Torrance, CA). Ellagic acid (0.0125–0.05 mg/mL) and quercetin (0.0078–0.0625 mg/mL) were used as external standards, and results were expressed as mg/100 g FW.

Proanthocyanidin (PAC) separation was performed according to the method of Wallace and Giusti (2010) using a normal phase Develosil Diol column, 250 mm × 4.6 mm with 5 μm particle size (Phenomenex). PAC components were identified with reference to standard PAC-B2 and monomeric and oligomeric PAC components were quantified with a calibration curve of PAC- B2 (0.1–0.4 mg/mL), and results were expressed as mg/100 g FW.

LC–MS analysis was used for phenolic compound identification, molecular formula determination and structural interpretation. A Shimadzu LC-ESI-TOF-MS^n (liquid chromatography–electrospray ionization ion-trap time-of-flight mass spectrometer) system was used for analysis (Shimadzu Scientific Instruments, Columbia, MD) This LC-ESI-TOF-MS^n system was equipped with a Prominence HPLC system and separation was performed using a Shim-pack XR-

ODS column (50 mm × 3.0 mm, with 2.2 μm particle size; Shimadzu), as described earlier (Kellogg et al., 2014).

2.4.3. Vitamin C content determination

Vitamin C (VitC) content was determined by HPLC according to our protocol (Van de Velde et al., 2012). The analyses were set up on a Konik KNK-500-A HPLC, with UV detector (Konik Instruments, Barcelona, Spain). Separations were achieved using a reversed-phase column, Gemini 5 μ C18, 250 mm × 4.6 mm, with 5 μm particle size (Phenomenex). Quantification was performed by ascorbic acid calibration curves (0.004–0.020 mg/mL) and the results were expressed as mg VitC/100 g FW.

2.5. Antioxidant capacity

2.5.1. Free radical scavenging (DPPH) assay

The antioxidant capacity of the samples was estimated spectrophotometrically by determining the free radical scavenging capacity evaluated with the stable radical DPPH, according to Sánchez-Moreno et al. (2003). The determinations were made in triplicate and results were calculated using a standard curve of ascorbic acid (AA) (41–207 mg AA/L) and expressed as mg AA equivalent/100 g FW.

2.5.2. Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was conducted as previously described (Vandekinderen et al., 2008) by monitoring the absorbance change at 593 nm caused by the reduction of the Fe^{3+}-TPTZ complex to the ferrous form at pH 3.6. FRAP values were obtained by comparing the absorbance change in the samples with those obtained from increasing concentrations of Fe^{2+} (0.1–0.9 mmol $FeSO_4$/L). Results were expressed as mmol Fe^{2+}/100 g FW.

2.6. Anti-inflammatory properties

2.6.1. Macrophage cell culture

The mouse macrophage cell line RAW 264.7 (ATCC TIB-71, obtained from American Type Culture Collection, Livingstone, MT) was maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Grand Island, NY), supplemented with 100 IU/mL penicillin/100 μg/mL streptomycin (Fisher) and 10% foetal bovine serum (Life Technologies) at a density not exceeding 5×10^5 cells/mL and maintained at 37 °C in a humidified incubator with 5% CO_2.

2.6.2. Cell viability assay and dose range determination studies

RAW 264.7 cells were seeded in a 96-well plate (5×10^4 cells/well) for the viability assay. Cell viability was measured after 24 h of exposure by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to Mosmann (1983) in triplicate and quantified spectrophotometrically at 550 nm using a Synergy H1 microplate reader (BioTek, Winooski, VT). Lyophilized extracts of blackberry were prepared in 95% ethanol at 50–100 μg/mL (dry weight/volume) dose range. The concentrations of test samples that showed no changes in cell viability compared with that of the vehicle (95% ethanol at a final concentration of 0.1%) were selected for further studies.

2.6.3. Anti-inflammatory in vitro *assay*

The *in-vitro* experiments were designed to quantify the relative amount of transcripts for target genes within the total RNA when cells were treated with each blackberry extract. Cells were cultivated in 24-well plates (5×10^5 cells/well) 24 h prior to treatment. Subsequently, cells were pretreated for 1 h with blackberry extracts at 50 μg/mL. Then, cells were elicited with lipopolysaccharide (LPS) at 1 μg/mL for an additional 6 h. For each assay, three controls were performed. A negative control (no LPS treatment, 95% ethanol at a final concentration of 0.1%) maintained a constant amount of transcripts for all constitutively expressed genes and served as a reference baseline, the induction control (treated with LPS) showed the maximum up-regulation of the marker genes, and the positive control (treated with LPS and dexamethasone at 10 μM) served as a reference for the effectiveness of the assay (Grace et al., 2014). Three replicates were made for both the treatments and the controls.

2.6.4. Total RNA extraction, purification, and cDNA synthesis

The total RNA was isolated from RAW macrophages using TRIzol reagent (Life Technologies) following the manufacturer's instructions. RNA was quantified using the Synergy H1/Take 3 spectrophotometer (BioTek). The cDNAs were synthesized using 2 μg of RNA for each sample using a commercially available high-capacity cDNA Reverse Transcription kit (Life Technologies), following the manufacturer's protocol on an ABI Gene AMP 9700 (Life Technologies).

2.6.5. Quantitative PCR analysis

The resulting cDNA was amplified in duplicate by real-time quantitative PCR using SYBR green PCR Master Mix (Life Technologies) according to our protocol (Grace et al., 2014). To avoid interference due to genomic DNA contamination, only intron-overlapping primers were selected using Primer Express version 2.0 software (Applied Biosystems, Foster City, CA) as follows: β-actin, forward primer 5′-AAC CGT GAA AAG ATG ACC CAG AT-3′, reverse primer 5′-CAC AGC CTG GAT GGC TAC GT-3′; Cox-2, forward primer 5′-TGG TGC CTG GTC TGA TGA TG-3′, reverse primer 5′-GTG GTA ACC GCT CAG GTG TTG-3′; iNOS, forward primer 5′-CCC TCC TGA TCT TGT GTT GGA-3′, reverse primer 5′-TCA ACC CGA GCT CCT GGA A-3′; IL-6, forward primer 5′ −TAG TCC TTC CTA CCC CAA TTT CC-3′, reverse primer 5′ TTG GTC CTT AGC CAC TCC TTC-3′, and IL-1β, forward primer 5′- CAA CCA ACA AGT GAT ATT CTC CAT G-3′, reverse primer 5′-GAT CCA CAC TCT CCA GCT GCA-3. Quantitative PCR (qPCR) amplifications were performed on an ABI 7500 fast real-time PCR (Life Technologies) using 1 cycle at 50 °C for 2 min and 1 cycle of 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The dissociation curve was completed with 1 cycle of 1 min at 95 °C, 30 s at 55 °C, and 30 s at 95 °C. mRNA expression was analyzed using the ΔΔCT method and normalized with respect to the expression of the β-actin housekeeping genes using 7500 Fast System SDS software v1.3.0 (Life Technologies). A value <1 indicates transcriptional down-regulation (inhibition of gene expression) compared with LPS, which shows maximum genetic induction. Values >1 imply over expression of the particular gene in excess of LPS stimulation.

2.6.6. Nitric oxide (NO) assay

The production of nitrite, the stable end-product of NO generation in activated macrophages, was assayed by a colorimetric assay as described by Kellogg and Lila (2013). To 100 μL of cell culture medium were added 100 μL of Griess reagent and the mixture was incubated at room temperature for 10 min. The absorbance at 540 nm was read on a microplate reader (BioTek). The nitrite concentration was calculated using a sodium nitrite standard curve (0.78–50 μM). NO production levels for each treatment were normalized to the non-stimulated control (Ctl) and expressed as % Ctl.

2.6.7. Intracellular reactive oxygen species (ROS) production

To determine intracellular ROS generation in RAW 264.7 cells, a fluorescent dye protocol was used as previously described by Choi et al. (2007). Briefly, cells (seeded at a density of 5×10^5 cells/mL, 24 h before treatment) were charged with 50 μM of 2′,7′-

dichlorodihydrofluorescein diacetate acetyl ester (H_2DCFDA; Molecular Probes) for 30 min and washed twice with PBS. H_2DCFDA penetrated the membrane of viable cells and was deacetylated to dichlorofluorescein (DCFH) by endogenous cytosolic esterases. Cellular inflammation was then induced with 1 μL of 1 μg/mL LPS in serum-free media and co-treated with either dexamethasone (3.9 μg/mL, 10 μM, positive control), and blackberry extracts (50 μg/mL) for 24 h. A separate set of cells, used as a non-stimulated control (Ctl), was not treated with LPS or blackberry compounds. During the incubation, DCFH reacted with intracellular ROS to yield the fluorophore 2′,7′-dichlorofluorescein (DCF). At the end of the treatment period ROS formation was determined as a measure of DCF fluorescence intensity at $\lambda_{excitation}$ 485 nm and $\lambda_{emission}$ 520 nm using a SynergyHT microplate reader. ROS production levels for each treatment were normalized to the non-stimulated control (Ctl) and expressed as % Ctl.

2.7. Statistical analysis

All data were analyzed by a one-way ANOVA using the software STATGRAPHICS Centurion XV 15.2.06 (Statpoint Technologies, Inc., Warrenton, VA). Significant differences among means were determined by Tukey's test at 5% level of significance.

3. Results and discussion

3.1. Soluble solids, pH, and dry matter content of blackberries

The soluble solids (°Brix) and pH values for 'Jumbo', 'Black Satin', and 'Dirksen' blackberries are presented in Table 1, and no differences ($p > 0.05$) were observed among cultivars. In the same way, the dry matter content did not significantly differ between the 3 cultivars examined in this study (Table 1). Soluble solids and pH results were in accordance with those reported in mature blackberries by other authors (Acosta-Montoya et al., 2010; Rutz et al., 2012). The value of soluble solids (around 7 Brix) corresponds to an appropriate biosynthesis of sugars during the maturation process following the hydrolysis of starch, a process in which a plant's reserves of carbohydrates are transformed into simpler, water soluble sugars. Meanwhile, the pH value (around 2.9) at this full ripe stage corresponded with the decrease in acidity, probably caused by the oxidation of organic acids in the Krebs cycle during respiration, which is more pronounced in the initial maturation stages (Rutz et al., 2012).

3.2. Vitamin C content of blackberries

The VitC content for the three blackberry cultivars (Table 1) was slightly lower but comparable with the content previously reported in the literature (15 mg/100 g FW) by Davey et al. (2000) and Pantelidis et al. (2007). Vit C content for 'Dirksen' was 24% and 14% higher than the values exhibited by 'Black Satin' and 'Jumbo' fruits, respectively, proving that this cultivar provided the highest vitamin C content. Interestingly, Kafkas et al. (2006) revealed no detectable amounts of ascorbic acid in 'Jumbo' blackberries from Turkey, confirming that the Vit C content varies considerably among cultivars, ripeness and growing condition, and it is dependent on the harvest maturity, soil fertilization, irrigation, light intensity and day/night temperatures (Davey et al., 2000).

Table 1
Soluble solids, pH, dry matter, vitamin C content, radical scavenging capacity (DPPH) and ferric reducing capacity (FRAP) of three blackberry cultivars.

Item	Blackberry variety		
	'Jumbo'	'Blacksatin'	'Dirksen'
Soluble solids (°Brix)	5.5 ± 1.0^a	7.0 ± 1.0^a	6.9 ± 0.6^a
pH	2.87 ± 0.02^a	2.94 ± 0.05^a	2.92 ± 0.03^a
Dry matter (%)	11.4 ± 0.5^a	11.7 ± 0.6^a	10.6 ± 0.7^a
Vitamin C (mg/100 g FW)	8.2 ± 0.2^b	7.1 ± 0.6^a	9.6 ± 0.3^c
DPPH (mg AA/100 g FW)	800 ± 50^b	798 ± 20^b	714 ± 32^a
FRAP (mmol Fe/100 g FW)	24 ± 1^b	27 ± 2^b	20 ± 1^a

Different lower case letters within the same row are significantly different ($p \leq 0.05$) (one-way ANOVA, Tukey's post hoc test; $p \leq 0.05$ was considered significant). Vitamin C content quantified by HPLC using a reference standard.

3.3. Characterization of blackberry phenolic compounds

Phenolic compounds were characterized and identified by their LC retention times, UV–vis, MS, MS^2 spectra and by comparison with available references and reported literature. Considering that chromatograms of all cultivars showed a similar pattern, representative chromatograms obtained for 'Jumbo' blackberries recorded at 280, 360 and 520 nm are presented in Fig. 1A,–C, respectively.

Cyanidin-3-*O*-glucoside (peak **1**) with an *m/z* 449 $[M]^+$ and its characteristic MS^2 fragment ion at *m/z* 287, obtained after the loss of 162 amu (hexose moiety), was the major anthocyanin detected in all blackberry samples, representing more than 85% of the total anthocyanins and more than 65% of the total individual phenolics quantified by HPLC (Table 2). This finding is in agreement with the reports for other blackberry cultivars (Cuevas-Rodriguez et al., 2010a; Fan-Chiang and Wrolstad, 2005; Mertz et al., 2007), and with Jennings and Carmichael (1980) who stated that blackberries exclusively biosynthesize cyanidin glycosides as primary metabolites. Peak **4** with an *m/z* 419 $[M]^+$ and its corresponding MS^2 fragment ion at *m/z* 287, obtained after the loss of 132 amu (xylose moiety), was identified as cyanidin-3-xyloside, as reported by Cho et al. (2004) in 'Kiowa' blackberry and Fan-Chiang and Wrolstad (2005) in eighteen blackberry cultivars. Peak **5** and **6** were tentatively identified as acylated derivates of cyanidin-3-glucoside (Stintzing et al., 2002). Peak **5** was assumed to be cyanidin-3-*O*-(6-*O*-malonyl-glucoside) with an *m/z* 535 $[M]^+$ and MS^2 fragment ions at *m/z* 449 and 287 (first loss of 86 amu corresponding to the malonyl moiety, and the subsequent loss of 162 amu corresponding to the glucose moiety). Finally, taking into account the literature data, peak **6** was initially identified as cyanidin-3-*O*-dioxalyglucoside with an *m/z* 593 $[M]^+$ and the MS^2 cyanidin fragment at *m/z* 287, possibly obtained after the loss of 162 amu (glucose moiety) and 144 amu corresponding to the loss of the oxalic acid dimer moiety (Ogawa et al., 2008; Siriwoharn et al., 2004; Stintzing et al., 2002). However, further investigation of this compound using high-resolution MS data as well as NMR spectroscopy by Jordheim et al. (2011) in fruits of wild Norwegian blackberries and three blackberry cultivars ('Thornless Evergreen', 'Tupi', and 'Loch Ness') revealed that this acyl moiety would not correspond to the oxalic acid dimer but to 3-hydroxy-3-methylglutaroyl. Hence, the compound data was in accordance with those of cyanidin-3-*O*-β-(6″-(3-hydroxy-3-methylglutaroyl)-glucoside) and not in accordance with those of cyanidin-3-*O*-dioxalylglucoside (Jordheim et al., 2011). Therefore, peak **6** in the present work was then tentatively identified as cyanidin-3-*O*-β-(6″-(3-hydroxy-3-methylglutaroyl)-glucoside).

Table 2 shows no differences ($p > 0.05$) between 'Jumbo' and 'Black Satin' cultivars in the concentration of cyanidin-3-*O*-glucoside, cyanidin-3-*O*-(6-*O*-malonyl-glucoside), and cyanidin-3-*O*-(6″-(3-hydroxy-3-methylglutaroyl)-glucoside). However, 'Dirksen' variety had lower amounts of all anthocyanins except cyanidin-3-xyloside, whose concentration was in the mid-range of those recorded for 'Black Satin' and 'Jumbo' (Table 2).

Two compounds of maximum absorption at 240 nm were tentatively identified as ellagitannin derivatives (peak **2** and **3**). Ellagitannins are hydrolysable tannins since they are esters of

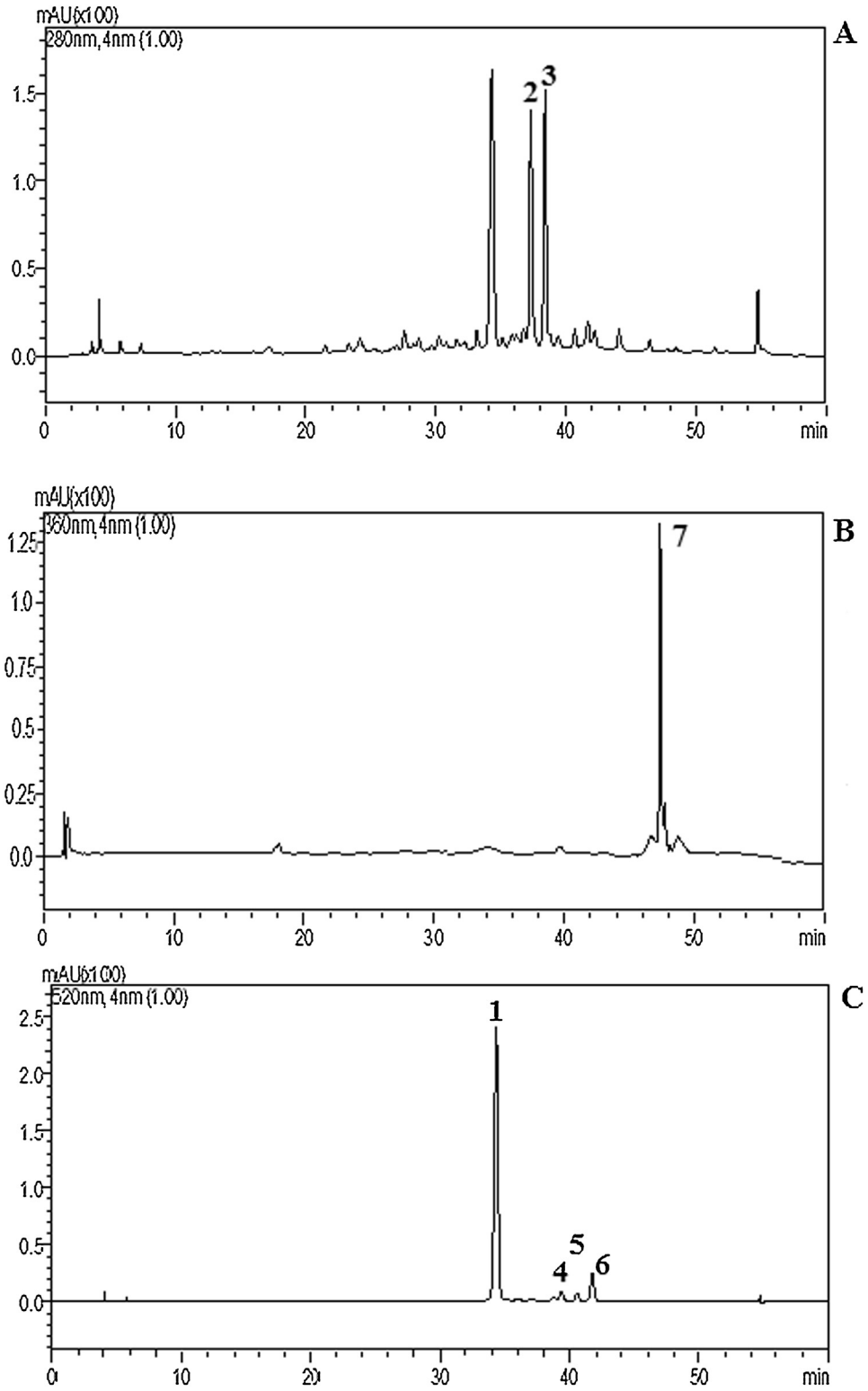

Fig. 1. Reversed-phase HPLC-UV chromatograms of 'Jumbo' blackberries at 280 nm (A), 360 nm (B), and 520 nm (C). Peak identification: (**1**) cyanidin-3-*O*-glucoside (**2**) lambertianin C, (**3**) dimer of galloyl-bis-HHDP-glucose (sanguiin H-6), (**4**) cyanidin-3-xyloside, (**5**) cyanidin-3-*O*-(6-*O*-malonyl glucoside), (**6**) cyanidin-3-*O*-β-(6″-(3-hydroxy-3-methylglutaroyl)-glucoside), and (7) quercetin-3-*O*-rutinoside (rutin).

hexahydroxydiphenic acid (HHDP: 6,6′-dicarbonyl-2,2′,3,3′,4,4′-hexahydroxybiphenyl moiety) and a polyol, usually glucose, and in some cases gallic acid (Häkkinen et al., 1999). Peak **2** with a double charged ion $[M - 2H]^{2-}$ at *m/z* 1401, giving a true mass of 2804, and with MS^2 fragment pattern ions at *m/z* 933, 633 and 301, confirmed the presence of lambertianin C. This compound was also identified by Hager et al. (2010) in 'Apache' blackberry and represented the compound with the highest mass identified in our samples. Meanwhile, peak **3** was identified as a dimer of galloyl-bis-HHDP-glucose (sanguiin H-6) with a double charged ion $[M-2H]^{2-}$ at *m/z*

Table 2
Polyphenolics profile content in three blackberry cultivars.

Phenolic compounds (mg/100 g FW)	Blackberry variety		
	'Jumbo'	'Blacksatin'	'Dirksen'
Flavonoids			
Anthocyanins			
Cyanidin-3-*O*-glucoside	107.3 ± 0.7^{b}	105 ± 2^{b}	91.4 ± 0.2^{a}
Cyanidin-3-*O*-xyloside	0.80 ± 0.02^{a}	2.9 ± 0.1^{c}	1.40 ± 0.01^{b}
Cyanidin-3-*O*-(6-*O*-malonyl glucoside)	7.10 ± 0.08^{b}	6.9 ± 0.2^{b}	5.80 ± 0.02^{a}
Cyanidin-3-*O*-β-(6″-(3-hydroxy-3-methylglutaroyl)-glucoside	9.1 ± 0.1^{b}	9.2 ± 0.2^{b}	8.40 ± 0.02^{a}
Total anthocyanins	124.3	124.0	107.0
Flavonols			
Quercetin-3-*O*-rutinoside	3.1 ± 0.2a	4.02 ± 0.04b	2.94 ± 0.09a
Hydrolysable tannins			
Ellagitannins			
Lambertianin C	4.7 ± 0.3^{a}	4.4 ± 0.4^{ab}	3.8 ± 0.3^{b}
Dimer of galloyl-diHHDP-glucose	5.6 ± 0.3^{b}	5.4 ± 0.6^{ab}	5.1 ± 0.1^{a}
Total ellagitannins	10.3	9.8	8.9
Condensed tannins			
Proanthocyanidins			
DP1	7.8 ± 0.3^{c}	7.1 ± 0.2^{b}	5.96 ± 0.03^{a}
DP2	5.0 ± 0.3^{b}	5.2 ± 0.1b	4.30 ± 0.02^{a}
DP3	5.0 ± 0.2^{b}	5.2 ± 0.1^{b}	4.30 ± 0.02^{a}
DP4	1.5 ± 0.1^{a}	1.70 ± 0.01^{b}	1.48 ± 0.01^{a}
DP5	1.3 ± 0.1b	1.38 ± 0.01^{b}	1.22 ± 0.05^{a}
DP6	1.17 ± 0.04^{b}	1.23 ± 0.01^{c}	1.10 ± 0.02^{a}
DP>10	2.3 ± 0.1^{a}	2.8 ± 0.4^{a}	2.62 ± 0.03^{a}
Total proanthocyanidins	24.1	24.6	21.0
Total phenolics	161.8	162.4	139.8

Different lower case letters within the same row are significantly different (one-way ANOVA, Tukey's post hoc test; $p \leq 0.05$ was considered significant). DP: degree of polymerization.

934, implying a true molecular weight of 1870, with major MS^2 fragments at 897, 633 and 301, in concordance with the reports of Mertz et al. (2007) and Hager et al. (2010) for *Rubus* sp. Ellagitannin content (lambertianin C plus sanguiin H-6) for all cultivars (ranging from 8.9 to 10.3 mg/100 g FW) was slightly lower than the content (18.9 mg/100 g FW) reported for 'Apache' blackberries by Hager et al. (2010). 'Jumbo' ellagitannin content was similar ($p > 0.05$) to 'Black Satin', but different ($p \leq 0.05$) from 'Dirksen'. Meanwhile, 'Black Satin' and 'Dirksen' ellagitannin contents were comparable ($p > 0.05$).

Quercetin-rhamnosyl-glucoside (quercetin-3-rutinoside, also known as rutin) was detected in the samples (peak **7**) and confirmed by its $[M-H]^-$ ion at *m/z* 609 and MS^2 fragment at 301 (−308 amu corresponding to the cleavage of rhamnose-glucose sugar; Del Rio et al., 2004). This compound was also identified by Cho et al. (2004) in 'Kiowa' blackberries. 'Black Satin' variety had

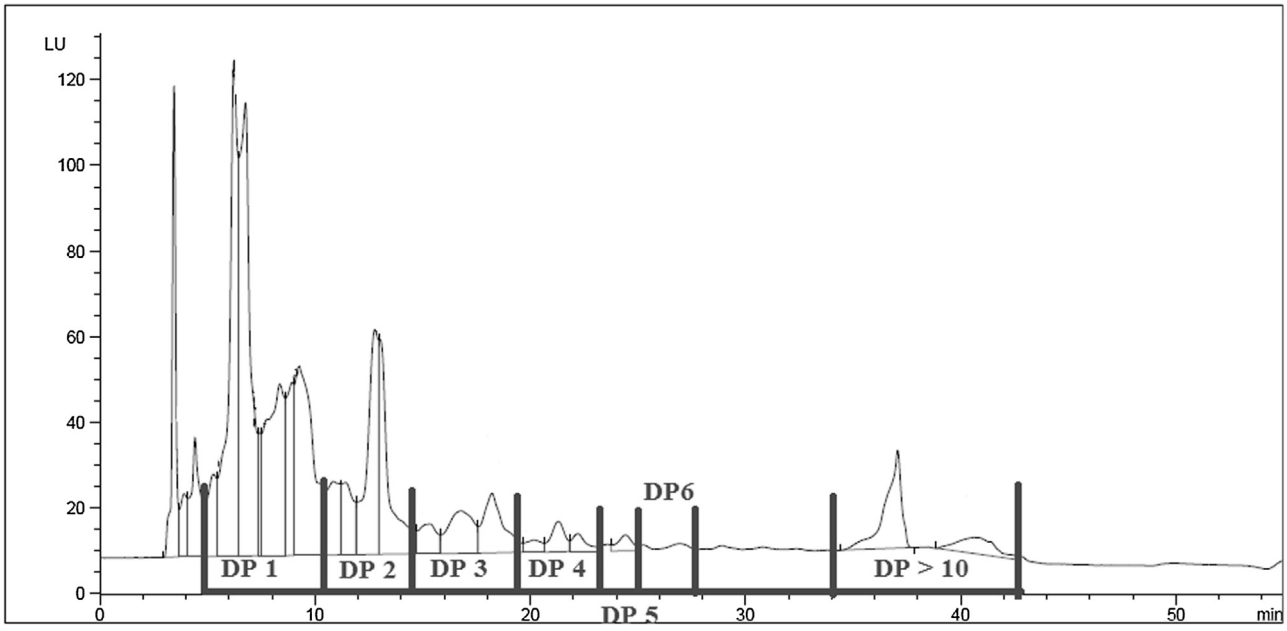

Fig. 2. Normal-phase HPLC-FLD chromatogram (excitation, 230 nm, emission, 320 nm) for proanthocyanidins of 'Jumbo' blackberries. DP = degree of polymerization.

the highest content of rutin among the three blackberry cultivars (Table 2).

Normal-phase HPLC with fluorescence detection was used to separate PAC components in the blackberry samples according to their degree of polymerization (Wallace and Giusti, 2010). PAC are mixtures of oligomers and polymers composed of flavan-3-ol units linked mainly through C4—C8 bonds and in minor proportion through C4—C6 bonds (both called B-type), or doubly linked by an additional ether bond between C2—O7 (A-type). Proanthocyanidins with B-type linkages were detected as the only PAC component in blackberry fruit as reported by Gu et al. (2003). Fig. 2 shows the PAC chromatogram obtained for 'Jumbo' blackberry, and similar chromatograms were obtained for 'Black Satin' and 'Dirksen' fruits (not shown). Compounds were successfully separated according to their degree of polymerization, showing strong signals for monomers through tetramers. Smaller but clearly identifiable signals were also obtained for pentamers and oligomers with polymerization degree >10 (Fig. 2). Total PAC content of the three blackberry cultivars obtained by normal-phase HPLC ranged from 21.0 to 24.6 mg/100 g FW (Table 2). PAC represented only 15% of the total blackberry phenolics and in general, 'Dirksen' had lower levels of each PAC than 'Black Satin' and 'Jumbo'. Gu et al. (2004) reported 27 mg/100 g FW PAC in blackberry fruit in agreement with our presented range.

The total phenolic (TP) content obtained as the sum of the individual phenolic compounds, as quantified by HPLC (Table 2) confirmed that 'Black Satin' and 'Jumbo' blackberries were comparable, and their TP contents were approximately 14% higher than 'Dirksen'. Therefore, the intake of polyphenolic compounds would be quantitatively higher with the consumption of blackberry cultivars 'Black Satin' and 'Jumbo'.

3.4. Antioxidant capacity

When assaying the antioxidant activity of natural antioxidants, it is recommended to use more than one antioxidant assay for a better understanding of the antioxidant properties of substances (Ciz et al., 2010). Therefore, in our study we used DPPH and FRAP assays expressing different aspects of the antioxidant action and providing a broader view of the antioxidant potential of blackberry extracts (Pérez et al., 2014). DPPH and FRAP antioxidant activity results for 'Black Satin', 'Jumbo' and 'Dirksen' blackberry extracts are presented in Table 1. Results were within ranges reported for both methodologies in the literature (Koca and Karadeniz, 2009; Zia-Ul-Haq et al., 2014). 'Black Satin' and 'Jumbo' blackberries exhibited approximately 15% higher antioxidant capacity (by both DPPH and FRAP assays) than 'Dirksen'; in accord with the highest phenolic compounds contents found for these two blackberry cultivars (Table 1 and 2). Polyphenols contains aromatic rings with —OH or OCH_3 substituents which together contribute to their biological activity, including antioxidant action. They have been shown to outperform well-known antioxidants, such as ascorbic acid and α-tocopherol, in *in-vitro* antioxidant assays because of their strong capacity to donate electrons or hydrogen atoms (Rodrigo and Libuy, 2014; Sharma et al., 2012). The mechanism behind the radical-scavenging activity of polyphenols such as flavonoids is thought to be hydrogen atom donation and the structural requirements include an *ortho*-dihydroxy substitution in the B ring, a C2=C3 double bond and a C4 carbonyl group in the C ring. The free hydroxyl groups on the B ring donate hydrogen to a radical, thus stabilizing it and giving rise to a relatively stable flavonoid radical (Amić et al., 2007). In agreement, other authors described the significant influence of phenolic compounds on the antioxidant capacity of *Rubus* sp., presenting ascorbic acid as a minimal contribution to the antioxidant potential of the fruits (Pantelidis et al., 2007).

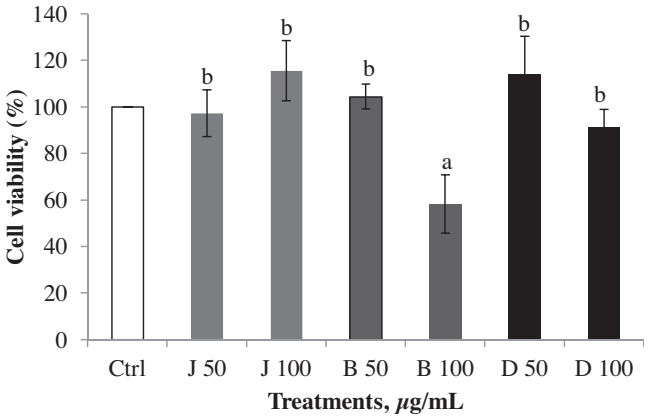

Fig. 3. Effects of blackberry crude extracts on the cell viability of murine RAW 264.7 macrophages. Cell viability was quantified spectrophotometrically by the MTT assay and is reported as the mean ± SD for three independent experiments. J: 'Jumbo', B: 'Black Satin', and D: 'Dirksen' blackberries. Means not sharing the same letter are significantly different (one-way ANOVA, Tukey's post hoc test, $p \leq 0.05$ was considered significant).

3.5. Anti-inflammatory properties

3.5.1. Effect of blackberry extracts on cell viability

The cytotoxic effects of the three blackberry extracts were evaluated by measuring RAW 264.7 macrophage viability by the MTT assay after 24 h of exposure (Fig. 3). 'Jumbo', 'Black Satin' and 'Dirksen' blackberry extracts in the 50–100 μg/mL dose range did not depress cell viability; only 'Black Satin' extract at 100 μg/mL reduced viability close to 60%. For that reason, only 50 μg/mL of each fruit extract was used in subsequent studies; an amount that is easily achievable in the gastrointestinal tract after consumption of berries such as blueberries (Kahle et al., 2006). Therefore, any inhibitory effect of the blackberry extracts at this concentration will not be attributable to cytotoxic effects on the macrophage cells but to phytochemical activity.

3.5.2. Effect of blackberry extracts on inflammatory markers, intracellular reactive oxygen species (ROS) and nitric oxide (NO) production

The physiology of various inflammatory diseases is a complex process mediated by inflammatory and immune cells such as macrophages and monocytes (Ingersoll et al., 2011). Chronic inflammation, as observed in many cardiovascular and autoimmune disorders, occurs when the low-grade inflammatory response fails to resolve with time. Because of the complexity of the chronic inflammatory disease, major efforts have focused on identifying novel anti-inflammatory agents and dietary regimes that prevent the pro-inflammatory process at the early stage of gene expression of key pro-inflammatory mediators and cytokines (Esposito et al., 2014). Many studies suggest that the exposure of mammalian cells to lipopolysaccharide (LPS), the principal component of the Gram-negative outer membrane bacteria, can lead to release of pro-inflammatory cytokines and in turn activate inflammatory cascades including cytokines such as interleukin-1β (IL-1β) and interleukin-6 (IL-6), and inflammatory enzymes such as inducible nitric oxide synthase (iNOS) and cyclooxynase-2 (Cox-2), both responsible for the synthesis and secretion of nitric oxide (NO) and prostaglandins, respectively (Grace et al., 2014; Shi and Pamer, 2011). These four well-known genetic biomarkers involved in the acute-phase response, inflammatory response, and humoral immune responses were investigated *in vitro* in the LPS-stimulated murine RAW 264.7 macrophage model. Moreover, the ability of the

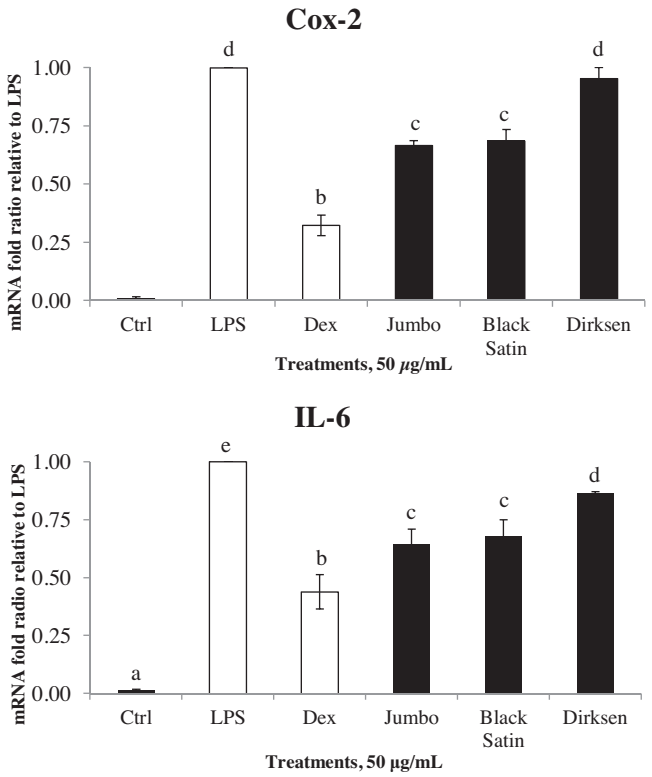

Fig. 4. Effects of crude blackberry extracts on pro-inflammatory gene expression profiles associated with chronic inflammatory response: (A) cyclooxygenase-2 (COX-2) and (B) cytokine interleukin-6 (IL-6). Fold changes in gene expression are reported as the mean ± SD relative to LPS controls. Ctrl, cells treatment with vehicle only; Dex, dexamethasone at 10 μM used as positive control. Means not sharing the same letter are significantly different (one-way ANOVA, Tukey's post hoc test, $p \leq 0.05$ was considered significant).

different blackberry extracts to inhibit the cellular generation of ROS and NO synthesis was also examined.

According to our results, 'Jumbo' and 'Black Satin' extracts significantly ($p \leq 0.05$) suppressed Cox-2 and IL-6 genes expression based on 30% or higher changes relative to the LPS-stimulated controls (Fig. 4). Meanwhile, 'Dirksen' extract did not show any inhibitory effect on the expression of both genes (Fig. 4). The latter behaviour mirrors the intracellular ROS production of the LPS stimulated RAW 264.7 macrophages after treatment with the blackberry extracts (Fig. 5), suggesting that 'Black Satin' and 'Jumbo' phytochemicals may play a role in the health maintenance by reducing oxidative stress. These results were in accordance with

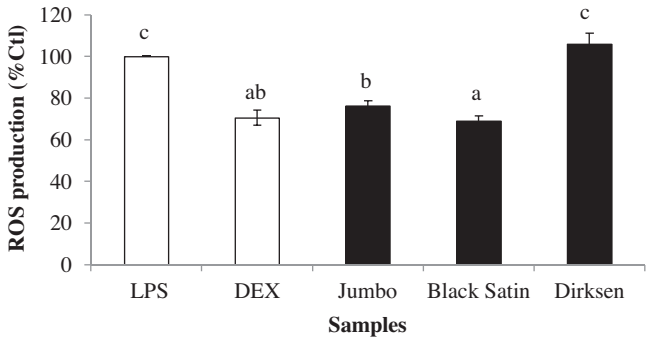

Fig. 5. Effects of crude blackberry extracts on reactive oxygen species (ROS) production. Changes in ROS are reported as the mean ± SD relative to LPS controls. Means not sharing the same letter are significantly different (one-way ANOVA, Tukey's post hoc test, $p \leq 0.05$ was considered significant).

the higher total phenolic contents, and antioxidant capacity values by DPPH and FRAP observed for 'Jumbo' and 'Black Satin' blackberries in comparison with 'Dirksen' (Table 1 and 2), suggesting their better bioactivity based on higher phenolic contribution. In agreement with our results, Azofeifa et al. (2015) reported that the induced intracellular ROS species were reduced by enriched-polyphenols fractions from blackberries in a dose-dependent manner. Meanwhile, Tattini et al. (2006) stated that polyphenols can modulate by acting as antioxidants against the damaging effects of increased ROS levels in cells.

On the other hand, none of the three blackberry cultivar extracts exhibited effective inhibition of LPS-elicited inducible cytokine IL-1β and nitric oxide synthase (iNOS) genes expression (data not shown). However, a slight suppression in the NO production (less than 5%) in the activated macrophages was observed by 'Jumbo' and 'Black Satin' extracts (Fig. 6), suggesting a higher NO suppression property by the polyphenols of these two blackberry varieties, in accordance with their higher polyphenols contents. In a similar study, Wang and Mazza (2002) described a very small suppression effect on NO production by 50 μg/mL blackberry crude extracts, which was attributed to the low concentration of phenolics in these extracts. Meanwhile, Cuevas-Rodríguez et al. (2010b) did not find any effect of wild and commercial Mexican blackberry extracts over NO production, and iNOS and Cox-2 genes expression suppression under the same conditions. However, both authors reported potent anti-inflammatory effects of the latter extracts after their phenolic fractionation/purification and polyphenolic enrichment, indicating that higher doses of phenolic fractions in contact with the cells were more effective in NO suppression and in the down-regulation of pro-inflammatory genes.

The anti-inflammatory activities of berries were mainly associated with their anthocyanin composition (Pérez et al., 2014). In this sense, Pergola et al. (2006) stated that at least some part of the anti-inflammatory activity of the enriched anthocyanin fraction from blackberry was due to the suppression of NO production by cyanidin-3-glucoside, which, as indicated before, is the main phenolic compound present in the blackberry extracts. According to Esposito et al. (2014) it is possible that the biological effect of a particular plant extract or fraction depends on the specific concentrations and molar ratios of bioactive compounds or the yet unknown interactions among the dietary bioactive compounds and the matrix itself.

Finally, it appears that polyphenols can interact with some receptors or transcription factors, trigger some signalling

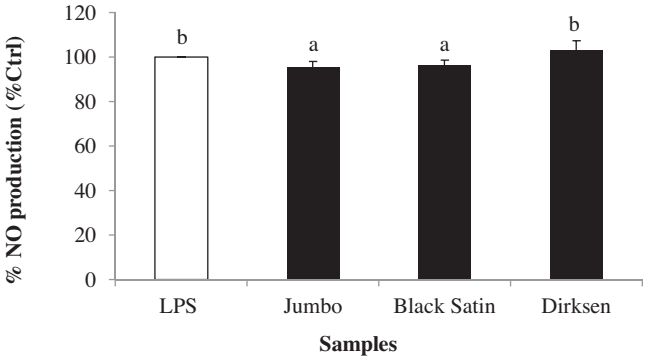

Fig. 6. Nitric oxide (NO) production in LPS-stimulated RAW 264.7 macrophage cells. Cultures were co-treated with 50 μg/mL fractions of 'Jumbo', 'Black Satin', and 'Dirksen' blackberries for 24 h. Fold changes in NO production are reported as the mean ± SD relative to LPS controls. Ctrl, cells treatment with vehicle only; Dex, dexamethasone at 10 μM used as positive control. Means not sharing the same letter are significantly different (one-way ANOVA, Tukey's post hoc test, $p \leq 0.05$ was considered significant).

pathways and modulate the expression of some key genes involved in the physiopathology of inflammatory mediated processes such as atherosclerosis (Auclair et al., 2009). Therefore, the underlying role of polyphenols from *Rubus* sp. in the protection of inflammatory process deserves to be further explored.

4. Conclusions

Blackberry cultivars 'Jumbo' and 'Black Satin' presented higher individual and total phenolic contents (more than 15%) than 'Dirksen'. However, 'Dirksen' fruits exhibited the highest VitC content, 24 and 14% higher than in 'Black Satin' and 'Jumbo' blackberries, respectively. Nevertheless, higher antioxidant capacity results were recorded for 'Black Satin' and 'Jumbo' blackberries by both DPPH and FRAP assays, and on intracellular ROS production, indicating that the antioxidant potential was more dependent on the polyphenols content rather than on ascorbic acid content. Moreover, both cultivars exhibited a moderate down-regulation (more than 30%) on Cox-2 and IL-6 genes expression, and slight NO production suppression, suggesting their role as anti-inflammatory agents. These finding suggest an inherent better bioactivity of blackberries 'Black Satin' and 'Jumbo' based on their higher polyphenols contribution. This information may help blackberry growers of the central-east of Argentina to select cultivars in relation to their higher potential health benefits.

Conflict of interest

The authors declare no conflict interest.

Acknowledgments

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