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Anti-inflammatory and wound healing properties of polyphenolic extracts from strawberry and blackberry fruits

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Abstract

The polyphenolic profiles by HPLC-TOF-MS of strawberry 'San Andreas' and blackberry 'Black Satin' crude extracts (CE) were analyzed. Anthocyanin-enriched fractions (AEFs) and proanthocyanidin-enriched fractions (PEFs) were prepared, and all samples were probed for *in vitro* anti-inflammatory and wound healing effects in a LPS-stimulated RAW 264.7 macrophage model and in a skin fibroblast migration and proliferation assay, respectively. Blackberry samples exhibited higher ROS reduction than strawberry's (up to 50% ROS suppression). Berries CEs exhibited 20% inhibition in Cox-2 gene expression, while AEFs and PEFs were inactive at the same concentration. Strawberry AEF and PEF were more active against IL-1β and IL-6 gene expressions than the similar fractions from blackberry, where PEF was more active than AEF (75 % suppression by strawberry PEF). Moreover, berry PEFs were the active polyphenol fraction against iNOS gene expression (50% and 65% gen suppression by strawberry and blackberry PEF, respectively), mirroring results of NO synthesis suppression. The cell migration potential of berry polyphenolics was associated with anthocyanins. AEFs showed fibroblast migration around 50% of that registered for the positive control. Results obtained in this work highlight the anti-inflammatory properties of berry polyphenolics, especially due to proanthocyanidins. Moreover, promising results were obtained about the effects of berry anthocyanins on wound healing.

Keywords: *Fragaria ananassa*; *Rubus fruticosus*, anthocyanins; proanthocyanidins; cyclooxygenase-2; interleukin-6; skin cell migration.

1. Introduction

Berries such as strawberry (*Fragaria ananassa*) and blackberry (*Rubus species*), among others, are popularly used in the human diet either fresh or in processed forms ((Nile & Park, 2014). It is documented that berries provide significant health benefits because of their high levels of polyphenols, vitamins, minerals, and fibers (Nile & Park, 2014; Afrin et al., 2016). The major polyphenolic compounds in berries are anthocyanins, hydrolyzable tannins (gallo- and ellagitannins), flavonols, and flavan-3-ols, including proanthocyanidins (Mertz et al., 2007).

There is convincing *in vitro* and *in vivo* evidence that suggest that polyphenols from berries have anti-inflammatory effects (Park et al., 2011; Joseph et al., 2014; Zia-Ul-Haq et al., 2014; Van de Velde et al., 2016a; Gasparrini et al., 2017; Alvarez-Suarez et al., 2017; Duarte et al., 2018). One common inflammatory agent used in different *in vitro* experimental models is the endotoxin lipopolysaccharide (LPS), which represent an outer membrane structure and an important virulence factor of the cell wall of Gram-negative bacteria (Giampieri et al., 2015; Gasparrini et al., 2017; Duarte et al., 2018). LPS can activate intracellular pathways and trigger the kinase cascade and nuclear transcription factors, like mitogen-activated protein kinases (MAPKs) and NF-kB. These molecules are involved in the up-regulation of inflammation-related genes such as the pro-inflammatory cytokines interleukin-1β (IL-1β) and interleukin-6 (IL-6), and the inflammatory enzymes inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (Cox-2), both responsible for the synthesis and secretion of nitric oxide (NO) and prostaglandins, respectively (Park et al., 2011; Gasparrini et al., 2017). In that sense, in a

previous work, the mRNA expression levels of Cox-2 and IL-6 were reduced consistently (more than 30%) by polyphenolic crude extracts from 'Black Satin' and 'Jumbo' blackberries in a LPS-stimulated RAW 264.7 macrophage model (Van de Velde et al., 2016a). However, the kind of polyphenolic compounds from blackberries responsible for these anti-inflammatory effects were not isolated and determined in that work. Previously, Cuevas-Rodríguez et al. (2010a) had reported that both anthocyanin and proanthocyanidin components were responsible for the anti-inflammatory properties observed in different blackberry genotypes. Nevertheless, it was recently revealed that pelargonidin-3-*O*-glucoside, the main anthocyanin component in strawberry, has important anti-inflammatory properties, and an anti-inflammatory molecular mechanism was proposed (Duarte et al., 2018). Therefore, the link between the anti-inflammatory effects provided by berries and one particular phenolic compound or group of them should be elucidated.

Related to the inflammatory process, wound healing is an important physiological process to maintain the integrity of skin after trauma, either by accident or by surgical procedures. The normal wound healing process involves three successive but overlapping phases, including hemostasis/inflammatory phase, proliferative phase, and remodeling phase (Wang et al., 2018). This process involves the coordinated efforts of a variety of cell types including keratinocytes, fibroblasts, endothelial cells, macrophages, and platelets (Nizamutdinova et al., 2009). The discovery of natural remedies which can improve the wound healing represents an important issue from clinical and economical points of view (Hemmati et al., 2018). Some *in vitro* and *in vivo* studies suggested that some African medicinal plants (Agyare et al., 2016), or even low-fat cow´s milk

(Hemmati et al., 2018) have significant effects on skin wound healing. However, there is a need for the isolation and characterization of bioactive compounds responsible for the wound healing properties of natural products (Agyare et al., 2016). In that sense, Nizamutdinova et al., (2009) stated that anthocyanins from black soybean seed coats might be used as an effective drug to potentiate wound healing in chronic wounds and thus attenuate the accompanying excessive inflammatory state. Therefore, taking into account previous reports where health-promoting benefits of anthocyanins and another phenolic compounds from berries were probed based on antioxidant and anti-inflammatory properties, the effect of polyphenols from berries on wound healing process deserves to be explored.

The aim of this work was, at first, to characterize and quantify the polyphenolic compound profile in crude and enriched-anthocyanin and proanthocyanidin extracts from 'San Andreas' strawberry and 'Black Satin' blackberry. Then, the anti-inflammatory properties of theses samples using an *in vitro* LPS-stimulated RAW 264.7 macrophage model, and their wound healing effects in an *in vitro* skin fibroblast migration and proliferation assay were studied.

2. Materials and Methods

2.1. Chemicals and reagents

Reference compounds pelargonidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside were purchased from Chromadex (Irvine, CA, USA). Ellagic, acid, quercetin-3-*O*-glucoside, caffeic acid, and procyanidin-B2 (PAC-B2) were from Sigma-Aldrich Inc., (St. Louis, MO, USA). All organic solvents were HPLC grade and obtained from VWR International (Suwanee, GA, USA).

2.2. Plant material

Blackberries (*Rubus fruticosus* L.) cultivar 'Black Satin' and strawberries (*Fragaria ananassa* Duch.) cultivar 'San Andreas' were collected from one planting at Coronda (31°58′00″S 60°55′00″W), Santa Fe, (Argentina) in 2017. Fruits were frozen at -80°C until lyophilization in a Flexy-dry freeze dryer (SP Scientific, NY). Phytochemical extractions were performed on the freeze-dried material, and results were calculated on a fresh weight (FW) basis by considering the water content in each sample.

2.3. Phytochemical determination

2.3.1. Preparation of polyphenolic-crude extracts

Extraction was conducted according to Van de Velde et al. (2016a) Briefly, 0.5 g of freeze-dried berries were mixed with 8 mL of 80% methanol: 20% water (0.5% acetic acid). The mixture was sonicated for 10 min, and then centrifuged at 5000 rpm (Sorvall RC-6 plus, Asheville, NC) for 10 min. Supernatants were separated, and the extraction of the pellet was repeated two more times. Finally, the combined extracts were brought to a final volume of 25 mL with the extraction solvent, and these crude extract (CE) were used for polyphenolic analysis. For cell culture assays, 2 mL of CEs were concentrated four times under reduced pressure using a Sorvall Legend RT centrifuge (Thermo Fisher Scientific, Langenselbold, Germany) and dried down completely by lyophilization before use. All extractions were made in triplicate.

2.3.2. Preparation of polyphenolic-enriched extracts

Strawberry and blackberry CEs (~ 25 mL) were evaporated to about 0.5 mL using a rotary evaporator at a temperature not exceeding 40 °C, and finally freeze-dried. The dried-materials were re-suspended in distilled water and loaded on an SPE cartridge

(Grace Pure^{TM} SPE C18-Max, 6 mL) preconditioned with ethyl acetate, methanol, and water. Cartridges were washed several times with acidified water (0.5% acetic acid) to remove free sugars and organic acids. Then, the polyphenolic mixture was eluted with 100% methanol, and the eluates were evaporated and freeze-dried to obtain the polyphenolic-enriched extracts.

2.3.2.1. Isolation of anthocyanin-enriched fractions and proanthocyanidin-enriched fractions from polyphenolic-enriched extracts

Strawberry and blackberry polyphenolic-enriched extracts were applied to a column packed with Sephadex LH-20 (30 x 3 cm), preconditioned with 20% methanol: 80% water (0.5% acetic acid), with an isocratic elution using the same solvent ratio. Six fractions (1-6) corresponding to anthocyanin-enriched fractions (AEFs), each of 50 mL, were collected starting when the colored material began to elute from the column. The column was then washed with 50 mL of 70% acetone: 30% water (fraction 7) to elute the proanthocyanidin-enriched fraction (PEF). Solvents were evaporated (<40 °C) and the aqueous extract was immediately freeze-died. Aliquots of the freeze-dried material were re-suspended in 80% methanol: 20% water (0.5% acetic acid) to analyze anthocyanin and proanthocyanidin concentrations, as explained below.

2.3.3. HPLC-PDA and LC-ESI-MS analysis

HPLC analysis for phenolic compounds was conducted using an Agilent 1200 HPLC (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). Quantification of anthocyanins was performed with reference to the external standard

method calibration curve obtained with cyanidin-3-*O*-glucoside (0.01 – 0.50 mg/mL) and pelargonidin-3-*O*-glucoside (0.1 – 1.0 mg/mL), and results were expressed as mg /100 g FW.

HPLC analysis for phenolic compounds was performed according to our protocol (Grace, et al., 2014). Ellagic acid (0.5 – 1.0 mg/mL) and quercetin-3-*O*-glucoside (0.25 – 1.00 mg/mL), and caffeic acid (0.5 – 1 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 & Giusti (2010). PAC components were identified with reference to standard PAC-B2 and monomeric and oligomeric PAC components were quantified with a calibration curve of procianidin- B2 (0.05 - 0.50 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-MSn (Liquid chromatography-electrospray ionization ion-trap time-of-flight mass spectrometry) system was used for analysis (Shimadzu Scientific Instruments, Columbia, MD, USA) This system was equipped with a Prominence HPLC system and separation was performed as described earlier (Kellogg et al., 2014).

2.4. Cell culture

2.4.1. Cell lines

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, USA), supplemented

with 100 IU/mL penicillin/100 µg/mL streptomycin (Thermo Fisher Scientific, Waltham, MA, USA), and 10% fetal bovine serum (Life Technologies, Grand Island, NY, USA) at a confluence not exceeding 80% and maintained at 37 °C in a humidified incubator with 5% CO_2.

Primary human dermal fibroblasts isolated from adult skin (HDFa, Invitrogen C-013-5C) were cultured in Medium 106 (Invitrogen M-106-500, Invitrogen, CA, USA) with Low Serum Growth Supplement (LSGS, Invitrogen S-003-10, Invitrogen, CA, USA) supplemented with Antibiotics Penicillin /Streptomycin Solution 100 IU/100 µg/mL (Fisher MT-30-002-CI). The cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

2.4.2. Cell viability and dose range determination

The cytotoxic activity against mouse macrophage and human fibroblast cells was evaluated after 24 h of exposure by using MTT ([3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide] colorimetric assay essentially as described by (Mosmann, 1983) and quantified spectrophotometrically at 550 nm using a microplate reader SynergyH1 (BioTek). Lyophilized extracts of samples were prepared in 80% ethanol in a concentration range from 1 to 250 µg/mL (dry weight/volume). The concentrations of test reagents that showed no changes in cell viability compared with that of the vehicle (80% ethanol) were selected for further studies.

2.4.3. Reactive oxygen species (ROS) assay

For determining *in vitro* reactive oxygen species (ROS), an adapted fluorescent dye protocol was used (Choi et al., 2007). Briefly, RAW 264.7 macrophage cells were seeded at a concentration of approx. 5×10^5 cells/well, final volume: 1 mL, into a 24-well plate

and incubated overnight at 37 °C. Fresh fluorescent medium was added to cells [5 µL of a solution of dichlorodihydrofluorescein diacetate acetylester (H_2DCFDA) 5 mg/mL prepared in sterile phosphate-buffered saline (PBS), (Molecular Probes, Eugene, OR, USA), reaching a concentration of 50 µM in each well] for 30 min. After aspiration of medium, cells were exposed to 1 µL of sample extract (Final concentration: 50 µg/mL) and 10 µL of 100 µg/mL lipopolysaccharide (LPS, from Escherichia coli 0127:B8) (final concentration: 1 µg/mL) and incubated for 24 h. The fluorescence of 2′, 7′-dichlorofluorescein (DCF) was measured at 485 nm (excitation) and 515 nm (emission) on a microplate reader (Synergy H1, Biotech, Winooski, VT, USA). Dexamethasone (DEX) was used as a positive control at 10 µM. Resulting production levels for each treatment were normalized to the non-stimulated control and expressed as % Control.

2.4.4. Nitric oxide radical inhibition assay

The ability of test samples to inhibit nitric oxide radical formation was determined in macrophage RAW 264.7 cells. Briefly, cells were seeded at a concentration of 5×10^5 cells/well, final volume: 1 mL, into a 24-well plate, and were exposed to 1 µL of sample extract (final concentrations: 50 µg/mL) and 10 µL of 100 µg/mL lipopolysaccharide (LPS, from Escherichia coli 0127:B8) (final concentration: 1 µg/mL) and incubated for 24 h. The production of nitrite, the stable end-product of NO generation by macrophages, was assayed colorimetrically as described by (Kellogg & Lila, 2013). To 100 µL of cell culture medium was added 100 µL of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine in 5% phosphoric acid), and the mixture was incubated in the dark at room temperature for 10 min. The absorbance at 540 nm was recorded (Synergy

H1, Biotech, Winooski, VT, USA), and a calibration curve of sodium nitrite (0-100 µM) was used to express results as µM of nitric oxide concentration.

2.4.5. Anti-inflammatory *in vitro* assay

The anti-inflammatory activity of samples was evaluated by treating macrophage cells with test samples followed by RNA extraction, purification and cDNA synthesis and quantitative PCR analysis. Initially cells were subcultured by TrypLE™ (Life Technologies, Grand Island, NY, USA) when dishes reached up to 90% confluence seeded in 24-well plates (5×10^5 cells/well, final volume: 1 mL) 24 h prior to treatment. The cells were treated with test samples at predetermined dose (final concentration: 50 µg/mL) for 1 h before elicitation with 10 µL of 100 µg/mL lipopolysaccharide (LPS, from Escherichia coli 0127:B8) LPS (final concentration: 1 µg/mL) for an additional 4 h. Positive (cells treated with DEX, at 10 µM) and negative controls (cells treatment with vehicle) were included in every experiment. Three replicates were made for both the treatments and the controls. At the end of the treatment period, cells were harvested in TRIzol reagent for subsequent cellular RNA extraction which was quantified spectrophotometrically using the SynergyH1/Take 3 Reader (Biotek, Winooski, VT, USA). The cDNA synthesis was conducted by using 2 µg of RNA for each sample and a high-capacity cDNA Reverse Transcription kit (Life Technologies, CA, USA), according to the manufacturer's protocol on an ABI GeneAMP 9700 (Life Technologies, CA, USA). The resulting cDNA was amplified by real-time quantitative PCR using SYBR green PCR Master Mix (Life Technologies, CA, USA). Quantitative PCR (qPCR) amplifications were performed on an ABI 7500 Fast Real Time PCR (Life Technologies, CA, USA) as described by Grace et al. (2014).

2.4.5.1. Quantitative PCR analysis.

The resulting cDNA was amplified by real-time quantitative PCR using SYBR green PCR Master Mix (Life Technologies). To avoid interference due to genomic DNA contamination, only intron-overlapping primers were selected using the Primer Express version 2.0 software (Applied Biosystems, Foster City, CA, USA) 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-1*β*, forward primer 5′- CAA CCA ACA AGT GAT ATT CTC CAT G-3′, reverse primer 5′-GAT CCA CAC TCT CCA GCT GCA-3. Samples were subjected to a melting curve analysis to confirm the amplification specificity. The relative change in the target gene with respect to the endogenous control gene was determined using $2^{-\Delta\Delta CT}$ method (Winer et al., 1999).

2.4.6. *In vitro* skin fibroblast migration and proliferation assay

HDFa fibroblasts were seeded into 96-well Oris™ plate (Platypus Technologies, LLC, Madison, WI, USA) at a concentration of 3×10^5 cells/ mL and cultured to nearly confluent cell monolayers. The cells were labeled with NucBlue® Live Cell Stain and CellTracker™ Red CMTPX (at 1 μM) fluorescent dyes. After confluence was reached, the well inserts were subsequently removed to form an unseeded region (2 mm in diameter) at the center of each well. Then, growth media was removed and free cellular

debris was removed by washing cells once with sterile phosphate buffered saline (PBS). Fresh growth medium containing vehicle (ethanol 80%), positive control (0.5% FBS), or extracts (final concentrations: 1 – 50 µg/mL) were added to a set of 4 wells per dose and incubated up to 48 h at 37 °C with 5% CO_2. The progress of cell migration was materialized by the cell movement into the unseeded region located at the center of each well and was monitored after 0, 24, and 48 h after sample addition by measuring the fluorescence at excitation/emission wavelength of 360/460 nm and 577/605 nm on a microplate reader (Synergy H1, Biotech, Winooski, VT, USA). Bright field and fluorescent images were observed using EVOS® FL Auto Cell Imaging System (Life Technologies). Images were captured as at center of well for consistency purposes. Three representative images of the areas from each well under each condition were photographed at 0 and 48 h to estimate the wound closure.

2.5. Statistical analysis

Statistics were performed using the software GraphPad Prism v6 (GraphPad Software Inc., La Jolla, CA). All data were analyzed by one-way ANOVA. Significant differences among means were determined by Tukey´s test at 5% level of significance. All samples were obtained in triplicate and analyzed in three repetitions (N=9), unless specified. All results are expressed as means ± standard deviation (SD).

3. Results and discussion

3.1. Characterization of phenolic compounds in the crude extracts

Phenolic compounds were characterized and identified by their LC retention times, UV-Vis absorption, MS fragmentation patterns and by comparison with available standard compounds and reported literature.

Table 1 presents the phenolic compound characterization in strawberry CE. Thirteen phenolic compounds were tentatively identified in strawberry 'San Andreas'. Peaks **2**, **3**, and **8** (Table 1) correspond to ellagitannins. Peaks **2** and **3** were identified as bis-HHDP-glucose isomers (also known as pedunculagin isomers). Peak **8** was identified as a dimer of galloyl-bis-HHDP-glucose (sanguiin H-6). This compound was proposed to be a chemotaxonomic marker for *Fragaria* (Okuda et al., 1992).

Peaks **5**, **6**, **7**, **10**, and **11** (Table 1) correspond to anthocyanins detected in the strawberry samples. Peak **5** and **6** correspond to cyanidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside, respectively. The last one was the major anthocyanin detected in the samples. Peaks **7**, **10**, and **11** (Table 1) correspond to rutinoside, malonylglucoside, and acetylglucoside of perlargonidin, respectively.

Quercetin-3-*O*-glucuronide (peak **12**) and Kaempferol-3-*O*-glucoside (peak **13**) were also detected in strawberry samples (Table 1). Peaks **1** and **4** (Table 1) were tentatively identified as hydroxycinnamic acid derivatives. Caffeoyl hexose (peak **1**) and *p*-coumaroyl hexoside (peak **4)** were confirmed in the samples. Peak **9** was identified as cinnamoyl hexoside (Table 1).

Table 2 shows the average content of the individual phenolic compounds quantified in 'San Andreas' strawberry CE. Results were in agreement with reported concentrations of phenolic compounds for other strawberry cultivars (Buendía et al., 2010; Gasparrini et al., 2017; Duarte et al., 2018).

Normal phase HPLC with fluorescence detection was able to separate proanthocyanidin (PAC) components in the strawberry CE according to their degree of polymerization. Proanthocyanidins with B-type linkages were detected as the only PAC component in

strawberries. Several peaks corresponding to dimers, trimers, tetramers, and oligomers of PAC were distinguished in the chromatograms of the extracts (data not shown). Table 2 shows the average content of strawberry PACs. According to the results, total PAC concentration represented the highest contribution to the total phenolic compounds, being near to 50% (Table 2).

Table 3 presents the phenolic compound characterization in the CE of blackberry cultivar 'Black Satin'. Fifteen phenolic compounds were identified in the blackberry extracts by HPLC-PAD-ESI-MS. The profile of phenolic compounds of this blackberry cultivar harvested in 2014 was previously determined by Van de Velde et al., (2016a). Some extra ellagitannins and flavonols were observed in this cultivar harvested in 2017 in relation to the fruit harvested in 2014. Peaks **1** to **7** (Table 3) correspond to ellagitannins. Peaks **1**, **2** and **4** were identified as bis-HHDP-glucose isomers (also known as pedunculagin isomers). Peak **3** corresponded to an ellagitannin derivative, peak **6** was identified as a trimer of galloyl-bis-HHDP-glucose (lambertianin C), and peak **7** was identified as a dimer of galloyl-bis-HHDP-glucose (sanguiin H-6/lambertianin A). Peaks **5**, **8**, **9**, and **10** (Table 3) correspond to anthocyanins. Peak **5** was identified as cyanidin-3-*O*-glucoside, the major anthocyanin detected in the samples. Peak **8** corresponded with cyanidin-3-*O*-xyloside, and peaks **9** and **10** were identified as cyanidin-3-*O*-(6-O-malonyl glucoside) and cyanidin-3-*O*-β"-(3-hydroxy-3-methylglutaroyl)-glucoside, respectively. Five peaks (peaks **11** to **15)** were identified as flavonols, of which 4 peaks corresponded to quercetin derivatives and 1 peak corresponded to a kaempferol derivative. Peak **11**, **12**, **13**, **14** and **15** corresponded to quercetin-3-*O*-pentoside, quercetin-3-*O*-rutinoside, quercetin-(6'(-(3-

hydroxy-3-methylglautaroyl)-galactoside, quercetin-glucoside malonate, and kampferol-3-*O*-glucoside, respectively.

Table 4 presents the average content of phenolic compounds in the CE of blackberry cultivar 'Black Satin'. As it can be seen, anthocyanins represented the highest contribution (66.4%) to the total phenolic compounds, being cyanidin-3-*O*-glucoside 84.7% of the total anthocyanins registered in the samples (Table 4).

PAC components with different degree of polymerization and B-type linkages were detected as the only PAC component in blackberry 'Black Satin' (Table 4). Total PAC recorded as the sum of different PAC components represented 18.2% of the total polyphenols in the blackberry samples, a lower contribution compared with the value observed for strawberry samples.

3.2. Polyphenol-enriched extracts

A first cleanup procedure was conducted on blackberry and strawberry CEs using C18-SPE cartridges to remove sugars, pectins, and lipophilic material and to concentrate polyphenolic compounds. Then, these polyphenol-enriched extracts were loaded separately on columns packed with Sephadex LH-20 (second cleanup) and eluted (fractions 1 - 6) with 20% methanol: 80% water (0.5% acetic acid), to produce anthocyanin-enriched fractions (AEFs) with different level of anthocyanins, and with 70 % acetone: 30% water (fraction 7), to produce a proanthocyanidin-enriched fraction (PEF). The AEFs (fraction 4 for strawberry and fraction 6 for blackberry) had the highest anthocyanin concentrations between the other fractions, while having the same relative composition of anthocyanin components observed in the CEs (Table 1 and 3), and were used for the cell culture assays. In the same way, the PEFs (fraction 7 for both fruits)

were used for cell culture assays. Table 5 shows the content of total anthocyanins and total PACs in the CEs and purified polyphenolic-enriched extracts of strawberry and blackberry, on a dry weight basis. As it can be seen, two cleanup procedures in the strawberry CE increased the anthocyanin content (AEF) by approximately 170-fold, and proanthocyanidins (PEF) by 17-fold. For blackberry CE, cleanup procedures increased the anthocyanin concentration (AEF) by approximately 37-times, and PAC (PEF) increased by 2-times, on a dry weight basis.

According to the results, the total anthocyanins registered in the CE of blackberry, on a dry weight basis, resulted 8-fold higher content than in the CE of strawberry (Table 5). Moreover, total PAC, calculated on a dry weight basis, was1.1 times higher in blackberry CE than in strawberry CE (Table 5). The same result was observed for both fruits but on a fresh weight basis (Table 2 and 4). After the polyphenolic extract enrichment, the level of total anthocyanins (AEF) in blackberry was 1.6-times higher than in strawberry (Table 5). However, the level of PAC (PAF) was almost 8-times higher in strawberry than in blackberry (Table 5).

CEs, AEFs, and PEFs for both fruits were tested in cell culture assays.

3.3. Anti-inflammatory properties

3.3.1. Effects of strawberry and blackberry extracts on cell viability

The viability of cells incubated with different treatment concentrations was evaluated through the MTT assay after 24 h of exposure. Crude extract of strawberry and blackberry, and AEF and PEF of both fruits in the range 50 – 250 µg/mL did not depress the cell viability ($p > 0.05$). Only the AEF of strawberry at 250 µg/mL reduced cell viability close to 65% (Figure supplementary material 1). For that reason, all subsequent

experiments in this work were performed at 50 µg/mL, a non-toxic concentration that is easily achievable in the gastrointestinal tract after consumption of berries (Kahle et al., 2006). Therefore, any inhibitory effect of the extracts at treated concentrations was not attributable to cytotoxic effects on the macrophage cells, but instead to phytochemical activity.

3.3.2. Effects of strawberry and blackberry extracts on intracellular reactive oxygen species (ROS) and nitric oxide (NO) production

The ability of phytochemical compounds from CE of strawberry and blackberry (50 µg/mL), and AEF and PEF obtained from both fruits (50 µg/mL) to inhibit the intracellular ROS production and NO synthesis was investigated *in vitro* in a LPS-stimulated murine RAW 264.7 macrophage model.

'San Andreas' strawberry CE exhibited a 20% reduction in ROS production compared to cells treated only with LPS ($p \leq 0.05$) (Figure 1). This result was in line with Gasparrini et al. (2017), whom reported a significant decrease (close to 35%) in the amount of ROS in macrophages treated with 'Alba' strawberry crude extract at 100 µg/mL.

AEF from strawberry did not show any inhibitory effect on ROS production ($p > 0.05$). However, PEF from the same fruit showed ROS reduction around 35% ($p \leq 0.05$) (Figure 1). On the other hand, the CE from 'Black Satin' blackberry exhibited a 30% reduction in the ROS amount compared with cells treated with only LPS ($p \leq 0.05$) (Figure 1), as was previously observed for this blackberry cultivar harvested in 2014 by Van de Velde et al., 2016a). Meanwhile, ROS reduction was around 23% and 50% when cells were treated with AEF and PEF from blackberry, respectively ($p \leq 0.05$) (Figure 1).

According to the results, phytochemicals from blackberry CE and polyphenol fractions exhibited higher ROS reduction than phytochemicals from strawberry samples. Moreover, PEF from both fruits showed the highest ROS reduction among treatments, suggesting that proanthocyanidin components may be more active than anthocyanins against the oxidative stress.

Jung et al. (2015) reported an approximate 60% decrease in the intracellular ROS levels in RAW 264.7 cells treated with an anthocyanin rich-fraction obtained from Korean blackberries at 20 μg/mL. However, the effectiveness of blackberry proanthocyanidins against the oxidative stress was not reported by the authors. In agreement with our study, the capacity of a proanthocyanidin-rich extract from a deciduous tree *Cassia abbreviata* to reduce the ROS production in early larval stage wild type worms to 61.73%, compared to control group, was recently revealed (Sobeh et al., 2018). The mechanism by which phytochemicals from berries such as strawberry reduce ROS production in LPS-stimulated RAW 264.7 macrophages was suggested for the first time due to the increase in endogenous antioxidant enzyme activities such as glutathione reductase, glutathione transferase and superoxide dismutase (Gasparrini et al., 2017).

In addition, Ávila et al. (2017) stated that the polyphenolic-enriched extracts from Chilean berries exerted protective effects on human epithelial gastric cells against free radical-induced damage. Such effects were related to a direct scavenging activity of polyphenols towards free radicals and to the modulation of pivotal intracellular mechanisms such as glyoxalase I and glutathione s-transferases activities, and carboxymethyl lysine and malondialdehyde levels.

As shown in Figure 2, CE from strawberry and blackberry showed a slight suppression on the NO synthesis (lower than 10%). In agreement, Van de Velde et al. (2016a) found a slight suppression in NO production (less than 5%) in activated macrophages by the crude extract of 'Black Satin' blackberries harvested in 2014. Contrary, Gasparrini et al. (2017) reported a reduction close to 50% in the NO synthesis in activated macrophages treated with 100 µg/mL of 'Alba' strawberry crude extracts.

Strawberry AEF did not suppress NO synthesis, however, strawberry PEF showed an NO synthesis suppression near to 35.6%, even higher than the NO inhibition observed for the positive control DEX (~ 30%) (Figure 2). As shown, PAC components from strawberry 'San Andreas' were more active in NO suppression of LPS-stimulated macrophages than anthocyanins.

On the other hand, activated macrophages treated with blackberry AEF showed 14% of inhibition of NO production compared with cells treated with only LPS, while only ~ 5% of inhibition was observed for the CE. Meanwhile, blackberry PEF exhibited NO synthesis inhibition near to 25% (Figure 2). As observed for strawberry, blackberry PEF exhibited higher NO synthesis inhibition than blackberry AEF. In a study of the inhibition of NO synthesis in raw 264.7 macrophages by polyphenol fractions from a wide number of wild Mexican blackberry genotypes, authors revealed that the anthocyanin-rich fraction from one genotype (WB-10) and the proanthocyanidin-rich fraction from another genotype (UM-601) exhibited the highest NO inhibitory activities (Cuevas-Rodríguez et al., 2010a). Therefore, taking into consideration the latter results and results obtained in this work, as suggested by Esposito et al. (2014), the effect on the suppression of NO synthesis and other anti-inflammatory effects 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.

3.3.3. Effects of strawberry and blackberry extracts on inflammatory markers

'San Andreas' strawberry and 'Black Satin' blackberry CEs significantly ($p \leq 0.05$) suppressed Cox-2 gene expression based on 30% change relative to LPS stimulated control (Figure 3a). AEFs and PEFs from both fruits did not show any significant reduction in Cox-2 gene expression, suggesting that activity of CEs is due to a synergistic contribution of both anthocyanin and proanthocyanidin components, together in the same treatment, on the gene modulation.

The expression of the iNOS gene, responsible for the secretion of NO, showed a suppression of nearly 20% by the CEs of strawberry and blackberry (Figure 3b). AEFs for both fruits did not inhibit gene expression at the tested concentration (50 µg/mL). However, the gene expression suppression of this biomarker of inflammation was around 50% and 65% reduction by the PAF from strawberry and blackberry, respectively (Figure 3b). The same behavior was observed for suppression of NO synthesis (Figure 2).

Meanwhile, the inhibition of IL1-β gene expression was only due to AEFs and PEFs of both fruits (Figure 3c). CEs did not show any inhibitory effect on this acute inflammatory biomarker at least at the treated concentration (50 µg/mL). Strawberry AEF and PEF exhibited a stronger effect on gene inhibition compared with polyphenol fractions from blackberry (Figure 3c).

Finally, all strawberry and blackberry CEs and polyphenol fractions suppressed the expression of the chronic pro-inflammatory gene IL-6 (Figure 3d). The CE from

strawberry inhibited IL-6 expression based on 60% change relative to LPS stimulated control (Figure 3d). Meanwhile, AEF and PEF from the same fruit suppressed IL-6 gene expression about 40 and 75%, respectively, relative to LPS stimulated control (Figure 3d). As it can be seen, the inhibition on this biomarker of inflammation was more dependent on proanthocyanidin components than anthocyanins from 'San Andreas' strawberry extracts. On the other hand, blackberry CE showed a reduction on the IL-6 gene expression around 40%, while the inhibition of this gene expression was around 33 % by the AEF and slightly higher (43%) by the PEF of this fruit.

Strawberry and blackberry phytochemicals exhibited different behaviors on gene expression of pro-inflammatory mediators and cytokines. For instance, the phytochemical compounds from strawberry were more active against IL-1 β and IL-6 gene expressions than blackberry phytochemicals, and the PEF was more active than AEF. Moreover, strawberry and blackberry PEFs proved to be the active polyphenol fraction against iNOS gene expression.

Our results are in agreement with the data obtained in other studies achieved on RAW macrophages, in which the expression of anti-inflammatory mediators and cytokines induced by LPS was reduced by strawberry crude extracts (Liang et al., 2012; Liu & Lin, 2013; Gasparrini et al., 2017) and by blackberry crude extracts (Van de Velde et al., 2016a), and blackberry polyphenol fractions (Cuevas-Rodríguez et al., 2010a).

The molecular mechanism of the inhibition of inflammatory mediators and cytokines by anthocyanins from berry fruits was recently reported (Lee et al., 2014; Limtrakul et al., 2015; Gasparrini et al., 2017; Duarte et al., 2018). As it is known, LPS activates intracellular pathways and trigger the kinase cascade and nuclear transcription factors,

like MAPKs and NF-kB, both responsible for the up-regulation of inflammation-related genes such as pro-inflammatory cytokines and inflammatory enzymes. It was observed that pelargonin-3-*O*-glucoside and cyanidin-3-*O*-glucoside interfere with the NF-kB pathway by inhibiting IkB-α phosphorylation (Nf-kB inhibitor), and in turn, avoiding IkB-α proteaosomal degradation. Thus, the nuclear translocation of NF-kB to induce the production of inflammatory cytokines such as IL-1β, IL-6 is prevented, along with other process (Zhang et al., 2010; Duarte et al., 2018). Moreover, anthocyanins have anti-inflammatory action in LPS stimulated macrophages, due to the decrease of the phosphorylation of ERK, p38 and JNK, proteins related to the MAPKs. Thus, the activation of the transcription factor AP-1 is inhibited, which in turn, prevent the gene expression of inflammatory mediators such as IL-6 and TNF-α (Mansell & Jenkins, 2013; Limtrakul et al., 2015; Duarte et al., 2018). As it was described, the molecular mechanism by which anthocyanins from berries exerted anti-inflammatory action was demonstrated. However, as revealed in the present work, the enriched-proanthocyanidin extracts from strawberry and blackberry exerted more activity against pro-inflammatory gene expression than the corresponding enrich-anthocyanin-enriched extracts. In agreement, in a recent study, PACs from purple maize demonstrated anti-inflammatory effects inhibiting 66% of iNOS and 89% of Cox-2 activities. Moreover, an iNOS inhibitory kinetic study showed that PACs from purple maize and the anthocyanin cyanidin-3-*O*-glucoside acted as non-competitive inhibitors (Chen et al., 2017), but a molecular mechanism in which PACs exert the anti-inflammatory effects was not revealed by the authors. Therefore, a molecular mechanism which describes the anti-

inflammatory effects of these phytochemicals needs to be proposed and future research should be focalized in that direction.

3.4. Wound healing properties

3.4.1. Effects of strawberry and blackberry extracts on cell viability

The viability of HDFa fibroblasts incubated with different treatment concentrations was evaluated through the MTT assay after 24 h of exposure. CE of strawberry and blackberry in the range 50 – 250 µg/mL did not depress the cell viability ($p > 0.05$) (Figure supplementary material 2). AEF and PEF of both fruits in the range 50 – 250 µg/mL depressed the cell viability ($p \leq 0.05$) to different degrees. For instance, samples at 50 µg/mL reduced cell viability close to 60% (Figure supplementary material 2). For that reason, all subsequent experiments in this work were performed at not toxic concentrations: 50 µg/mL for CEs, and 1 - 5 µg/mL for AEFs and PEFs.

3.4.2. Effect of strawberry and blackberry extracts on skin fibroblast migration

Strawberry CE enhanced cell migration after 48 h in a percentage equal to 45% of the migration registered for the positive control FBS (Figure 4). Blackberry CE did not exhibit cell migration into the unseeded region after 48 h of treatment, and its migration was not different to that observed for cells treated with the sample vehicle (ethanol 80%) (Figure 4). Strawberry and blackberry AEFs, at the lowest treated concentration, 1 µg/mL, showed a fibroblast migration around 50% of that registered for FBS. Both strawberry and blackberry AEFs at 5 µg/mL showed lower migration, around 35% relative to FBS cell migration (Figure 4). Only strawberry PEF at 1 µg/mL exhibited fibroblast migration around 30% relative to FBS. The other treatments with PEF samples

did not show any enhancement in the cell migration compared with the sample vehicle (Figure 4).

According to the results, cell migration potential of polyphenolic compounds from strawberry and blackberry was associated to the anthocyanin content. In agreement, Nizamutdinova et al. (2009) reported that a 48 h treatment with 50 and 100 µg/mL of anthocyanins isolated from black soybean, consisted of cyanidin-3-glucoside (72%), delphinidin-3-glucoside (20%), and petunidin-3-glucoside (6%), induced fibroblast and keratinocyte migration in an *in vitro* cell migration assay. Moreover, authors found that anthocyanins also increased the production of the vascular endothelial growth factor, VEGF (an important cytokine that is involved in wound healing), and increased the wound-induced cell migration at 24 h post wound formation in an *in vitro* scratch-wound assay. Therefore, the authors speculated that anthocyanins might potentiate wound healing through the increase of VEGF production. Similarly, San Miguel et al. (2011) studied the promotion of the proliferation and migration of human oral fibroblasts by bioactive compounds such as resveratrol, ferulic acid, phloretin and tetrahydrocurcuminoid. Authors revealed that some concentrations (10^{-3} and 10^{-5} M) of these compounds may have beneficial effects on functional mechanisms regulating fibroblast migration and proliferation during gingival healing or periodontal repair. In addition, the pre-incubation of HDFa fibroblasts with crude extracts of Andean blackberries and Andean blueberries significantly improved the markers of oxidative damage (intracellular ROS levels, catalase, superoxide dismutase and glutathione activities, lipid peroxidation, protein oxidation and ATP and nitrite levels) compared with the stressed cells group (Alarcón-Barrera et al., 2018).

Because discovery of natural remedies which can improve the wound healing is an important issue from clinical and economical points of view, novel results obtained about the potential effects of anthocyanins from strawberry and blackberry on fibroblast migration, research in this area deserves to be further explored.

4. Conclusions

The polyphenolic profile of 'San Andreas' strawberry and 'Black Satin' blackberry was characterized and quantified. CE, AEF and PEF from blackberry exhibited higher ROS reduction than samples from strawberry (almost 50% of ROS suppression by blackberry PEF). PEF from both fruits showed the highest ROS reduction among treatments, suggesting that proanthocyanidin components may be more active than anthocyanins against the oxidative stress. Polyphenolic extracts from strawberry were more active against IL-1β and IL-6 gene expressions than blackberry's, having been PEF more active than AEF (75% gene expression suppression on IL-1β and IL-6 by strawberry PEF). Moreover, strawberry and blackberry PEFs were the active polyphenol fraction against iNOS gene expression (50% and 65% gene expression by strawberry and blackberry PEF, respectively). The cell migration potential of polyphenolic compounds from strawberry and blackberry could be only associated with the anthocyanins. Strawberry and blackberry AEFs, at 1 μg/mL, showed a fibroblast migration around 50% of that registered for the positive control, FBS.

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Conflict of Interest

The authors declare no conflict of interest.

References

Afrin, S., Gasparrini, M., Forbes-Hernandez, T. Y., Reboredo-Rodriguez, P., Mezzetti, B., Varela-López, A., … Battino, M. (2016). Promising Health Benefits of the Strawberry: A Focus on Clinical Studies. *Journal of Agricultural and Food Chemistry*, *64*(22), 4435–4449. https://doi.org/10.1021/acs.jafc.6b00857

Agyare, C., Boakye, Y. D., Bekoe, E. O., Hensel, A., Dapaah, S. O., & Appiah, T. (2016). Review: African medicinal plants with wound healing properties. *Journal of Ethnopharmacology*, *177*, 85–100. https://doi.org/10.1016/j.jep.2015.11.008

Alarcón-Barrera, K. S., Armijos-Montesinos, D. S., García-Tenesaca, M., Iturralde, G., Jaramilo-Vivanco, T., Granda-Albuja, M. G., … Alvarez-Suarez, J. M. (2018). Wild Andean blackberry (Rubus glaucus Benth) and Andean blueberry (Vaccinium floribundum Kunth) from the Highlands of Ecuador: Nutritional composition and protective effect on human dermal fibroblasts against cytotoxic oxidative damage. *Journal of Berry Research*, *8*(3), 223–236. https://doi.org/10.3233/JBR-180316

Ávila, F., Theoduloz, C., López-Alarcón, C., Dorta, E., & Schmeda-Hirschmann, G. (2017). Cytoprotective Mechanisms Mediated by Polyphenols from Chilean Native Berries against Free Radical-Induced Damage on AGS Cells. *Oxidative Medicine and Cellular Longevity*, *2017*. https://doi.org/10.1155/2017/9808520

Buendía, B., Gil, M. I., Tudela, J. A., Gady, A. L., Medina, J. J., Soria, C., … Tomás-

Barberán, F. A. (2010). HPLC-MS Analysis of Proanthocyanidin Oligomers and Other Phenolics in 15 Strawberry Cultivars †. *Journal of Agricultural and Food Chemistry*, *58*(7), 3916–3926. https://doi.org/10.1021/jf9030597

Chen, C., Somavat, P., Singh, V., & Gonzalez de Mejia, E. (2017). Chemical characterization of proanthocyanidins in purple, blue, and red maize coproducts from different milling processes and their anti-inflammatory properties. *Industrial Crops and Products*, *109*(April), 464–475. https://doi.org/10.1016/j.indcrop.2017.08.046

Choi, S.-Y., Hwang, J.-H., Ko, H.-C., Park, J.-G., & Kim, S.-J. (2007). Nobiletin from citrus fruit peel inhibits the DNA-binding activity of NF-κB and ROS production in LPS-activated RAW 264.7 cells. *Journal of Ethnopharmacology*, *113*(1), 149–155. https://doi.org/10.1016/j.jep.2007.05.021

Cuevas-Rodríguez, E. O., Dia, V. P., Yousef, G. G., García-Saucedo, P. A., López-Medina, J., Paredes-López, O., … Lila, M. A. (2010). Inhibition of Pro-inflammatory Responses and Antioxidant Capacity of Mexican Blackberry (Rubus spp.) Extracts. *Journal of Agricultural and Food Chemistry*, *58*(17), 9542–9548. https://doi.org/10.1021/jf102590p

Duarte, L. J., Chaves, V. C., Nascimento, M. V. P. dos S., Calvete, E., Li, M., Ciraolo, E., … Dalmarco, E. M. (2018). Molecular mechanism of action of Pelargonidin-3-O-glucoside, the main anthocyanin responsible for the anti-inflammatory effect of strawberry fruits. *Food Chemistry*, *247*(December 2017), 56–65. https://doi.org/10.1016/j.foodchem.2017.12.015

Esposito, D., Chen, A., Grace, M. H., Komarnytsky, S., & Lila, M. A. (2014). Inhibitory

effects of wild blueberry anthocyanins and other flavonoids on biomarkers of acute and chronic inflammation in vitro. *Journal of Agricultural and Food Chemistry*, *62*(29), 7022–7028. https://doi.org/10.1021/jf4051599

Gasparrini, M., Forbes-Hernandez, T. Y., Giampieri, F., Afrin, S., Alvarez-Suarez, J. M., Mazzoni, L., … Battino, M. (2017). Anti-inflammatory effect of strawberry extract against LPS-induced stress in RAW 264.7 macrophages. *Food and Chemical Toxicology*, *102*, 1–10. https://doi.org/10.1016/j.fct.2017.01.018

Giampieri, F., Forbes-Hernandez, T. Y., Gasparrini, M., Alvarez-Suarez, J. M., Afrin, S., Bompadre, S., … Battino, M. (2015). Strawberry as a health promoter: an evidence based review. *Food & Function*, *6*(5), 1386–1398. https://doi.org/10.1039/C5FO00147A

Grace, M. H., Esposito, D., Dunlap, K. L., & Lila, M. A. (2014). Comparative Analysis of Phenolic Content and Profile, Antioxidant Capacity, and Anti-inflammatory Bioactivity in Wild Alaskan and Commercial *Vaccinium* Berries. *Journal of Agricultural and Food Chemistry*, *62*(18), 4007–4017. https://doi.org/10.1021/jf403810y

Grace, M. H., Guzman, I., Roopchand, D. E., Moskal, K., Cheng, D. M., Pogrebnyak, N., … Lila, M. A. (2013). Stable Binding of Alternative Protein-Enriched Food Matrices with Concentrated Cranberry Bioflavonoids for Functional Food Applications. *Journal of Agricultural and Food Chemistry*, *61*(28), 6856–6864. https://doi.org/10.1021/jf401627m

Hemmati, A. A., Larki-Harchegani, A., shabib, S., Jalali, A., Rezaei, A., & Housmand, G. (2018). Wound healing property of milk in full thickness wound model of rabbit.

International Journal of Surgery, *54*(March), 133–140. https://doi.org/10.1016/j.ijsu.2018.04.030

Joseph, S. V, Edirisinghe, I., & Burton-Freeman, B. M. (2014). Berries: Anti-inflammatory Effects in Humans. *Journal of Agricultural and Food Chemistry*, *62*(18), 3886–3903. https://doi.org/10.1021/jf4044056

Jung, H., Lee, H. J., Cho, H., Lee, K., Kwak, H. K., & Hwang, K. T. (2015). Anthocyanins in Rubus fruits and antioxidant and anti-inflammatory activities in RAW 264.7 cells. *Food Science and Biotechnology*, *24*(5), 1879–1886. https://doi.org/10.1007/s10068-015-0246-1

Kahle, K., Kraus, M., Scheppach, W., Ackermann, M., Ridder, F., & Richling, E. (2006). Studies on apple and blueberry fruit constituents: Do the polyphenols reach the colon after ingestion? *Molecular Nutrition & Food Research*, *50*(4–5), 418–423. https://doi.org/10.1002/mnfr.200500211

Kellogg, J., Grace, M., & Lila, M. (2014). Phlorotannins from Alaskan Seaweed Inhibit Carbolytic Enzyme Activity. *Marine Drugs*, *12*(10), 5277–5294. https://doi.org/10.3390/md12105277

Kellogg, J., & Lila, M. A. (2013). Chemical and in Vitro Assessment of Alaskan Coastal Vegetation Antioxidant Capacity. *Journal of Agricultural and Food Chemistry*, *61*(46), 11025–11032. https://doi.org/10.1021/jf403697z

Lee, S. G., Kim, B., Yang, Y., Pham, T. X., Park, Y.-K., Manatou, J., … Lee, J.-Y. (2014). Berry anthocyanins suppress the expression and secretion of proinflammatory mediators in macrophages by inhibiting nuclear translocation of NF-κB independent of NRF2-mediated mechanism. *The Journal of Nutritional*

Biochemistry, *25*(4), 404–411. https://doi.org/10.1016/J.JNUTBIO.2013.12.001

Liang, L., Wu, X., Zhao, T., Zhao, J., Li, F., Zou, Y., … Yang, L. (2012). In vitro bioaccessibility and antioxidant activity of anthocyanins from mulberry (Morus atropurpurea Roxb.) following simulated gastro-intestinal digestion. *Food Research International*, *46*(1), 76–82. https://doi.org/10.1016/j.foodres.2011.11.024

Limtrakul, P., Yodkeeree, S., Pitchakarn, P., & Punfa, W. (2015). Suppression of inflammatory responses by black rice extract in RAW 264.7 macrophage cells via downregulation of NF-kB and AP-1 signaling pathways. *Asian Pacific Journal of Cancer Prevention*, *16*(10), 4277–4283. https://doi.org/10.7314/APJCP.2015.16.10.4277

Liu, C. J., & Lin, J. Y. (2013). Anti-inflammatory effects of phenolic extracts from strawberry and mulberry fruits on cytokine secretion profiles using mouse primary splenocytes and peritoneal macrophages. *International Immunopharmacology*, *16*(2), 165–170. https://doi.org/10.1016/j.intimp.2013.03.032

Mansell, A., & Jenkins, B. J. (2013). Dangerous liaisons between interleukin-6 cytokine and toll-like receptor families: A potent combination in inflammation and cancer. *Cytokine & Growth Factor Reviews*, *24*(3), 249–256. https://doi.org/10.1016/J.CYTOGFR.2013.03.007

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, *65*(1–2), 55–63. https://doi.org/10.1016/0022-1759(83)90303-4

Nile, S. H., & Park, S. W. (2014). Edible berries: Bioactive components and their effect on human health. *Nutrition*, *30*(2), 134–144.

https://doi.org/10.1016/j.nut.2013.04.007

Nizamutdinova, I. T., Kim, Y. M., Chung, J. Il, Shin, S. C., Jeong, Y. K., Seo, H. G., … Kim, H. J. (2009). Anthocyanins from black soybean seed coats stimulate wound healing in fibroblasts and keratinocytes and prevent inflammation in endothelial cells. *Food and Chemical Toxicology*, *47*(11), 2806–2812. https://doi.org/10.1016/j.fct.2009.08.016

Okuda, T., Yoshida, T., Hatano, T., Iwasaki, M., Kubo, M., Orime, T., … Naruhashi Okayama University, Tsushima, Okayama 700 (Japan)), N. (Faculty of P. S. (1992). Hydrolysable tannins as chemotaxonomic markers in the Rosaceae. *Phytochemistry (United Kingdom)*.

Park, C. M., Park, J. Y., Noh, K. H., Shin, J. H., & Song, Y. S. (2011). Taraxacum officinale Weber extracts inhibit LPS-induced oxidative stress and nitric oxide production via the NF-κB modulation in RAW 264.7 cells. *Journal of Ethnopharmacology*, *133*(2), 834–842. https://doi.org/10.1016/J.JEP.2010.11.015

San Miguel, S. M., Opperman, L. A., Allen, E. P., Zielinski, J., & Svoboda, K. K. H. (2011). Bioactive antioxidant mixtures promote proliferation and migration on human oral fibroblasts. *Archives of Oral Biology*, *56*(8), 812–822. https://doi.org/10.1016/j.archoralbio.2011.01.001

Sobeh, M., Mahmoud, M. F., Abdelfattah, M. A. O., Cheng, H., El-Shazly, A. M., & Wink, M. (2018). A proanthocyanidin-rich extract from Cassia abbreviata exhibits antioxidant and hepatoprotective activities in vivo. *Journal of Ethnopharmacology*, *213*, 38–47. https://doi.org/10.1016/J.JEP.2017.11.007

Van de Velde, F., Grace, M. H., Esposito, D., Pirovani, M. T., & Lila, M. A. (2016a).

Quantitative comparison of phytochemical profile, antioxidant, and anti-inflammatory properties of blackberry fruits adapted to Argentina. *Journal of Food Composition and Analysis*, *47*. https://doi.org/10.1016/j.jfca.2016.01.008

Van de Velde, F., Grace, M. H., Pirovani, M. T., & Lila, M. A. (2016b). Impact of a new postharvest disinfection method based on peracetic acid fogging on the phenolic profile of strawberries. *Postharvest Biology and Technology*, *117*. https://doi.org/10.1016/j.postharvbio.2016.03.005

Wallace, T. C., & Giusti, M. M. (2010). Extraction and normal-phase HPLC-fluorescence-electrospray MS characterization and quantification of procyanidins in cranberry extracts. *Journal of Food Science*, *75*(8), 690–696. https://doi.org/10.1111/j.1750-3841.2010.01799.x

Wang, P. H., Huang, B. S., Horng, H. C., Yeh, C. C., & Chen, Y. J. (2018). Wound healing. *Journal of the Chinese Medical Association*, *81*(2), 94–101. https://doi.org/10.1016/j.jcma.2017.11.002

Winer, J., Jung, C. K. S., Shackel, I., & Williams, P. M. (1999). Development and Validation of Real-Time Quantitative Reverse Transcriptase–Polymerase Chain Reaction for Monitoring Gene Expression in Cardiac Myocytesin Vitro. *Analytical Biochemistry*, *270*(1), 41–49. https://doi.org/10.1006/ABIO.1999.4085

Zhang, Y., Lian, F., Zhu, Y., Xia, M., Wang, Q., Ling, W., & Wang, X. D. (2010). Cyanidin-3-O-β-glucoside inhibits LPS-induced expression of inflammatory mediators through decreasing IκBα phosphorylation in THP-1 cells. *Inflammation Research*, *59*(9), 723–730. https://doi.org/10.1007/s00011-010-0183-7

Zia-Ul-Haq, M., Riaz, M., De Feo, V., Jaafar, H., & Moga, M. (2014). Rubus Fruticosus

L.: Constituents, Biological Activities and Health Related Uses. *Molecules*, *19*(8), 10998–11029. https://doi.org/10.3390/molecules190810998

Figure captions

Figure 1. Effects of strawberry and blackberry crude extracts (CEs), anthocyanin-enriched fractions (AEFs) and proanthocyanidin-enriched fractions (PEFs) on the 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).

Figure 2. Effects of strawberry and blackberry crude extracts (CEs), and anthocyanin-enriched fractions (AEFs) and proanthocyanidin-enriched fractions (PEFs) on nitric oxide (NO) production. Fold changes in NO production are reported as the mean ± SD relative to LPS controls. 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).

Figure 3. Effects of strawberry and blackberry crude extracts (CEs), and anthocyanin-enriched fractions (AEFs) and proanthocyanidin-enriched fractions (PEFs) on pro-inflammatory gene expression profile: (**A**) cyclooxygenase-2 (Cox-2), (**B**) iNOS, (**C**) cytokine interleukin-6 (IL-6), and (**D**) cytokine interleukin-1β (IL-1β). Fold changes in gene expression are reported as the mean ± SD relative to LPS controls. 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).

Figure 4. Effects of strawberry and blackberry crude extracts (CEs), and anthocyanin-enriched fractions (AEFs) and proanthocyanidin-enriched fractions (PEFs) on skin fibroblast migration. Data are reported as the mean ± SD. FBS, fetal bovine serum at 0.5 % 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).

Figure supplementary material 1. Effects of strawberry and blackberry crude extracts (CE), anthocyanin-enriched fractions (AEFs) and proanthocyanidin-enriched fractions (PEFs) 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. Means not sharing the same letter are significantly different (one-way ANOVA, Tukey's post hoc test, $p \leq 0.05$ was considered significant).

Figure supplementary material 2. Effects of strawberry and blackberry crude extracts (CE), anthocyanin-enriched fractions (AEFs) and proanthocyanidin-enriched fractions (PEFs) on the cell viability of HDFa fibroblasts. Cell viability was quantified spectrophotometrically by the MTT assay and is reported as the mean ± SD for three independent experiments. Means not sharing the same letter are significantly different (one-way ANOVA, Tukey's post hoc test, $p \leq 0.05$ was considered significant).

Figure supplementary material 3. Progress of skin HDFa fibroblast migration after 48 h exposure with strawberry and blackberry crude extracts (CE), anthocyanin-enriched fractions (AEFs) and proanthocyanidin-enriched fractions (PEFs). Bright field and fluorescent images were observed using EVOS® FL Auto Cell Imaging System (Life Technologies). FBS: fetal bovine serum at 0.5 % used as positive control, Full cells correspond to wells seeded without stopper.

Table 1. Characterization of phenolic compounds in strawberry 'San Andreas' using HPLC-PDA-ESI-MS

Peak	Rt (min)	λ_{max} (nm)	MW	MS (m/z)	MS^2 ions (m/z)[a]	Tentative identification	Ref.[b]
1	1.2	248	342	341 $[M-H]^-$	**179**, 161	caffeoyl hexose	2, 6, 7
2	7.0	229	784	783 $[M-H]^-$	481, **301**	Bis HHDP glucose	1, 2
3	8.5	229	784	783 $[M-H]^-$	481, **301**	Bis HHDP glucose	1, 2
4	9.6	313	326	325 $[M-H]^-$	145	*p*-coumaroyl hexose	2, 6, 7
5	11.5	515	449	449 $[M]^+$	287	Cyanidin-3-*O*-glucoside	4
6	13.30	501	433	433 $[M]^+$	271	Pelargonidin-3-*O*-glucoside	2, 4, 5
7	15.0	502	579	579 $[M]^+$	433, **271**	Pelargonidin-3-*O*-rutinoside	2, 9
8	16.5	237	1870	934 $[M-2H]^{2-}$	1567, 1265, **897**, 633, **301**	Lambertianin A (Sanguiin-H6)	3, 4
9	17.28	281	310	355 $[M+HCO_2]^-$	**309**, 207, 147	Cinnamoyl glucose	4, 7, 8
10	19.37	502	519	519 $[M]^+$	271	Pelargonidin-3-*O*-malonylglucoside	2, 4
11	21.0	503	475	457 $[M]^+$	271	Pelargonidin-3-*O*-acetylglucoside	2, 4
12	22.2	255, 355	478	477 $[M-H]^-$	301	Quercetin-3-*O*-glucuronide	2, 3, 6
13	24.4	345	448	447 $[M-H]^-$	285	Kaempferol-3-*O*-glucoside	2, 3, 6

[a]The most abundant ions are shown in bold.

[b]Literature were the compounds have been characterized by MS analysis: 1: Seeram et al. (2006) , 2: Kajdžanoska et al. (2010), 3: Buendía et al. (2010), 4: Aaby et al. (2012), 5: Van de Velde, et al. (2016b) , 6: Määttä-Riihinen et al. (2004), 7: Aaby et al. (2007), 8: Lunkenbein et al. (2006), 9: Milala, et al. (2017).

Table 2. Phenolic content in 'San Andreas' strawberries, using HPLC-PDA

Phenolic compounds	mg/100 g FW
Flavonoids	
Anthocyanins	
Cyanidin-3-*O*-glucoside	1.0 ± 0.1
Pelargonidin-3-*O*-glucoside	22.1 ± 1.2
Pelargonidin-3-*O*-rutinoside	1.6 ± 0.1
Pelargonidin-3-*O*-malonylglucoside	0.5 ± 0.1
Pelargonidin-3-*O*-acetylglucoside	0.2 ± 0.1
Flavonols	
Quercetin-3-*O*-glucuronide	7.8 ± 0.2
Kaempferol-3-*O*-glucoside	2.4 ± 0.1
Phenolic acids	
Hydroxycinnamic acid derivatives	
p-Coumaroyl hexose	0.5 ± 0.1
Cinnamoyl hexoside	3.1 ± 0.1
Caffeoyl hexose	9.5 ± 0.1
Hydrolysable tannins	
Ellagitannins	
Bis HHDP glucose	0.3 ± 0.1
Bis HHDP glucose	0.3 ± 0.1
Sanguiin H-6 (dimer of galloyl-bis-HHDP-glucose)	0.08 ± 0.01
Condensed tannins	
Proanthocyanidins	
DP_1	12.6 ± 0.9
DP_2	6.8 ± 0.3
DP_3	5.0 ± 0.3
DP_4	4.0 ± 0.2
DP_5	3.7 ± 0.2
DP_6	3.5 ± 0.2
DP_7	3.3 ± 0.1
$DP_{>10}$	8.2 ± 0.9
Total phenolics	96.6 ±4.9

DP: degree of polymerization

Table 3. Characterization of phenolic compounds in blackberry 'Black Satin' using HPLC-PDA-ESI-MS

Peak	Rt (min)	λ_{max} (nm)	MW	(MS) (m/z)[a]	$(MS)^2$ ions (m/z)	Tentative identification	Ref.[b]
1	3.5	229	784	783 $[M-H]^-$	481, **301**	bis-HHDP glucose	1, 2, 3
2	5.5	229	784	783 $[M-H]^-$	481**, 301**	bis-HHDP glucose	1, 2, 3
3	6.5	234	952	951 $[M-H]^-$	907, 783	Ellagitannin derivate	1
4	7.5	229	784	783 $[M-H]^-$	481, **301**,	bis-HHDP glucose	1, 2, 3
5	11.7	515	449	449 $[M]^+$	287	Cyanidin-3-*O*-glucoside	4, 5, 6, 7
6	12.4	235	2805	1401$[M-2H]^{2-}$	1567, 1250, 935, **633**, 301	Lambertianin C	1, 3, 4
7	12.7	237	1870	934 $[M-2H]^{2-}$	1567, 1265, **897**, 633, **301**	Lambertianin A (Sanguiin-H6)	1, 3, 4
8	15.9	514	419	419 $[M]^+$	287	Cyanidin-3-*O*-xyloside	4, 8
9	18.0	515	535	535 $[M]^+$	449, **287**	Cyanidin-3-*O*-(6-O-malonyl glucoside)	4, 0
10	18.1	515	593	593 $[M]^+$	287	Cyanidin-3-*O*-β"-(3-hydroxy-3-methylglutaroyl)-glucoside	4, 9
11	21.5	360	434	432 $[M-H]^-$	301	Quercetin-3-*O*-pentoside	10
12	21.9	609	610	609 $[M-H]^-$	301	Quercetin-3-*O*-rutinoside	4, 10
13	23.0	355	608	607 $[M-H]^-$	**463**, 301	Quercetin-(6'(-(3-hydroxy-3-methylglautaroyl)-galactoside	10
14	23.4	254, 353	550	505 $[M-HCO_2]^-$	301	Quercetin-glucoside malonate	6
15	24.8	348	448	447[M-H]-	285	Kampferol-3-*O*-glucoside	6

[a]The most abundant ions are shown in bold.

[b]Literature were the compounds have been characterized by MS analysis: 1: Hager et al. (2008), 2: Hager et al. (2010), 3: Kosmala et al. (2017), 4: Van de Velde et al. (2016a), 5: Fan-Chiang and Wrolstad (2005), 6: Mertz et al. (2007), 7: Cuevas-Rodríguez et al. (2010b), 8: Cho et al. (2004), 9: Jordheim et al. (2011), 10: Oszmiański et al. (2015).

Table 4. Phenolic content in 'Black Satin' blackberries, using HPLC-PDA

Phenolic compounds	mg/100g FW
Flavonoids	
Anthocyanins	
Cyanidin-3-*O*-glucoside	180 ± 2
Cyanidin-3-*O*-xyloside	8.1 ± 0.1
Cyanidin-3-*O*-(6-*O*-malonyl glucoside)	5,6 ± 0.1
Cyaniding-3-*O*-*β*-(6''-(3-hydroxy-3-methylglutaroyl)-glucoside)	18,7 ± 0.3
Flavonols	
Quercetin-3-*O*-rutinoside	2.9 ± 0.2
Quercetin-3-*O*-pentoside	10.0 ± 0.2
Quercetin-(6'(-(3-hydroxy-3-methylglautaroyl)-galatoside	6.7 ± 0.2
Quercetin-glucoside malonate	2.3 ± 0.1
Kampferol-3-*O*-glucoside	2.9 ± 0.2
Hydrolysable tannins	
Ellagitannins	
di-HHDP glucose	1.4 ± 0.02
di-HHDP glucose	0.6 ± 0.1
Ellagitannin derivate	1.2 ± 0.1
di-HHDP glucose	1.7 ± 0.1
Lambertianin C	7.1 ± 0.9
Lambertianin A (Sanguiin H6)	12.3 ± 0.6
Condensed tannins	
Proanthocyanidins	
DP1	20.1 ± 0.9
DP2	13.3 ± 0.3
DP3	5.7 ± 0.1
DP4	4.5 ± 0.2
DP5	3.8 ± 0.1
DP6	3.7 ± 0.2
DP>10	7.1 ± 0.2
Total phenolics	319.7 ±0.3

DP: degree of polymerization

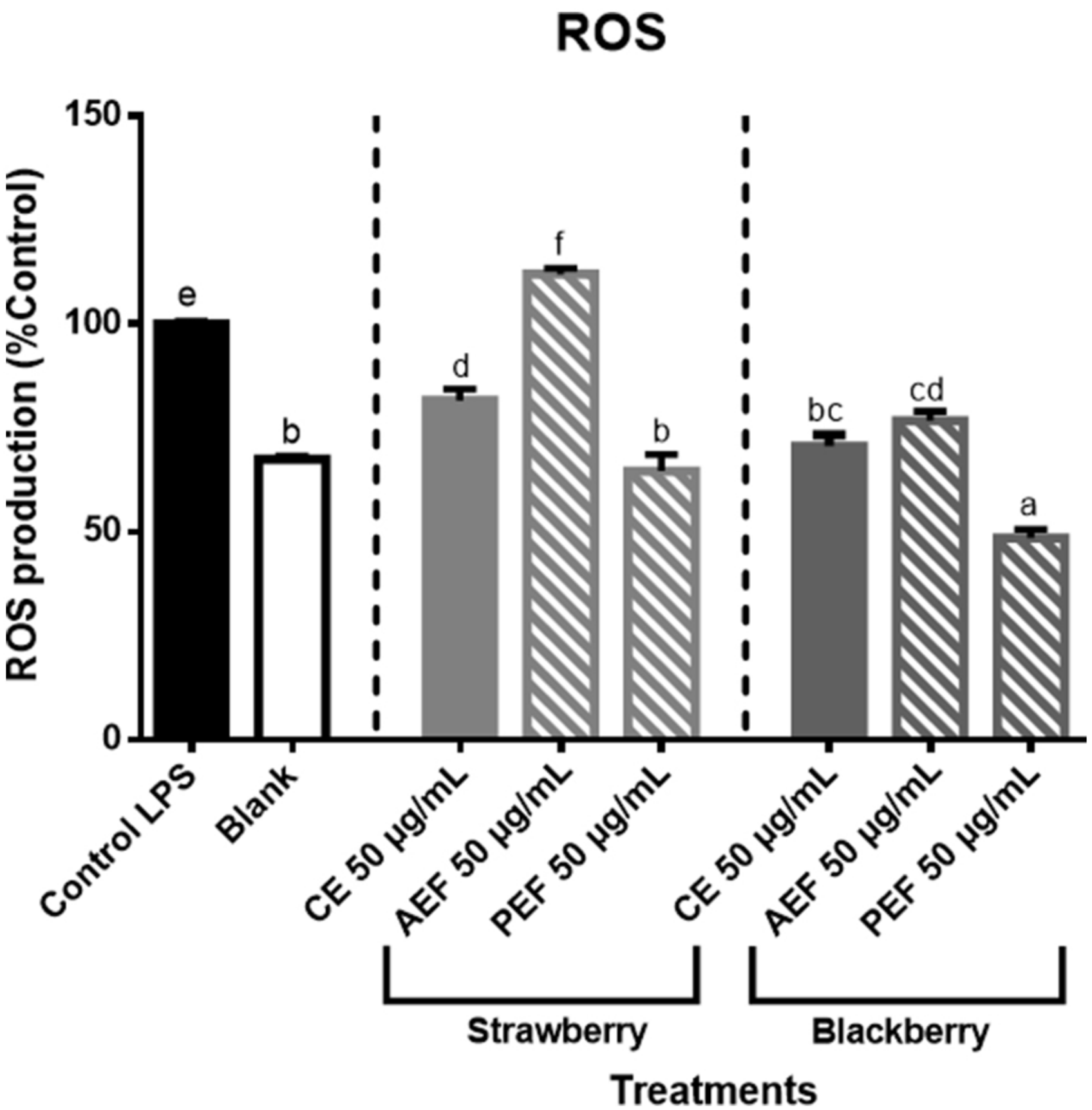
Table 5. Anthocyanins and proanthocyanidin contents in crude extract and polyphenolic-enriched extracts of strawberry and blackberry samples

Fruit	Extract		Total anthocyanins[A]	Total proanthocyanidins[B]
Strawberry 'San Andreas'	Crude extract (mg/g DW)		2.5 ± 0.1	4.7 ± 0.2
	Polyphenolic-enriched extract	AEF (mg/g DW	435 ± 9	1.5 ± 0.5
		PEF (mg/g DW)	2.6 ± 0.5	79 ± 9
Blackberry 'Black Satin'	Crude extract (mg/g DW)		18.2 ± 0.2	5.0 ± 0.1
	Polyphenolic-enriched extract	AEF (mg/g DW	681 ± 11	2.3 ± 0.3
		PEF (mg/g DW)	0.3 ± 0.1	10 ± 1

AEF: anthocyanin-enriched fraction, PEF: proanthocyanidin-enriched fraction.
[A]Total anthocyanins quantified by HPLC.
[B]Total proanthocyanidins quantified by HPLC.

Highlights

- Blackberry samples exhibited higher ROS reduction than strawberry's.
- Berries crude extracts exhibited 20% inhibition in Cox-2 gene expression.
- Results highlighted anti-inflammatory effects of berries due to proanthocyanidins.
- AEFs showed fibroblast migration around 50% of that registered for positive control.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ROS production (%Control)
150
100
50
0
e
b
d
f
b
bc
cd
a
Control LPS
Blank
CE 50 µg/mL
AEF 50 µg/mL
PEF 50 µg/mL
CE 50 µg/mL
AEF 50 µg/mL
PEF 50 µg/mL
Strawberry
Blackberry
Treatments

Figure 1

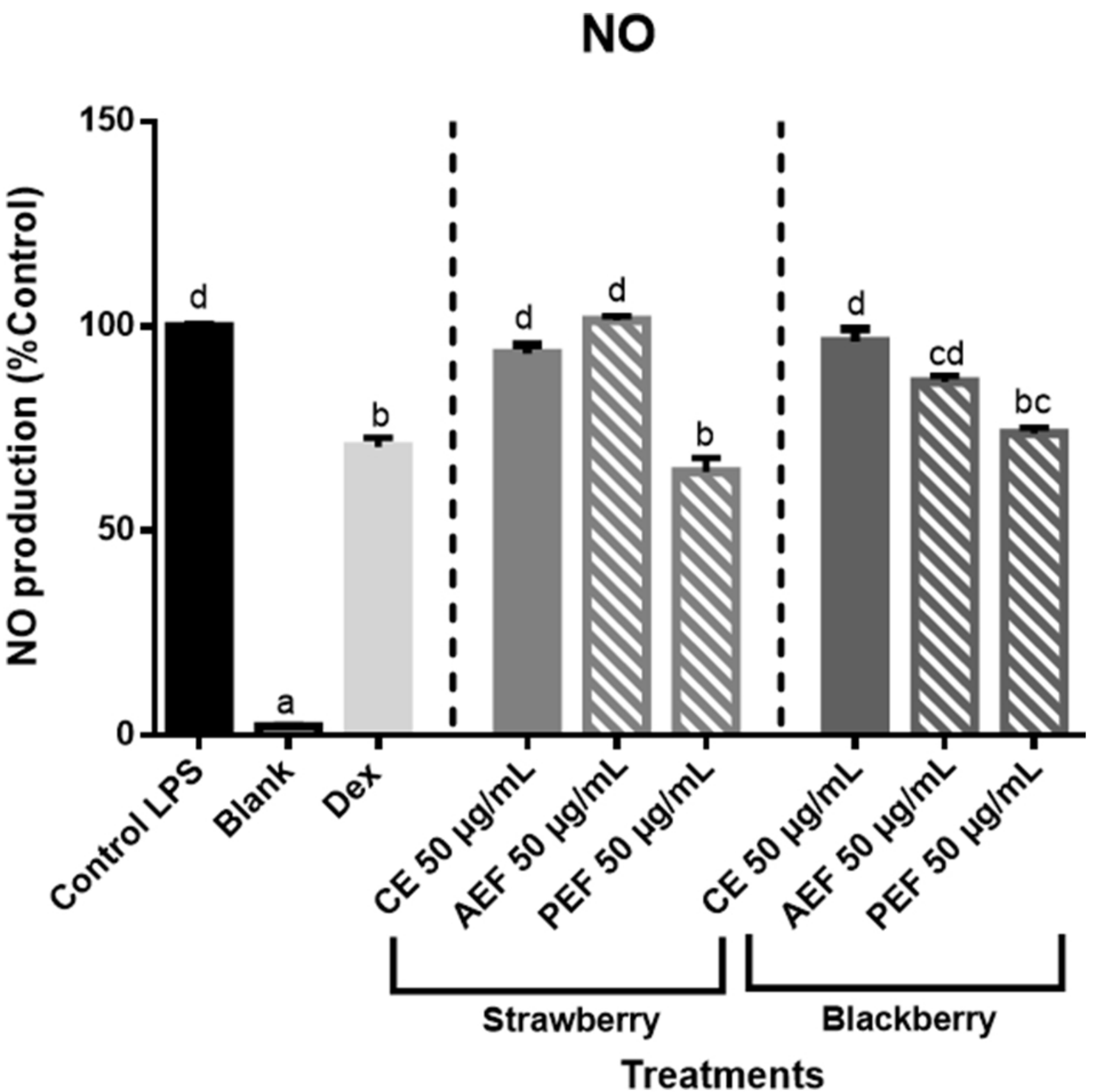

NO
NO production (%Control)
150
100
50
0
d
a
b
d
d
b
d
cd
bc
Control LPS
Blank
Dex
CE 50 µg/mL
AEF 50 µg/mL
PEF 50 µg/mL
CE 50 µg/mL
AEF 50 µg/mL
PEF 50 µg/mL
Strawberry
Blackberry
Treatments

Figure 2

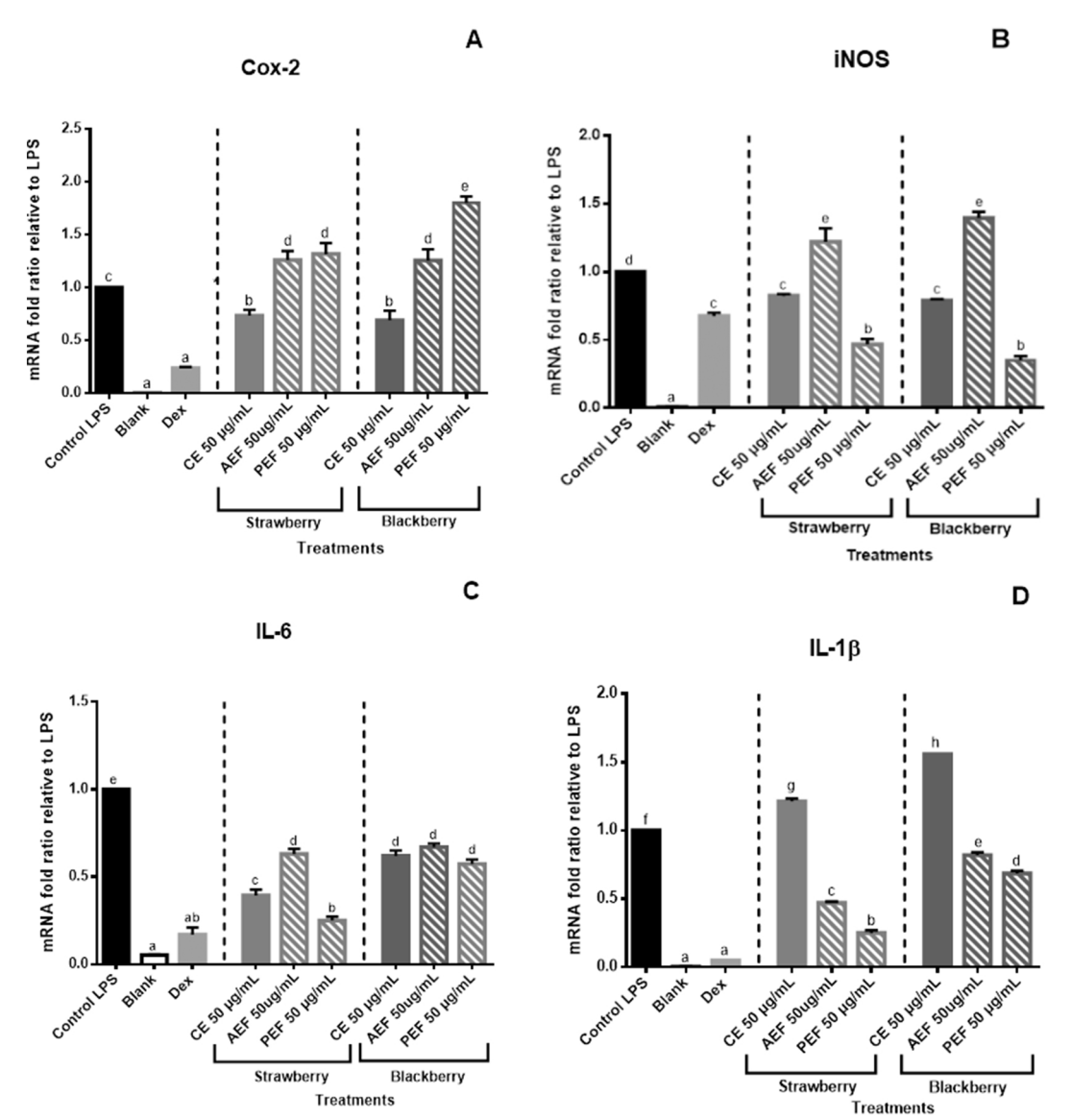
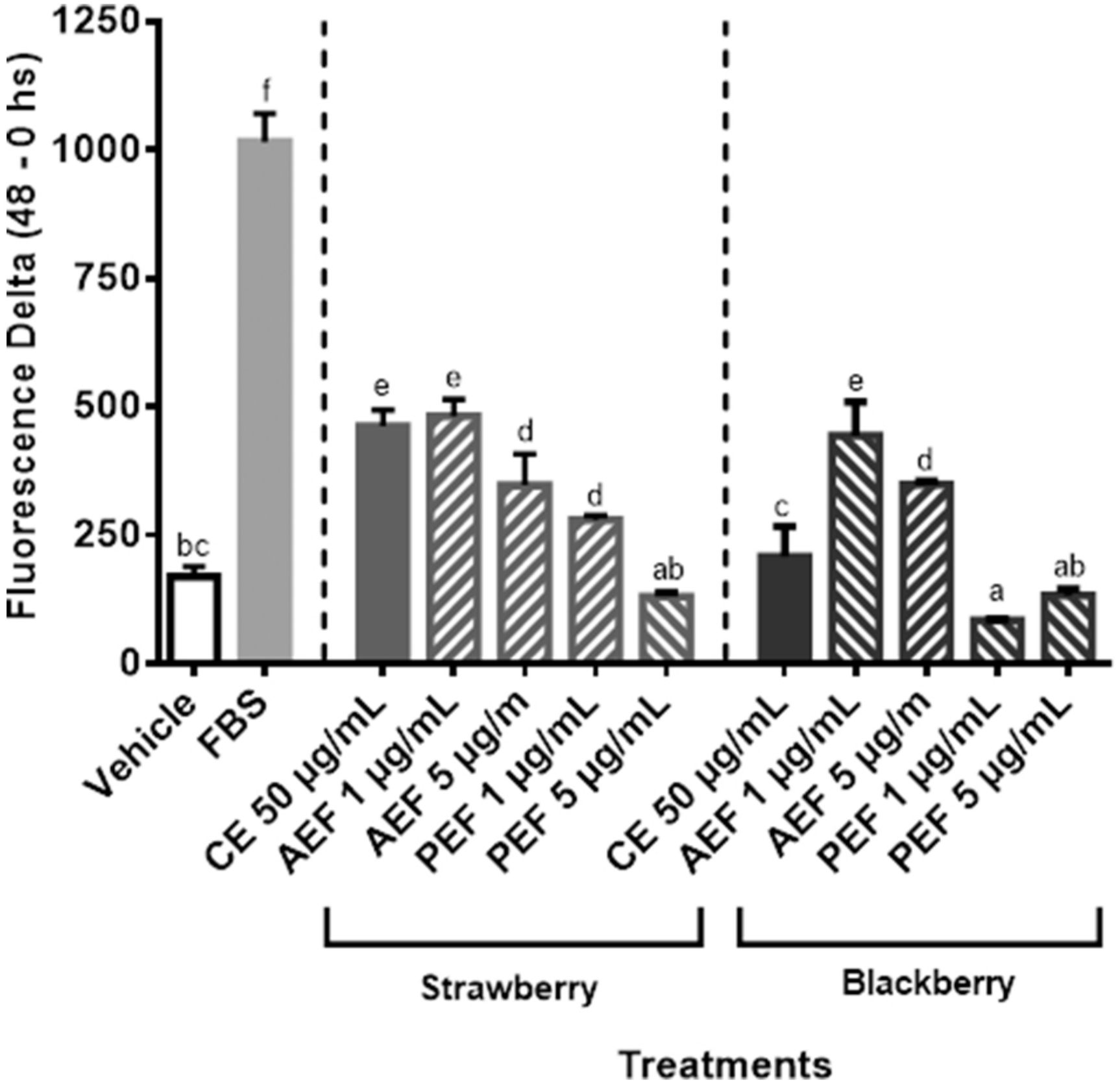

A
Cox-2
mRNA fold ratio relative to LPS
Control LPS
Blank
Dex
CE 50 µg/mL
AEF 50ug/mL
PEF 50 µg/mL
Strawberry
Blackberry
Treatments
B
iNOS
C
IL-6
D
IL-1β

Figure 3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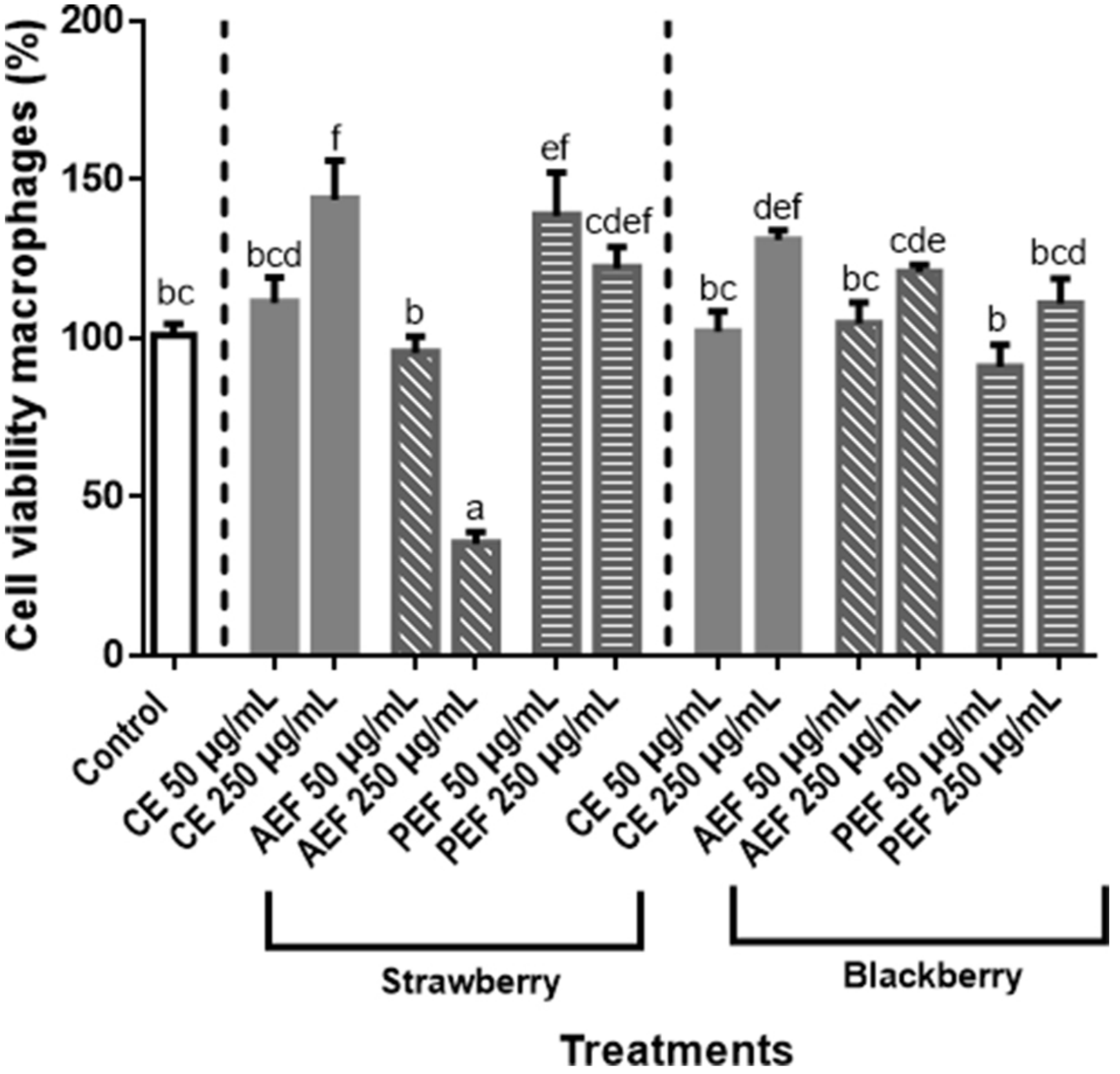
750
500
250
0
bc
f
e
e
d
d
ab
c
e
d
a
ab
Vehicle
FBS
CE 50 µg/mL
AEF 1 µg/mL
AEF 5 µg/m
PEF 1 µg/mL
PEF 5 µg/mL
CE 50 µg/mL
AEF 1 µg/mL
AEF 5 µg/m
PEF 1 µg/mL
PEF 5 µg/mL
Strawberry
Blackberry
Treatments

Figure 4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200
150
100
50
0
bc
bcd
f
b
a
ef
cdef
bc
def
bc
cde
b
bcd
Control
CE 50 µg/mL
CE 250 µg/mL
AEF 50 µg/mL
AEF 250 µg/mL
PEF 50 µg/mL
PEF 250 µg/mL
CE 50 µg/mL
CE 250 µg/mL
AEF 50 µg/mL
AEF 250 µg/mL
PEF 50 µg/mL
PEF 250 µg/mL
Strawberry
Blackberry
Treatments

Figure 5

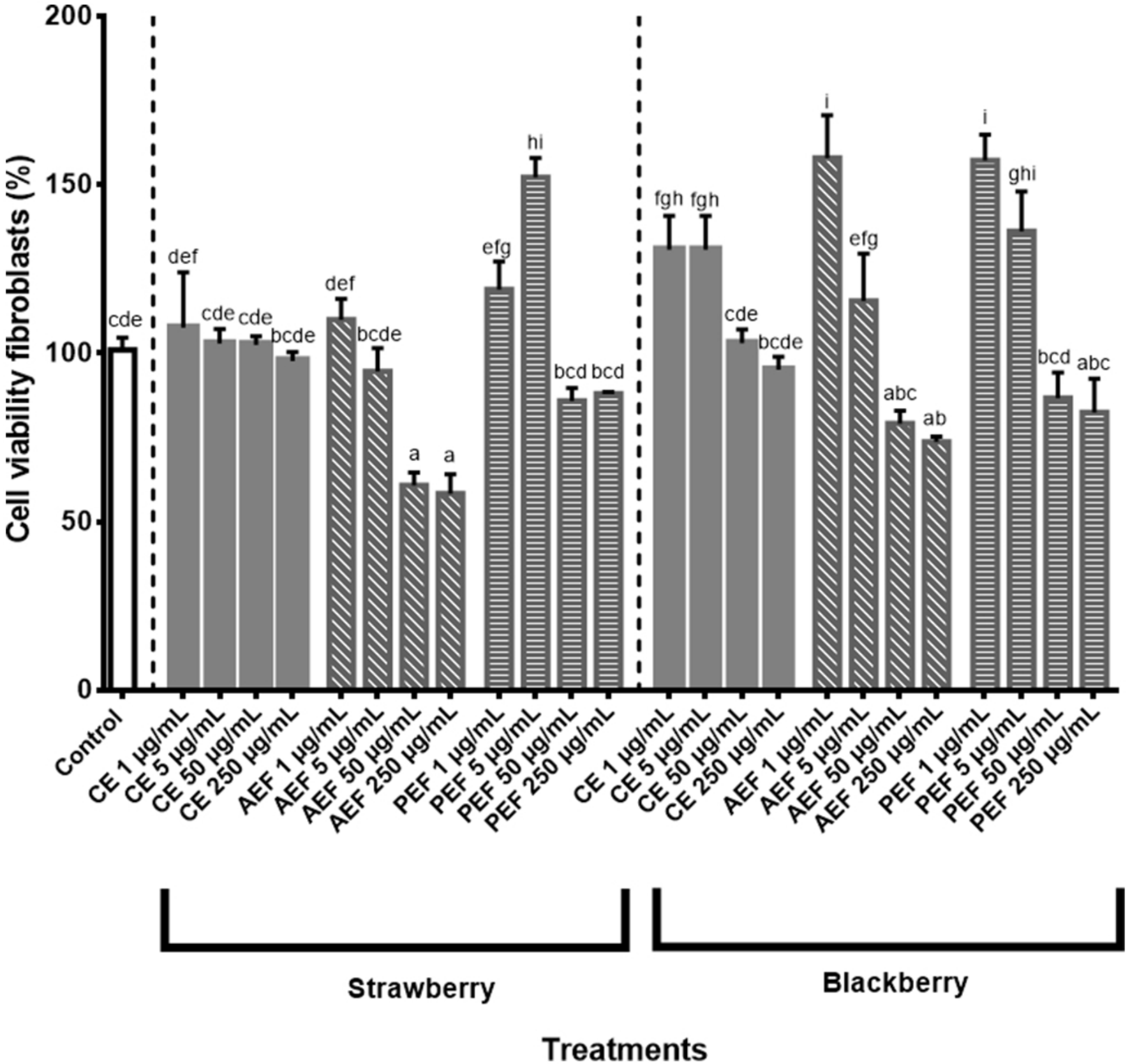
Cell viability fibroblasts (%)
200
150
100
50
0
cde
def
cde
cde
bcde
def
bcde
a
a
efg
hi
bcd
bcd
fgh
fgh
cde
bcde
i
efg
abc
ab
i
ghi
bcd
abc
Control
CE 1 µg/mL
CE 5 µg/mL
CE 50 µg/mL
CE 250 µg/mL
AEF 1 µg/mL
AEF 5 µg/mL
AEF 50 µg/mL
AEF 250 µg/mL
PEF 1 µg/mL
PEF 5 µg/mL
PEF 50 µg/mL
PEF 250 µg/mL
CE 1 µg/mL
CE 5 µg/mL
CE 50 µg/mL
CE 250 µg/mL
AEF 1 µg/mL
AEF 5 µg/mL
AEF 50 µg/mL
AEF 250 µg/mL
PEF 1 µg/mL
PEF 5 µg/mL
PEF 50 µg/mL
PEF 250 µg/mL
Strawberry
Blackberry
Treatments

Figure 6

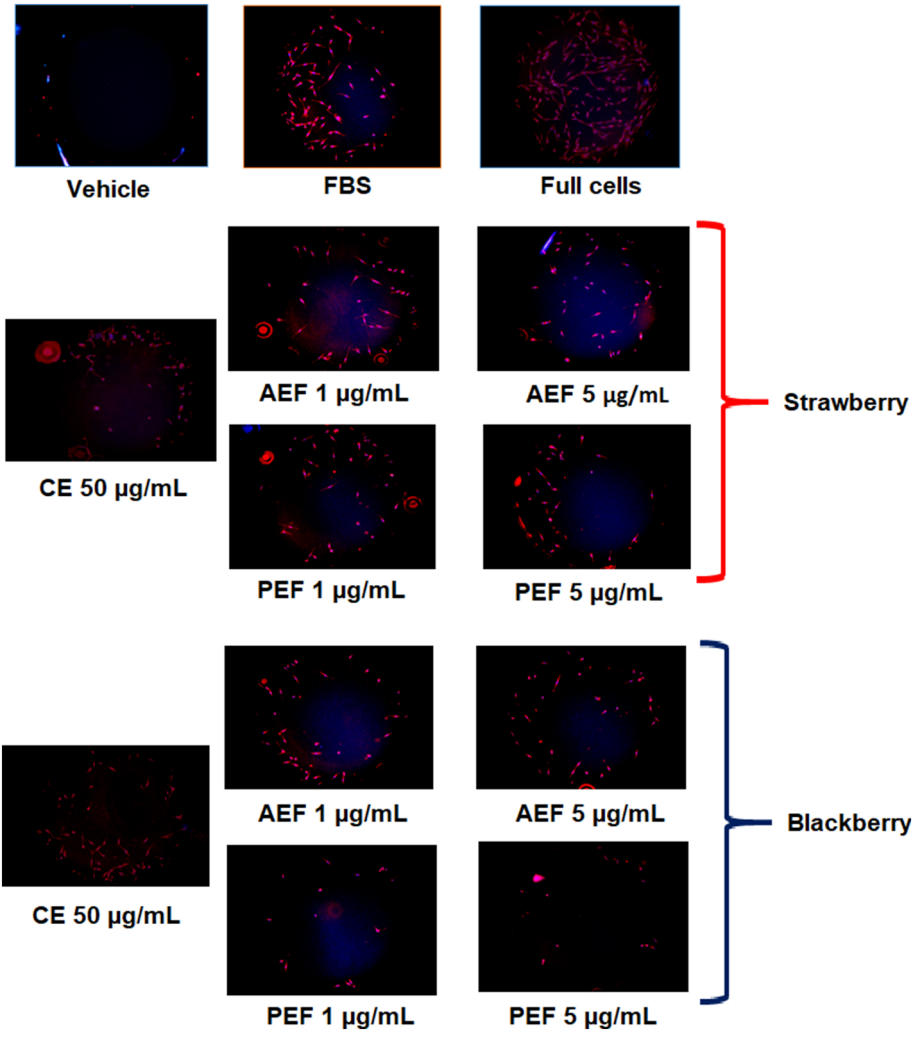
Vehicle
FBS
Full cells
AEF 1 µg/mL
AEF 5 µg/mL
CE 50 µg/mL
Strawberry
PEF 1 µg/mL
PEF 5 µg/mL
AEF 1 µg/mL
AEF 5 µg/mL
CE 50 µg/mL
Blackberry
PEF 1 µg/mL
PEF 5 µg/mL

Figure 7