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Research Article

Phytochemical Content, Antioxidant, Anti-inflammatory Activities and Wound Healing Properties of Freeze-Dried Fruits

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Abstract

This work evaluated the phytochemical composition of freeze-dried acerola, camu-camu, apple and pineapple extracts, and correlated it with their cell-based *in vitro* antioxidant, anti-inflammatory and wound healing activities. All freeze-dried fruit extracts at 50 µm/mL significantly inhibited ($p<0.001$) reactive oxygen species (ROS) up to 70%, but only acerola and camu-camu extracts at 150 µm/mL decreased ($p<0.001$) the nitric oxide (NO) production (up to 33%) in LPS-stimulated RAW 264.7 macrophages. When selected mRNA biomarkers of acute inflammation were evaluated, all freeze-dried fruit extracts inhibited the expression of IL-6 and IL-1β genes, but only acerola reduced the COX-2 expression ($p<0.01$). In addition, freeze-dried acerola extract (50 µm/mL) significantly enhanced human dermal fibroblasts (HDFa) migration by 1.5-fold compared to the control after 36 h. Based on our results, we hypothesize that the high concentration of ascorbic acid (9454.3 mg/100 g) and anthocyanin contents (14.0 mg /100 g) of freeze-dried acerola may play an important role on the *in vitro* biological results.

Keywords: Ascorbic Acid; Inflammation; Acerola; Wound Healing; Edible Fruits

Abbreviations

ROS: Reactive Oxygen Species; Ac: Acerola; Cc: Camu-Camu; Ap: Apple; Pi: Pineapple; GAE: Gallic Acid Equivalentes; DTT: Dithiothreitol; DPPH: 2,2-diphenyl-1-picrylhydrazyl; FRAP: Ferric Reducing Antioxidant Power; FBS: Fetal Bovine Serum; HDFa: Human Dermal Fibroblasts; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium Bromide; NO: Nitric Oxide; LPS: lipopolysaccharide; DEX: Dexamethasone; TE: Trolox Equivalents; CYA: cyanidin-3-glucoside; SFE: Ferrous Sulphite Equivalents; TNFα: Tumor Necrosis Factor; iNOS: Inducible Nitric Oxide Synthase; COX-2: Cyclooxygenase; IL-1β: Interleukin; IL-6: Interleukin; RFU: Relative Fluorescence Unit.

Introduction

Increased levels of reactive oxygen species (ROS) damage important biomolecules, disrupt homeostasis and can lead to the development of pathological conditions such as cardiovascular disease and diabetes [1]. Furthermore, there is evidence that chronic inflammation is at the pathogenesis of several diseases, and an intricate relationship between oxidative stress and inflammation has been revealed [2]. It is also true that unhealthy diets impact nutrient metabolism, by promoting a cascade of body reactions that ultimately lead to dysregulated metabolism, and high production of pro-inflammatory and pro-oxidant molecules [3]. Therefore, finding natural dietary agents capable of both fighting the inflammatory process at early stages and mitigating oxidative conditions would be of great interest [3,4].

Solid scientific data has demonstrated that fruits are natural sources of phytochemicals with potent antioxidant, anti-inflammatory and health promoting activities [3,5-7]. In particular, the health benefits of ascorbic acid, a key nutrient found in fruits and vegetables, are well-documented [8]. Although the consumption of fresh whole fruits is desired, many times it is not easily accomplished or feasible. As a result, in order to overcome fruit seasonality and natural perishability and enable their consumption all year long, several fruit-based products with extended shelf life have been developed. For example, dried fruits have become a popular segment of the food industry due to their convenience and portability. However, the quality of dried products vary and it is well known that drying can decrease the content of ephemeral, sensitive bioactive compounds [9,10]. Among the most popular drying techniques, freeze-drying is a mild processing method that uses low temperature and pressure that allows the delivery of preserved

phytoactives [10]. In fact, freeze dried commercial fruit products have a successful marketing appeal and are now found in local retail stores and on-line commerce [9], with highly claimed phytochemical content and biological effects.

Therefore, in this study we have the objective of investigating selected commercial freeze-dried fruits with regard to their phytochemical composition (ascorbic acid, anthocyanins, total phenolic compounds and phenolic acids) and in vitro antioxidant and cell-based biological activities. Specifically, we selected acerola and camu-camu fruits, two of the world's most abundant sources of ascorbic acid [11,12] and two fruits with comparatively low ascorbic acid content (apple and pineapple) with the objective of evaluating their effect on the in vitro antioxidant capacity, anti-inflammatory markers and fibroblast migration and correlating these effects with their phytochemical composition.

Materials and Methods

Material

Four experimental groups consisting of freeze-dried fruits were evaluated in this study: Ac - acerola (*Malpighia emarginata,* Microingredients, Diamond Bar, CA), Cc - camu-camu (*Myrciaria dubia*, Feel Good Organics, Seattle, WA), Ap - apple (*Malus domestica*, Mother Earth Products, Harrisonburg, VA) and Pi - pineapple (*Ananas comosus*, Natierra Nature's All Food, Van Nuys, CA).

Preparation of extracts

The methanolic extracts were prepared according to Hoskin., *et al.* [5]. Briefly, freeze-dried samples (1 g) were eluted three consecutive times with 8 mL of 2% acetic acid in 80% methanol in water. After sonication for 10 min at room temperature, the tubes were centrifuged for 10 min at 4,500 rpm and 4°C (Thermo Sorvall Legend TR, Waltham, MA). Finally, the supernatants were pooled to a final volume of 25 mL in a volumetric flask and constituted the extracts used for total phenolic content, total anthocyanins and radical scavenging (DPPH and FRAP) assays.

For HPLC analyses, the extracts prepared as described above were filtered (PFTE membrane, 0.2 um, Fischer Scientific, Pittsburg, PA) prior to injection. For cell culture assays (MTT, NO, ROS, PCR and wound healing), 1 mL of the extracts prepared as described above were dried down using a Speed Vac Concentrator (DNA 120, Thermo Fischer Scientific, Waltham, MA) and diluted to selected concentrations (25, 50, 150 or 250 µg/mL) using ethanol 80%. The extracts were stored at -20°C until further use.

Total phenolic content

It was determined according to an adapted microplate method [13]. Briefly, 25 µL of extract, standard or blank was mixed with 75 µL of distilled water and 25 µL of Folin-Ciocalteu phenol reagent (diluted 1:1). After 6 minutes, 100 µL of Na_2CO_3 (7.5%) was added and the absorbance was measured (Spectramax Plus 384, Molecular Devices, Sunnyvale, CA). Results were expressed as mg of gallic acid equivalents (GAE) per gram (mg GAE/g) using a standard gallic acid curve (0 to 250 mg/L).

HPLC analyses: ascorbic acid, total anthocyanins and phenolic acids

The ascorbic acid content was determined according to Grace., *et al.* [14]. Initially, freeze-dried samples (0.5 g) were diluted in 8 mL of extraction solution consisting of meta-phosphoric acid 3% and acetic acid 8% in deionized water. After sonication for 5 min at room temperature, the tubes were centrifuged for 20 min at 4,000 rpm and 4°C (Thermo Sorvall Legend TR, Waltham, MA). The procedure was repeated two additional times and the supernatants were pooled to a final volume of 25 mL in a volumetric flask. An aliquot (800 µL) was filtered (cellulose membrane, 0.2 mm) and 200 µL of dithiothreitol (DTT) 20 g/L was added. The mixture was kept in the dark for 2 h prior to the injection (20 µL) to convert any DHA (L-dehydroascorbic acid) to ascorbic acid. A HPLC equipment (Agilent 1200 series, Agilent Technologies, Santa Clara, CA) coupled to a reverse phase column (Phenomenex Synergi, 4 µm hidro-RP 80 Å (250 mm x 4.6 mm x 5 µm, Torrance, CA.) was used. The isocratic elution was conducted with a mobile phase of deionized acidified water P^H 2.2 at constant flow of 1.2 mL/min and peaks were registered at 245 nm. Results expressed as mg/100 g were obtained using an ascorbic acid calibration curve (1 to 100 µg/mL).

Total anthocyanins were determined using a photodiode array detector HPLC equipment (Agilent 1200 series, Agilent Technologies, Santa Clara, CA, USA) according to a previous protocol [14]. Separation was performed using a RP Supelcosil-LC-18 column (250 mm × 4.6 mm × 5 µm, Supelco, Bellefonte, PA, USA). The mobile phases consisted of 5% formic acid in deionized water (A) and 100% methanol (B). The flow rate was set as 1 mL/min with a step gradient of 10, 15, 20, 25, 30, 60, 10, and 10% of solvent B at 0, 5, 15, 20, 25, 45, 47, and 60 min, respectively at 30 °C. Peaks were registered at 520 nm and the Chemstation software was used to manage the HPLC data. Results were expressed as mg cyanidin-3-O-glycoside equivalents per 100 gram using a standard calibration curve (0.1 to 1.0 mg/mL).

The phenolic acids were determined by HPLC using a reverse phase column Phenomenex Synergi 4 µm hydro-RP 80 Å (250 mm × 4.6 mm × 5 µm, Supelco, Bellefonte, PA, USA) and a pre-column (Phenomenex cartridge, AQC 18.4 x 3.0 mm). The mobile phases consisted of 0.1% formic acid in deionized water (A) and 100% methanol (B). The automatic sampler tray was kept at 10oC, the column oven at 35oC and the elution flow rate of 1 mL/min. A step gradient of 20% B (0-10 min), 40% B (10-25 min) and 90% B (25-30 min) was set. Aliquots (10 µL) in six different concentrations (0.5, 0.25, 0.125, 0.0625 and 0.03125 mg/mL) of gallic, ellagic,

p-coumaric, ferrulic, chlorogenic, cafeic and hydrobenzoic acids standards were injected as internal standards. Peak areas recorded at 326 nm generated calibration curves for each standard and results were expressed as mg/100 g of sample.

Radical scavenging assays

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical assay

It was determined according to a microplate method by Bobo-Garcia., *et al*. [15]. Briefly, 20 µL of extract and 180 µL of DPPH solution 150 µmol/L in methanol 80% v/v were mixed and the absorbance was read at 515 nm. Results were expressed as µmol of Trolox (6-hydroxy-2.5.7.8-tetramethyl chromane-2-carboxylic acid) equivalents per gram (µmol TE/g) using a standard calibration curve with Trolox (100 to 500 µM concentration range).

Ferric reducing antioxidant power (FRAP) assay

A modified microplate method based on Torre., *et al.* [16] was used. Initially, the FRAP reagent was prepared (25 mL of acetate buffer 3 M, 2.5 mL of ferric chloride 20 mM and 2.50 mL of TPTZ 10 mM). Extracts (30 µL), distilled water (30 µL) and the FRAP reagent (90 µL) were mixed and incubated at 37°C for 30 min. The absorbance was read at 595 nm (Genesys 10S UV-VIS spectrophotometer, Thermo Scientific, Waltham, MA, USA) and results were expressed as µmol of ferrous sulphite equivalents per gram (µmol SFE/g) based on a calibration curve built with SFE 2 mM (500 to 2,000 mM concentration range).

Cell lines

Mouse macrophage RAW 264.7 cells (ATCC TIB-71) were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Grand Island, NY), supplemented with a 1% solution of penicillin 100 IU/mL/streptomycin 100 µg/mL (Fisher MT-30-002-CI) and fetal bovine serum (FBS, Life Technologies, Long Island, NY) 10% (v/v) solution at a confluence not exceeding 80%. Human dermal fibroblasts (HDFa, Invitrogen C-013-5C) isolated from adult skin were kept in Medium 106 (M106, Invitrogen M-106-500) supplemented with a 1% solution of penicillin 100 IU/mL/streptomycin 100 µg/mL (Fisher MT-30-002-CI) and low serum growth supplement (LSGS, Invitrogen S-003-10). All cell lines were kept in a humidified incubator (5% CO_2) at 37°C.

Cell viability assay

RAW 264.7 and HDFa cells were seeded in 96-well plates, treated with different concentrations of freeze-dried samples (RAW 264.7: 25, 50, 150 and 250 µg/mL; HDFa: 50 and 150 µg/mL) and exposed to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) for 24 h according to a previous protocol [5].

In vitro reactive oxygen species (ROS) assay

It was conducted according to Choi., *et al*. [17] RAW 264.7 macrophage cells (approx. 5 × 10^5 cells/well) seeded into 24-well plates were incubated overnight in a humidified incubator (5% CO_2) at 37°C. The cells were exposed to fresh fluorescent medium consisting of 50 µM solution of dichlorodihydrofluorescein diacetate acetylester (H2DCFDA) in ethanol for 30 min. After aspiration of medium, cells received 1 µL of sample extract (50 µg/mL final concentration) and 1 µL of a lipopolysaccharide solution (LPS, from *Escherichia coli* $O_2$6:B6, 100 µg/mL) and plates were incubated again in a humidified incubator (5% CO_2) at 37°C 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). Dexamethasone (DEX) solution 10 µM was used as positive control. Results were expressed as percentage of ROS production (%) relative to LPS induction.

Nitric oxide (NO) inhibition assay

It was determined by the Griess colorimetric assay [5]. RAW 264.7 macrophage cells (approx. 5 × 10^5 cells/well) seeded into 24-well plates were incubated overnight in a humidified incubator (5% CO_2) at 37°C. Cells received 1 µL of sample extract (50 µg/mL or 150 µg/mL final concentration) and 10 µL of a LPS solution (from *Escherichia coli* $O_2$6:B6, 100 µg/mL). Positive (DEX solution 10 µM) and negative (vehicle ethanol 80%) controls were included in every assay. The plates were kept for 18 h in a humidified incubator (5% CO_2) at 37°C and 100 µL of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine in 5% phosphoric acid) was added to 50 µL of RAW 264.7 cell culture medium. After incubation for 10 min in the dark at room temperature, the absorbance was read at 520 nm (microplate reader Synergy H1, Biotech, Winooski, VT). Results were expressed as percentage of NO production (%) relative to LPS induction using a calibration curve built with serial dilutions of sodium nitrite (0 to 100 µM, R^2 = 0.9992).

Anti-inflammatory *in vitro* assay

It was determined according to Esposito., *et al*. [18] RAW 264.7 cells seeded into sterile 24-well plates (up to 5 x 10^5 cells/well) and incubated for 24h in a humidified incubator (5% CO_2) at 37°C. The cells treated with sample extracts (50 µg/mL final concentration) received 1 µL of LPS solution (from Escherichia coli $O_2$6:B6, 1 mg/mL) and plates were kept for 6h in a humidified incubator (5% CO_2) at 37°C. Positive (DEX solution 10 µM) and negative (ethanol 80%) controls were included in every assay. After treatment, cells harvested with TRIzol reagent (ref. 15596026, Thermo Fisher Scientific Inc. Waltham, MA) were used for further cellular RNA extraction.

Total RNA extraction, purification, and cDNA synthesis

Initially, extraction of the total RNA was performed with the TRIzol reagent, following the manufacturer's instructions. The RNA was measured spectrophotometrically (microplate reader Synergy H1, Biotech, Winooski, VT). The synthesized cDNAs were performed using the commercially available high capacity cDNA reverse transcription kits (Life Technologies, Grand Island, NY) and 2 µg of RNA for each sample. The procedure followed the protocol of the manufacturer ABI GeneAMP 9700 (Life Technologies, Grand Island, NY).

Quantitative PCR analysis

The resulting cDNA was amplified by quantitative real-time PCR (qPCR) using the PowerUp™ SYBR™ Green Master Mix (ref.

A257424367659, Thermo Fisher Scientific Inc. Waltham, MA) SYBR Green Master Mix (ref. 4367659, Life Technologies, Grand Island, NY). The following intron overlapping primers for β-actin (housekeeping gene) and the biomarkers of acute inflammation (COX-2, iNOS, IL-6, IL-1β, TNF-α5') were selected using Primer Express version 2.0 Software (Applied Biosystems, Foster City, CA): 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'; COX2: 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'; 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' and TNF-α5': forward primer: 5'-GTT CTA TGG CCC AGA CCC TCA CA-3'; reverse primer: 5' -TAC CAG GGT TTG AGC TCA GC - 3'. The qPCR amplifications were performed on an ABI 7500 Fast Time PCR (Life Technologies, Grand Island, NY) using a cyclic program: 1 cycle at 50°C for 2 min; 1 cycle of 95°C for 10 min; 40 cycles at 95°C for 15 s and 1 min at 60°C. The dissociation curve was supplemented with 1 cycle of 1 min at 95°C, 30 s at 55 °C and 30 s at 95°C. The mRNA expression (using the ΔΔCT mode) and the normalized relative expression of the β-actin manipulation genes were analyzed using 7500 Fast System SDS Software v1.3.0 (Life Technologies, Grand Island, NY). LPS was set as the maximum genetic induction (equal to 1), therefore, values lower than 1 indicate inhibition of gene expression, while values higher than 1 indicate overexpression of the particular gene. Fusion curves were used to confirm the amplification of the transcripts. All samples were tested in duplicate.

Wound healing assay - *In vitro* fibroblast migration test

It was evaluated using an exclusion zone-based wound healing procedure (Oris® Migration Assay) using 96-well microplates (Platypus Technologies, WI, USA) and HDFa cells according to a protocol previously described by our group [5]. Initially, each well received silicone-based inserts to inhibit cell adherence to a central zone. Then HDFa cells were seeded at 3 x 10^5 cells/mL until close to total confluence and labeled with fluorescent dye NucBlue® Live Cell Stain (R37605, Thermo Fischer Scientific, Waltham, MA) solution at 1 µM. Once confluence was reached, the silicone inserts were removed and a cell-free central zone was exposed to allow fibroblast migration. After the addition of fresh M106 growth medium, controls (positive: 0.5% fetal bovine serum, FBS; negative: vehicle 80% ethanol) and extracts (50 µg/mL, 4 wells/each) were prepared and plates incubated in a humidified incubator (5% CO_2) at 37°C for 48 h. The fibroblast migration was measured by excitation (360 nm) and emission (460 nm) wavelength fluorescence in a microplate reader (Synergy H1, Biotech, Winooski, VT, USA). Bright field and fluorescent images were analyzed using EVOS® FL Auto Cell Imaging System and EVOS® software (Life Technologies, Grand Island, NY) to obtain the relative fluorescence (expressed as relative fluorescence units, RFU). The difference between the data captured at the center of each well after 18, 24, 36 and 48h and the beginning of the experiment (T0) was used to estimate the influence of freeze-dried fruit extracts on the progress of fibroblast migration relevant to wound closure/healing.

Statistical analyses

Statistics were performed using GraphPad Prism 4.0 (GraphPad Software Inc., La Jolla, CA) and Statistica 7.0 (StatSoft, CA, USA) software. All data were analyzed by one-way ANOVA. Post hoc analyses of differences between the composition of freeze dried extracts was conducted by Tukey's multiple comparison test, while the analyses of *in vitro* biological assays were performed by Dunnett's multiple comparison test ($p<0.05$, $p < 0.01$, $p < 0.001$, or $p < 0.0001$). All samples were analyzed at least in triplicate, unless specified. All results were expressed as means±SEM.

Results

Phytochemical analyses and radical scavenging assays

Results for phytochemical compounds and antioxidant activity are presented in Table 1. The ascorbic acid content followed the order acerola > camu-camu > apple > pineapple. Freeze-dried camu-camu presented higher total phenolic content when compared to the other groups ($p<0.05$), but similar anthocyanin content to apple and pineapple. Acerola, a red berry-like fruit, showed superior anthocyanin content when compared to the other freeze-dried extracts ($p<0.05$). This study also identified seven natural antioxidant phenolic acids in freeze-dried camu-camu extracts (gallic, hydrobenzoic, chlorogenic, cafeic, p-coumaric, ferrulic and ellagic acids; Table 1). The most abundant phenolic acid detected in freeze-dried apple was chlorogenic acid, while ellagic acid was the most prevalent in freeze-dried camu-camu, pineapple and the only phenolic acid found in acerola samples. Freeze-dried camu-camu and acerola samples had the highest antioxidant capacity measured by both DPPH radical scavenging activity and FRAP reducing power (Table 1).

Cell viability

In order to evaluate possible cytotoxicity of freeze-dried acerola, camu-camu, apple and pineapple extracts, the viability of each cell line was evaluated after 24h of exposure to MTT and different concentrations of the extracts (RAW 264.7 macrophages: 25, 50, 150 and 250 µg/mL; HDFa cells: 50 µg/mL and 150 µg/mL). None of the extracts reduced cell viability below 80% compared to the control (ethanol 80%, data not shown) and concentrations of 50 µg/mL (all experiments) and 150 µg/mL (for NO assay only) were selected for further studies.

Intracellular reactive radical species (ROS) and nitric oxide (NO) assays

In this study, all freeze-dried fruit extracts (Ac, Cc, Ap and Pi) administered at 50 µg/mL were able to reduce the production of ROS when compared to the LPS-activated macrophage cells ($p<0.001$, Figure 1A). On the other hand, when evaluating the effect of fruit extracts at 50 µg/mL on the production of nitric oxide (NO), no inhibition was observed (data not shown). Therefore, a concentration

	Acerola	Camu-camu	Apple	Pineapple
Ascorbic acid (mg/100 g)	9454.3 ± 63.8^{a}	5830.5 ± 19.9^{b}	944.6 ± 60.9^{c}	442.5 ± 23.3^{d}
Total phenolic content (mg GAE/100 g)	4035.1 ± 2.2^{b}	9295.3 ± 2.9^{a}	1468.3 ± 0.2^{c}	351.5 ± 0.2^{d}
Total anthocyanin (mg CYA/100 g)	14.0 ± 0.4^{a}	5.4 ± 0.4^{c}	6.7 ± 0.6^{b}	5.9 ± 0.2^{bc}
DPPH (µmol TE/g)	366.8 ± 8.0^{b}	756.1 ± 15.2^{a}	72.4 ± 4.2^{c}	10.9 ± 0.2^{d}
FRAP (µmol SFE/g)	101.1 ± 1.9^{b}	111.1 ± 1.9^{a}	109.3 ± 0.5^{ab}	49.5 ± 0.9^{c}
Phenolic acids, mg GAE/100g				
Gallic acid	ND	6.6 ± 0.6	ND	ND
Hydrobenzoic acid	ND	2.3 ± 0.5	ND	ND
Chlorogenic acid	ND	4.4 ± 0.1^{b}	15.4 ± 0.5^{a}	0.7 ± 0.1^{c}
Cafeic acid	ND	1.6 ± 0.1^{a}	1.2 ± 0.1^{b}	ND
p-coumaric acid	ND	0.7 ± 0.1^{a}	ND	0.3 ± 0.0^{b}
Ferrulic acid	ND	1.1 ± 0.1^{a}	0.3 ± 0.0^{b}	ND
Ellagic acid	2.4± 0.0^{b}	13.6 ± 0.8^{a}	ND	2.2 ± 0.0^{b}

Table 1: Concentration of ascorbic acid, total phenolics, total anthocyanins, antioxidant activity (DPPH and FRAP) and phenolic acids in freeze-dried acerola, camu-camu, apple and pineapple extracts. Results expressed as mean±SD. Total phenolic content and phenolic acids (quantified by HPLC) calculated as mg gallic acid equivalent (GAE), total anthocyanins quantified by HPLC as cyanidin-3-glucoside equivalents (CYA), DPPH as micromoles of Trolox equivalents (TE), FRAP as micromoles of ferrous sulphite equivalents (SFE) per gram. a,b,c,d: Different letters mean statistical difference by Tukey's test ($p < 0.05$). ND: not detected.

of 150 µg/mL was tested to allow detection of the NO inhibition induced by freeze-dried fruit extracts. Only freeze-dried acerola and camu-camu (samples with higher ascorbic acid content, Table 1) at 150 µg/mL were able to significantly ($p<0.001$) inhibit the production of nitric oxide (Figure 1B).

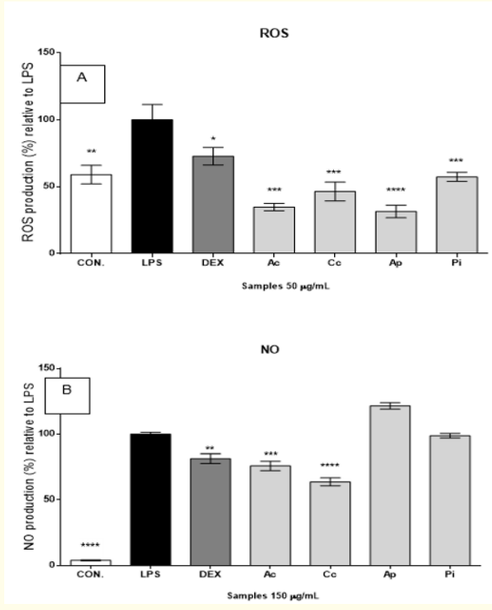

Figure 1: Effect of freeze-dried acerola (Ac), camu-camu (Cc), apple (Ap) and pineapple (Pi) extracts on the production of (A) Reactive oxygen species (ROS) at 50 µm/mL and (B) Nitric oxide (NO) at 150 µm/mL in LPS-induced RAW 264.7 cells. CON: vehicle ethanol 80% (negative control); DEX: dexamethasone (positive control), LPS - lipopolysaccharide. Results expressed as mean±SD. Samples marked with asterisks are significantly different compared to LPS according to * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

Anti-inflammatory *in vitro* assay

In the present work, five markers (TNFα, iNOS, COX-2, IL-1β and IL-6) were selected to evaluate the effect of extracts of freeze-dried acerola, camu-camu, apple and pineapple extracts on the in vitro inflammatory process. None of the fruit extracts interfered with the expression of tumor necrosis factor (TNFα) and inducible nitric oxide synthase (iNOS) markers (data not shown).

Only freeze-dried acerola extract at 50 µg/mL decreased the expression ($p<0.001$) of COX-2 (Figure 2A). Freeze-dried fruit extracts at 50 µg/mL inhibited IL-1b expression ($p<0.05$) in the order Ap > Ac > Cc > Pi (Figure 2B). Results also show that extracts at 50 µg/mL were effective in downregulating IL-6 expression ($p<0.05$) and acerola was, again, the main inhibitor (Figure 2C). Remarkably, only freeze-dried acerola extracts were able to efficiently downregulate the expression of COX-2, IL-1β and IL-6 markers in LPS-activated cells (Figure 2).

Wound healing assay - *In vitro* fibroblast migration test

Only freeze-dried acerola extracts at 50 µg/mL enhanced fibroblasts cell migration when compared to the control ($p<0.05$ or higher significance). After only 24 hours, the positive effect of freeze dried acerola extracts in accelerating fibroblast migration was already clear, and it reached its maximum after 36 hours, when the increase in cell migration reached 1.5 fold compared to the control treatment (Figure 3).

Discussion

Both *in vitro* and *in vivo* studies have demonstrated the presence of a wide range of phytochemicals in fruits and their related health benefits [3-7,18]. The ascorbic acid values shown here were higher than previous studies [19,20] that analyzed freeze-dried ac-

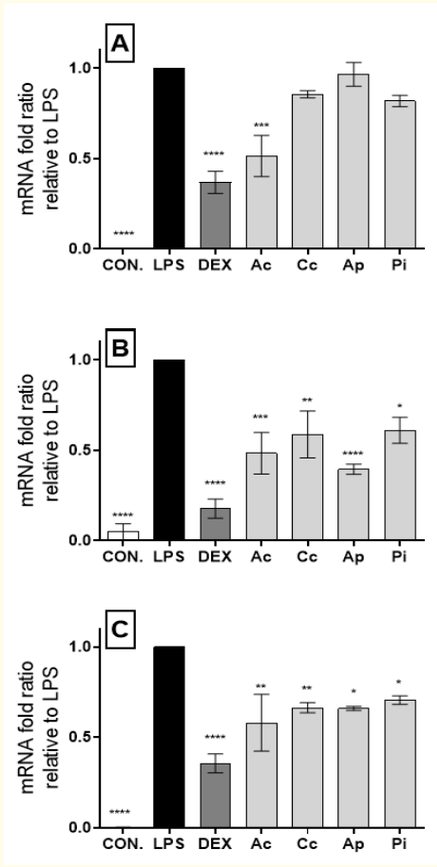

Figure 2: Effect of freeze-dried acerola (Ac), camu-camu (Cc), apple (Ap) and pineapple (Pi) extracts on the anti-inflammatory gene markers in LPS-induced RAW 264.7 cells at 50 µm/mL. (A) Cyclooxygenase-COX-2; (B) Interleukin IL-1β; (C) Interleukin IL-6. CON: 80% ethanol (negative control); DEX - Dexamethasone (positive control), LPS - lipopolysaccharide. Results expressed as mean±SD. Samples marked with asterisks are significantly different compared to LPS according to * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001.

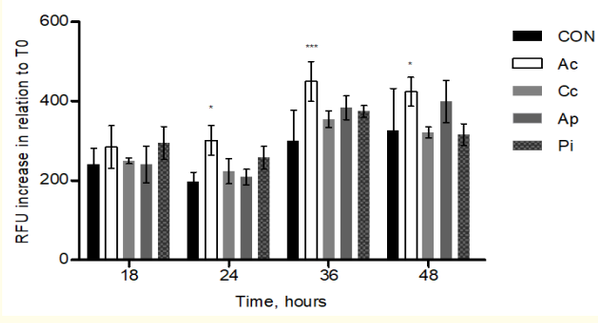

Figure 3: Effect of freeze-dried acerola (Ac), camu-camu (Cc), apple (Ap) and pineapple (Pi) extracts on the migration of adherent HDFa cell lines at 50 µm/mL. RFU: Relative fluorescence unit in relation to the initial time (T0). CON: ethanol 80% (control). Results expressed as mean±standard deviation. Samples marked with asterisks are significantly different compared to the vehicle according to * p<0.05; *** p<0.001.

erola (220.8 to 462.8 mg/100 g and 1201 mg/100 g, respectively). Due to low temperatures and reduced oxygen levels, freeze-drying is effective in preserving the natural bioactive compounds such as ascorbic acid. Actually, ascorbic acid is an ephemeral nutrient not synthetized or stored in the human body that quickly degrades when exposed to oxidative conditions [21]. In another study, camu-camu, as well as acerola, had higher total phenolic content when compared to other phytochemical-rich tropical fruits such as cashew (*Anacardium occidentale*), jaboticaba (*Myrciaria dubia*), and jambolan (*Syzygium cumini*) [11].

Phenolic acids are important non-flavonoid phenolics found in a large number of fruits and vegetables with a diverse range of health benefits. These biomolecules are readily absorbed in the human gastrointestinal tract and present consistent antioxidant, anti-inflammatory, antidiabetic, antimicrobial and other health-related activities [22]. In this study, several phenolic acids were identified in the freeze-dried samples. For example, the camu-camu extract presented a remarkable concentration of important phenolic acids such as gallic and ellagic acids (Table 1). Previously, chlorogenic and p-coumaric acids were identified in pineapple [23], and chlorogenic and caffeic acids in freeze-dried apple [24].

The total antioxidant activity results from the presence of individual bioactive compounds, as well as their interaction and synergy and can be exerted by different mechanisms [25]. The antioxidant activity of acerola samples measured by the DPPH method was higher, whereas FRAP values were relatively close (8.41 and 144.91 µmol TE/g, respectively) to another study that investigated edible parts of acerola [20]. In reality, different compounds found in fruit extracts can lead to different values in antioxidant assays. For example, hydrophilic phenolic compounds and ascorbic acid are known to give higher values in DPPH method when compared to FRAP [26].

Lipopolysaccharide (LPS) is a major biological endotoxin able to increase ROS and NO productions, as well as the expression of inflammatory markers in cells and biological systems [27-30]. ROS and NO intracellular production measurements have proved to be useful tools to evaluate the effect of natural extracts in modulating the stress response in *in vitro* biological systems [5,7,18,28,29]. While NO is an important biomolecule that promotes vasodilation and consequent increase of blood flow during the inflammatory process, the exaggerated production of ROS promote oxidative stress in cells, damage biostructures such as DNA, cell membranes and cellular proteins, and can cause system dysfunction and even cell death [31]. Collectively, our results suggest that freeze-dried camu-camu and acerola are the most potent inhibitors of both ROS and NO productions (Figure 1). Actually, these two fruits contain high concentrations of antioxidant bioactive compounds (ascorbic acid, total phenolics and total anthocyanins) when compared to the other freeze-dried fruit extracts ($p<0.05$). In this regard, the positive effect of ascorbic acid in mitigating the intracellular levels of ROS has been demonstrated either in the form of fruit extracts [28,29] and isolated compound [32,33]. In addition, anthocyanins

found in berry extracts showed efficient ROS and NO suppression when administered to LPS-induced macrophages [5] and human dermal fibroblasts [28]. Also, ellagic acid, detected in both acerola and camu-camu extracts, has shown potent antioxidant activity in previous reports [34].

The inflammatory process is a complex phenomenon that includes cascade reactions involving enzymes and inflammatory cytokines, such as interleukins IL-1β and IL-6 linked by complicated relationships [28,35,36]. For example, the production of NO is associated with the expression of iNOS [35,36]. In this study, none of the extracts at 50 µg/mL was able to suppress the activity of neither one of these markers, which suggests that our freeze-dried fruit extracts at this concentration do not activate this specific anti-inflammatory route. Only freeze-dried acerola extract at 50 µg/mL decreased the expression ($p < 0.001$) of COX-2 (Figure 2A), a key enzyme that is upregulated in response to inflammatory stimuli to produce pro-inflammatory prostaglandins, causing inflammation and pain [37]. Freeze-dried fruit extracts at 50 µg/mL inhibited IL-1b expression ($p<0.05$) in the order Ap > Ac > Cc > Pi (Figure 2B). Results also show that extracts at 50 µg/mL were effective in downregulating IL-6 expression ($p<0.05$) and acerola was, again, the main inhibitor (Figure 2C). Altogether, only freeze-dried acerola extracts were able to efficiently downregulate the expression of COX-2, IL-1β and IL-6 markers in LPS-activated cells (Figure 2). Our hypothesis is that the combination of high ascorbic acid and anthocyanin contents found in acerola extracts (Table 1) is responsible for the observed results. In fact, literature reports have already demonstrated the potent anti-inflammatory activity of ascorbic acid and anthocyanins [4,36,38-40]. For example, Duarte., *et al.* [36] have reported that the anthocyanin-rich crude extracts of strawberry fruits exerted potent anti-inflammatory effect in both *in vitro* and *in vivo* studies. Likewise, important signaling routes and inflammatory pathways have been readily suppressed by plant extracts containing phenolics and related antioxidant compounds [35,41]. In this regard, the effects of polyphenol compounds on human health may not rely merely on direct antioxidant mechanisms. Zhang, Virgous and Si [42] discuss the combination of different phytochemicals/foods as a way to efficiently fight chronic inflammation and diet-related diseases. Indeed, reliable scientific findings confidently support the use of dietary sources to combat inflammation and oxidative stress in a more natural, drug-free fashion.

Wound healing is a complex physiological process activated right after an injury as part of a survival mechanism. It is performed through well-coordinated and overlapping stages that would ultimately lead to wound closure and tissue integrity restoration [43]. The exact mechanism by which natural extracts interfere on fibroblast migration, related to wound healing, is yet unknown, but there is preliminary evidence showing that antioxidant, and anti-inflammatory agents might facilitate the skin healing process [44]. Therefore, once again, we speculate that the association of high concentration of ascorbic acid and anthocyanins of freeze-dried acerola (Table 1) partially explains the enhanced fibroblast migration (Figure 3). Our hypothesis is based on the fact that ascorbic acid is a powerful antioxidant molecule, an essential cofactor for multiple enzymatic reactions and has been shown to suppress pro-inflammatory processes and skin photodamage [44,45]. Besides, ascorbic acid and certain flavonoids play an important role in the metabolism and regulation of collagen, a key protein in the regeneration of skin cells and body structure strengthening [44,46,47]. Moreover, treatment with anthocyanins from black soybean seed coats inhibited ROS production, stimulated the migration of human fibroblasts, and proved to be a successful strategy to promote wound healing and prevent inflammation [48]. In a previous study conducted by Hoskin., *et al.* [5], anthocyanin-rich freeze-dried protein-polyphenol aggregates were able to suppress inflammation and ROS production, while significantly enhancing human dermal fibroblasts migration [5].

Conclusion

Fruit extracts obtained from freeze-dried acerola, camu-camu, apple and pineapple were able to reduce the production of reactive oxygen species and suppress the gene expression of important inflammatory markers, IL-1β and IL-6. However, only freeze-dried acerola and camu-camu were able to reduce the production of reactive nitrogen species (NO), while only acerola inhibited the expression of COX-2 and significantly enhanced the migration of HDFa cells. We hypothesize that the high ascorbic acid and anthocyanin contents detected in freeze-dried acerola explains the consistent antioxidant, anti-inflammatory activities and enhanced fibroblast migration observed in this study. Because they are commercial freeze-dried powders, the manufacturing parameters were not controlled, which creates prospects for future studies that involve monitoring of the production process that will unveil the full functional potential of these freeze-dried fruits. Overall, freeze-dried acerola would be a promising functional food ingredient that may be incorporated into food matrices to obtain value-added functional products.

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Conflict of Interest

The authors declare no conflict of interest.

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