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The data that support the findings of this study are available from the corresponding author, [GV], upon request

Regards
Giuseppe Valacchi

Gut-derived wild blueberry phenolic acid metabolites modulate extrinsic cutaneous damage

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Abstract

As the first line of defense, the skin is equipped with various physiological mechanisms positioned to prevent incoming oxidative damage from numerous environmental insults. With persistent exposure to the environment, understanding ways to augment the skin defenses is paramount in protecting from premature aging. In this study, we investigated the ability of five dietary phenolic metabolites, typically found in the bloodstream after wild blueberry consumption, to successfully defend the skin from UV light exposure in a novel ex vivo co-culture model of human skin explants and primary endothelial cells. Skin explants, placed in transwell inserts, were exposed to UV, and subsequently co-cultured with endothelial cells. When the endothelial cells had been pretreated with the bioactive metabolites at physiological concentrations (hippuric acid 3000 nM, isoferulic acid 1000 nM, salicylic acid 130 nM, benzoic acid 900 nM, α-hydroxyhippuric acid 400 nM) cutaneous damage was prevented on the co-cultured with UV-challenged skin explants. Co-culture with non-pretreated endothelial cells did not protect skin explants. Specifically, the pretreatment was able to reduce skin lipid peroxidation (measured as 4-hydroxynonenal protein adducts), and pro-inflammatory enzymes such as cyclooxygenase 2 (COX-2) and NADPH oxidase 4 (NOX-4). Furthermore, pretreatment with the metabolites prevented UV-induced release of inflammatory cytokines such as IL-1ß and IL-8 as well as nitric oxides (NO) levels. In addition, the metabolites showed an impressive ability to prevent the loss of cutaneous structural proteins including involucrin and collagen type 1. Of note, endothelial cells cultured with UV exposed skin explants exhibited increased oxidative stress demonstrated by heme oxygenase-1 (HO-1) up-regulation which was significantly prevented in the metabolite treated models. These findings highlight the ability of dietary polyphenolic metabolites to improve cutaneous defenses against extrinsic stressors.

Introduction

The skin is constantly exposed to the outside environment allowing for continuous physiological stress. Among the most noxious outdoor stressors, ultraviolet light (UV) has been recognized as the most aggressive environmental stressor which induces a variety of harmful effects particularly through the rampant generation of reactive oxygen species (ROS) leading to oxidative damage to cellular components including proteins, lipids, and nucleic acids.[1-2-3] Despite the skin being equipped with numerous endogenous antioxidant compounds and enzymes, these defensive mechanisms might be overwhelmed through chronic exposure to excessive levels of environmental pollutants.[3] Continued and intensive exposure to UV light has a variety of known detrimental skin health outcomes including photoaging and the development of skin disorders such as basal cell carcinoma, malignant melanoma and dermatitis.[4-5] The use of topical applications to improve skin health and ameliorate skin diseases have been extensively investigated and demonstrated.[6-7] However, there are some limitations to topical skin care due to the limited absorption, chemical stability of the compounds, and the need of a repetitive application.[8-9] Indeed, the barrier function of the skin acts as a limitation for topical skin care as it reduces the efficiency of absorption to the lower layers of the skin.[10] The physical appearance of skin has historically been used as a clinical indicator of nutritional deficiencies demonstrating its key role in skin health.[11-12-13] Recent research on nutraceuticals to supplement topical skincare routines have proven beneficial ensuring optimal delivery of nutrients and compounds to dermis and lower epithelial layers.[14] Better comprehension of the beneficial effects of dietary metabolites is critical to design nutritional interventions for skin health.

Characterized as a functional food, blueberries contain a variety of bioactive phytochemicals that are associated with amelioration of numerous diseases such as cardiovascular disease, type 2 diabetes mellitus and obesity.[15] The presence of bioactive phytochemicals and their antioxidant activity in vitro eludes to the plausible ability of their circulating dietary metabolites to improve skin health. Indeed, daily blueberry consumption has revealed beneficial effects on biomarkers related to oxidative stress and inflammation presumably due to the high levels of antioxidant compounds.[16] The topical application and dietary consumption of blueberry extracts have been demonstrated to improve skin health, increase cutaneous elasticity, and reduce skin roughness and inflammation.[17-18-19] While blueberries contain a variety of micronutrients such as vitamin C and E, particular interest has formed surrounding their polyphenolic compounds such

as anthocyanins which have exhibited anti-inflammatory, antioxidant, and immunomodulatory properties in vitro and in vivo.[20-21] When ingested these polyphenolic compounds undergo significant modification in small and large intestine generating a wide variety of phenolic metabolites which circulate at nanomolar to micromolar concentrations in the bloodstream varying with level of supplementation.[22-23-24] The role and ability of these dietary metabolites to induce skin benefits and modulate skin physiology is not well demonstrated.[25]

While some in vitro studies with keratinocytes and cutaneous fibroblasts have been conducted to understand the cutaneous protective role of specific blueberry polyphenolic against environmental stressors, little research has been conducted using physiological skin models, like human skin explants, in the presence of a combination of blueberry metabolites found in plasma after dietary supplementation.[25-26] In this work, a human umbilical vascular endothelial cell (HUVEC) model, in addition to a novel ex vivo co-culture model of human skin explants and HUVEC cells, were employed to investigate the ability of five predominant phenolic metabolites up-regulated with wild blueberry dietary supplementation, to reduce vascular inflammation and protect cutaneous physiological from damage induced by ultraviolet irradiation. The reduction of endothelial inflammation and cutaneous oxidative damage was determined through a variety of biomarkers related to oxidative stress, DNA damage, cutaneous extracellular matrix (ECM) and epidermal barrier formation, as well as the release of proinflammatory cytokines. This study provides a novel method for the investigation of dietary metabolites and their ability to protect cutaneous structures against environmental stressor induced oxinflammation and damage.[27] In addition, this study suggests a good methodology for investigating endothelial dysfunction induced from environmental pollutant exposure.

Materials and Methods

Metabolite Standard Preparation

A literature review of articles and clinical trials on wild and cultivated blueberry supplementation was conducted. Articles published until 2022 were retrieved from the Summons database. After review, 9 articles were selected for identification of serum metabolite concentrations. A standard metabolite mix was comprised of metabolites that were found in the greatest quantities in plasma after blueberry/wild blueberry consumption and qualitatively identified in all articles: hippuric acid, isoferulic acid, salicylic acid, benzoic acid, and α-hydroxyhippuric acid).[24-28-29-30-31-32-33-34-35] All metabolite standards were purchased from Sigma-Aldrich (St. Louis, MO, USA) or ChromaDex Standards (Longmont, CO, USA). Solvents were LC-MS grade and purchased from Fischer Scientific (Hampton, NH, USA). Concentrations of metabolites in the standard mix were averaged from quantitative serum values in clinical trials to maintain physiologically relevant concentrations in the model. The metabolite standard mix was stored at -20 °C until further use.

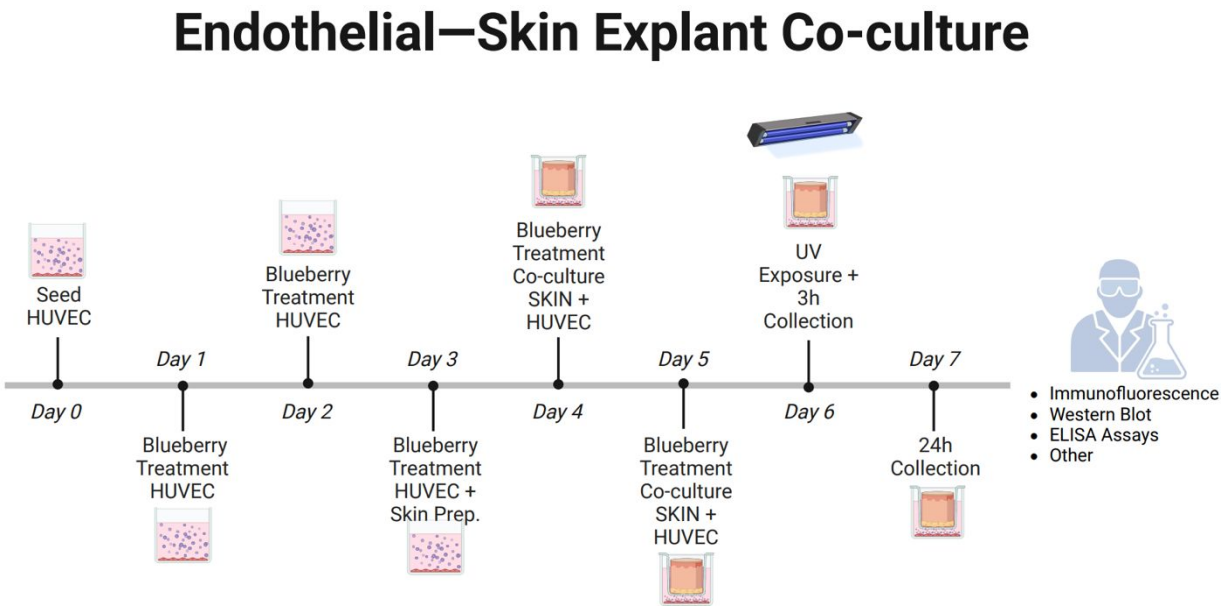
Table 1: Standard (Wild Blueberry) Metabolite Mixture Composition

Metabolite	Standard Concentration (nM)	Concentration Determination Reference(s)
hippuric acid	3000	Curtis, 2019; Curtis, 2022; Feliciano, 2016; Rodriguez-Mateos, 2013; Zhong; 2017
isoferulic acid	1000	Feliciano, 2016; Rodriguez-Mateos, 2013; Zhong, 2017
salicylic acid	130	Feliciano, 2016; Rodriguez-Mateos, 2013
benzoic acid	900	Curtis, 2019; Feliciano, 2016; Rodriguez-Mateos, 2013
α-hydroxyhippuric acid	400	Feliciano, 2016

Cell Culture, Skin Biopsies and Treatments

Human Umbilical Vascular Endothelial Cells (HUVEC) (cat. 354151, Corning, Manassas VA 20109) were cultured in Gibco Human Large Vessel Endothelial Cell Basal Medium (cat. M200500, ThermoFisher Scientific, Waltham, MA 02451) supplemented with Low Serum Growth Supplement (LSGS) (cat. S-003-10, ThermoFisher Scientific, Waltham, MA 02451) at 37 °C in 5% CO_2 in an incubator. HUVEC cells were seeded in 6-well plates at a density of 5000 cells/cm^2 and incubated overnight at 37 °C in 5% CO_2. The next day, cells were treated with media containing the five polyphenolic metabolites at the following concentrations: hippuric acid 3000 nM, isoferulic acid 1000 nM, salicylic acid 130 nM, benzoic acid 900 nM, α-hydroxyhippuric acid 400 nM for a total concentration of 5.43 μM. Control media was replaced and refreshed with media without metabolites. Treatments were repeated daily for three days to allow for appropriate cell confluence. On the third day, human skin explants used for co-culture were obtained from elective abdominoplasties and the protocol was approved by the Institutional Biosafety Committee (IBC) at North Carolina State University. All subcutaneous fat of the human skin explants was carefully removed using a scalpel and 20 mm biopsies were obtained. Biopsies were rinsed in phosphate-buffered saline (ref. 21-040-CV, Corning, Manassas, VA 20109) with 1% penicillin streptomycin (ref. 30-002-CI, Corning, Manassas VA 20109) and placed on transwell inserts in 6-well plates containing DMEM high-glucose media with 10% FBS, 1% penicillin streptomycin solution and incubated overnight at 37 °C in 5% CO_2. The following day, skin biopsies in transwell inserts were transferred to 6-well plates containing >90% confluent HUVEC cells and a 24-hour metabolite treatment was conducted before UV irradiation. The following day, the skin biopsies in transwell inserts were transferred into 6-well plate without HUVEC cells to be exposed to 200 mJ/cm^2 of UVA/UVB light (which can be considered as spending one day outdoor in a sunny day) using UVA/UVB Newport Oriel®, Sol1ATM, 1600W, Xenon Lamp, UVC & AM0 filters. UV doses were monitored and determined by radiometer ILT2400 Hand-Held Light Meter Optometer (International Light Technologies, INC., Peabody, MA, USA) as previously.[36] Directly after UV exposure, skin biopsies in transwell inserts were transferred back to 6-well plates containing HUVEC cells; no UV exposure was conducted on HUVEC cells (control). After indicated timepoints, co-culture media, skin biopsies, and HUVEC cells were collected for analysis.

Figure 1: Co-culture Treatment Timeline and Model Formation



MTT Viability Assay

HUVEC viability test upon the five-metabolite treatment was assessed by MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) which consists of the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheyltetrazolium bromide (MTT) to formazan as previously described.[37] The HUVEC cells were seeded in 96-well plates at a density of 10,000 cells per well in 200µL of phenol red free cell culture media. Cells were treated with a variety of doses of metabolites at 1.09µM, 2.72µM, 5.43µM, and 10.86µM concentrations for 24 and 48 hours. After the treatment period, media was removed and replaced with MTT solution (110µL 0.5mg/mL) and incubated for four hours. Solution was removed then, 100µL of DMSO was then added to dissolved insoluble formazan crystals at 37°C for 15 minutes. After shaking, the solution absorbance was measured at 590 nm and subtracted from 620 nm.

Tissue and Cell Immunofluorescence Staining

Human skin biopsies collected after conclusion of experiments were placed in cassettes and fixed in 10% neutral buffered formalin at room temperature for 24-hours and transferred into 70% ethanol for another 24-hours. Cassettes were then transferred into an automated Leica tissue

processor (Leica Biosystems, Deer Park, IL, USA) to dehydrate using the following steps: 30 minutes EtOH 70%, 30 minutes EtOH 80%, 60 minutes EtOH 95%, 90 minutes EtOH 100%, 90 minutes xylene, overnight paraffin. After dehydration, tissue samples were embedded in paraffin using Shandon Histocentre. Samples were then sectioned using a microtome (Leica Biosystems, Deer Park, IL, USA). Slides were heated at 60 °C for 20 minutes and deparaffinized using xylene and subsequently rehydrated using decreasing alcohol gradient solutions (100%, 80%, 70%, 30% EtOH). Slides were placed in distilled water and antigen retrieval was performed using citrate buffer (cat. C9999, Sigma-Aldrich, Merck Millipore, Burlington, MA, USA) (pH 6.0) in 95 °C heated water bath for 10 minutes. Following antigen retrieval, samples were left at room temperature for approximately 20-30 minutes to cool, washed twice in PBS, then blocked with 2% bovine serum albumin in PBS for 45 minutes at room temperature. Tissues were then incubated overnight at 4°C with primary antibodies: 4-HNE (AB5605, Millipore Sigma, Burlington, MA, USA), 8-OHdG (sc-393871, Santa Cruz Biotechnology Inc., Dallas, TX, USA), Involucrin (sc-21748, Santa Cruz Biotechnology Inc., Dallas, TX, USA), Collagen-1 (ab138492, abcam, Cambridge, UK) in 0.25% BSA in PBS. The following day, slides were washed three times in PBS for 5 minutes and subsequently incubated with corresponding fluorochrome-conjugated secondary antibodies (Alexa Fluor 568 A11057, Alexa Fluor 568 A11004, Alex Fluor A11008) at 1:500 dilution in 0.25% BSA/PBS) at room temperature for one hour. Slides were then washed another three times in PBS and nuclei were stained with DAPI (D1306, invitrogen, Thermo-Fisher Scientific, Waltham, MA, USA), and examined with a Zeiss Z1 AxioObserver LSM10 confocal microscope at 40x magnification. Images were then quantified using ImageJ software 1.53a (Java 1.8.0_172, National Institutes of Health, Bethesda, MD, USA).[38]

Medium was removed from 24-well plates containing HUVEC cells and rinsed with PBS briefly and fixed with 10% formalin at room temperature for 10 minutes. Cells were then washed three times with PBS for 5 minutes on an automated shaker 30 rpm and then permeabilized withA 0.2% triton x-100 at room temperature. After, blocking with 0.2% BSA/PBS was performed for 1 hour at room temperature the removed, primary antibodies (NFκB p65 (ref. 93H1, Cell Signaling Technology, Danvers, MA 01923), ASC (cat. NBP1-78977, Novus Biological, Littleton, CO, USA) were added and incubated overnight in 0.25% BSA/PBS. The following day, three PBS washes were performed, and samples were incubated with corresponding fluorochrome-

conjugated secondary antibodies (Alexa Fluor 568 A11008, Alexa Fluor 568 A11011) for two hours at room temperature. An additional three PBS washes were performed, and nuclei were stained with DAPI (D1306, invitrogen, Thermo-Fisher Scientific, Waltham, MA, USA), and examined with a Zeiss Z1 AxioObserver LSM10 confocal microscope at 63x magnification. Fluorescent intensity and nuclear translocation were quantified using ImageJ software 1.53a (Java 1.8.0_172, National Institutes of Health, Bethesda, MD, USA).

Protein Extraction and Western Blotting

Human skin biopsies and HUVEC pellets were homogenized in T-PER™ Tissue Protein Extraction Reagent (Thermo Fisher Scientific, USA) with 1% of phosphatase and protease inhibitor cocktails (Sigma, USA) with a Precellys tissue homogenizer (Bertin insturments) at 10,000 rpm at 4 °C for 20 seconds with 30 seconds breaks. This cycle was repeated three times and protein content of the samples were measured using the Quick start Bradford protein assay (Bio-Rad, USA). Following quantification, equivalent amounts of proteins were loaded into 10% polyacrulamide SDS gels and separated by molecular size. Gels were electroblotted onto nitrocellulose membranes, washed with TBS-T, and blocked with EveryBlot Blocking Buffer (Bio-Rad, USA) for five minutes Nitrocellulose membranes were then incubated at 4°C overnight on rocker at 60 rpm with primary antibodies: 4-HNE (AB5605, Millipore Sigma, Burlington, MA, USA), Collagen-1 (ab138492, abcam, Cambridge, UK), NOX-4 (cat. 14347-1-AP, ProteinTech Rosemont, IL), HO-1 (PA5-27338, Invitrogen, Thermo-Fisher Scientific, Waltham, MA, USA) diluted in EveryBlot Blocking Buffer. The next day, membranes were washed three times for ten minutes with TBS-T and incubated with secondary antibody for two hours (170–6515, 170–6516, 1721037, BioRad, USA). Detection of chemiluminescence was conducted with ChemiDoc (BioRad, USA). The membranes were then washed and incubated with β-actin (A3854, Sigma, USA) as control for protein loading. Bands were quantified using ImageJ software 1.53a (Java 1.8.0_172, National Institutes of Health, Bethesda, MD, USA).

ELISA for IL-1β and IL-8

IL-1β and IL-8 levels were measured in co-culture media collected at the indicated timepoints using IL-1β ELISA Kit (cat. ELH-IL1b RayBiotech, Peachtree Corners, GA 30092) and IL-8 ELISA Kit (cat. KAC1301 invitrogen by Thermo Fisher Scientific, Fredrick, MD 21704),

according to manufacturer protocol. The absorbance was measured with a spectrophotometer with a filter of 450 nm, using 570 nm wavelength as a reference. The Gen5 2.0 software (BioTek, Agilent, Santa Clara, CA, USA) was used for detection. Levels of IL-1β and IL-8 release were expressed as pg/mL in co-culture media according to manufacturer's protocol.

Nitric Oxide Measurement

Nitric Oxide levels in co-culture media at different timepoints were measured using Nitric Oxide (Nitrate/Nitrite) Assay Kit (cat. MA-NO-2 RayBiotech, Peachtree Corners, GA 30092) according to manual protocol. Optical density (OD) of samples were measured using a spectrophotometer with a filter of 540 nm. (BioTek, Agilent, Santa Clara, CA, USA) was used for detection.

Cutaneous Erythema

Cutaneous erythema was evaluated by DermaLab skin color probe (Cortex Technology ApS, Hadsund, Denmark); values represented as a ratio between the baseline measurement prior to UV exposure and after UV exposure.

Statistical Analysis

Appropriate statistical analyses were performed using GraphPad Prism version 10.1.1 (270) for macOS, GraphPad Software Boston, Massachusetts USA, www.graphpad.com. ELISA standard curves interpolated to asymmetric sigmoidal, 5PL model. Analyses performed include ordinary one-way ANOVA with subsequent Tukey multiple comparison for each variables/conditions tested. Data are expressed as mean with standard deviation of the mean (SEM) obtained in three independent experiments. Statistical significance was considered at * P < or equal to 0.05, ** P < or equal to 0.01, *** P < or equal to 0.001, **** P < or equal to 0.0001.

Results

Phenolic Acid Metabolite Standard Concentration and Cell Toxicity

To evaluate the ability of phenolic acid metabolites to modulate the cutaneous response to UV-exposure, a 5-metabolite standard (MIX) was created and subsequently diluted to mimic physiological concentrations of the metabolites following blueberry consumption.[24-28-29-30-31-32-33-34-35] The final concentrations present in the co-culture media treatments are depicted in Table 1. To assess the cytotoxic effect of the metabolite, MIX on HUVEC cells, a MTT assay was performed at two different time points, 24 and 48 hours after the treatment. As exhibited in Figure 2A and 2B, no cytotoxicity was observed at a range of concentrations analyzed in the HUVEC cells across the different time points.

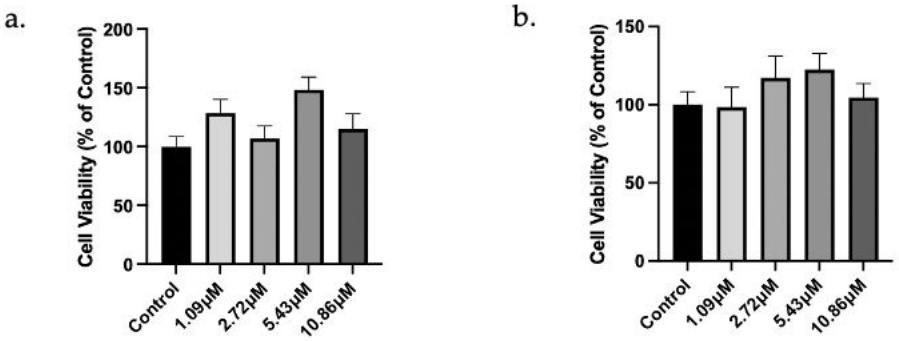


Figure 2. Cellular viability (A) 24-hour MTT toxicity assay after 5-metabolite MIX treatment in HUVEC media (B) 48-hour MTT toxicity assay after 5-metabolite standard treatment in HUVEC media

Metabolites MIX Prevents LPS-Induced NFkB Activation in HUVEC Cells

To test the anti-inflammatory properties of the metabolites MIX, HUVEC cells were challenged with lipopolysaccharide (LPS) to mimic an inflammatory response. The ability of LPS to activate NFκB signalling, a transcription factor involved in the regulation of a proinflammatory response, has been previously demonstrated.[39-40] As shown in Figure 3, LPS treated cells showed a clear nuclear translocation of NFκB, with significance after 30 minutes lasting up to 2 hours after the treatment while after 6 hours, NFκB nuclear levels return to baseline expression. LPS treatment induced a 1.46-fold increase in nuclear NFκB after 30 minutes and a 1.8-fold increase after 2 hours

compared to the control. This effect was significantly reduced at 30 minutes and 2 hours ($p<0.05$) when the cells were treated with metabolite MIX (1.43-fold and 1.29-fold respectively). Nuclear NFκB levels were comparable between LPS and metabolite MIX with LPS stimulation after 6 hours ($p=0.5592$). As a whole, when pre-treated with the metabolite MIX, an evident inhibition of NFκB translocation was observed in the HUVEC cells. The significance at both 30 minutes and 2 hours after the exposure to LPS confirming the anti-inflammatory ability of the metabolite MIX.

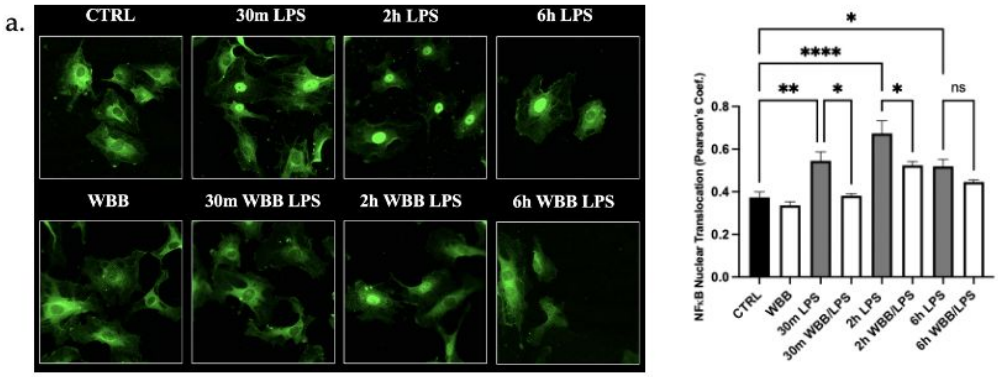


Figure 3. Immunofluorescence staining for NFκB (p65) in HUVEC cells challenged with 1µg/ml LPS. NFκB immunofluorescence staining and nuclear translocation in HUVEC cells treated with 1µg/ml LPS. Images were taken at 40× magnification and the fluorescent signal was quantified using the ImageJ software 1.53a (Java 1.8.0_172). The colocalization of NFκB (p65) is expressed as Pearson's correlation coefficient. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$; **** $p < 0.0001$

Metabolites MIX Inhibits UV-induced Cutaneous Lipid Peroxidation and DNA Damage

To assess the ability of the metabolites, MIX to protect the skin from UV damage, skin explants were exposed to UV light and subsequently co-cultured with the endothelial cells pre-treated with the metabolite MIX. As depicted in Figure 4A and 4B, after UV exposure there was a significant increase in 4-hydroxynonenal protein adducts (4-HNE/PA) cutaneous levels at 3 and 24 hours after the UV exposure (125.7% and 49% respectively compared to the control). The treatment with metabolite MIX reduced the formation of 4-HNE/PA from UV-irradiation at 3 and 24 hours by 48.3% and 34.5% respectively. Figure 4B, clearly indicates that the increased peroxidation levels occurred mainly in the epidermis. When the skin explants were co-cultured

with HUVEC cells pre-treated with the metabolite MIX and subsequently exposed to UV, there was an evident protective effect demonstrated by the significant decreased levels of 4-HNE/PA.

In addition, as depicted in Figure 4C, UV exposure significantly ($p<.0001$) induced the formation of 8-OHdG as seen through a 66.9% and 49.8% increase in fluorescent intensity after 3 and 24 hours respectively compared to the control. The UV-induced formation of 8-OHdG was significantly reduced in metabolite MIX treated co-culture models demonstrating comparable 8-OHdG fluorescent intensity to the basal condition. The metabolite MIX induced the decrease of 8-OHdG fluorescent intensity at 3- and 24-hours following UV exposure with a 44.7% and 45.9% decrease respectively. The formation of the 4-HNE/PA and 8-OHdG were significantly reduced in co-culture models treated with blueberry metabolites suggesting their ability to bolster cutaneous defences and protect the skin from incoming UV-induced oxidative damage.

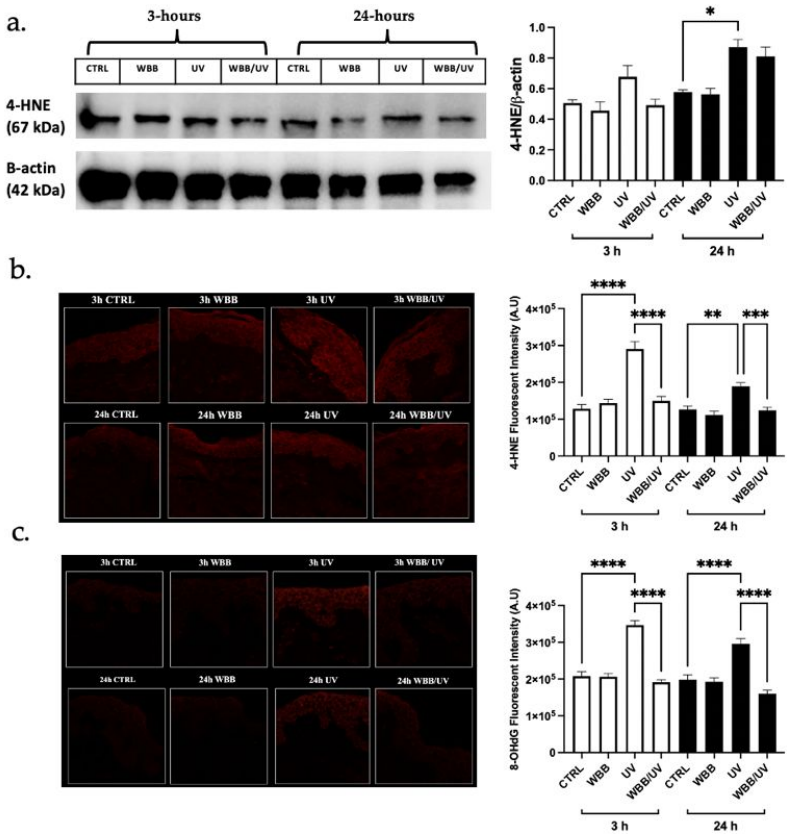


Figure 4. (A) 4-HNE/PA expression in skin biopsies exposed to UV light and co-cultured with HUVEC cells after indicated timepoints via Western blot (B) Immunofluorescence for 4-HNE/PA protein expression in skin biopsies exposed to UV light and co-cultured with HUVEC cells after indicated timepoints (C) Immunofluorescence for 8-OHdG protein expression in skin biopsies

exposed to UV light and co-cultured with HUVEC cells after indicated timepoints. Images were taken at 40× magnification and the fluorescent signal was quantified using the ImageJ software 1.53a (Java 1.8.0_172). * $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$; **** $p < 0.0001$

Metabolite MIX Prevents UV-Induced Cutaneous NADPH Oxidase and Inflammatory Mediators Expression

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases are membrane enzymes responsible for the endogenous production of ROS which act as powerful oxidative signaling molecules for downstream inflammatory genes including cyclooxygenase 2 (COX-2).[41-42] COX-2 is responsible for the production of numerous bioactive lipids such as prostaglandins which are involved in a variety of pathophysiological functions from inflammation to vasodilation.[43] Among the NAPDH oxidases isoforms, NOX-4 has been shown to play a key role in skin differentiation, wound healing and is responsible for the direct conversion of superoxide to hydrogen peroxide.[44-45] Figure 5A demonstrates the significant upregulation of COX-2 levels (1.64-fold) after UV-irradiation; this induction was prevented in metabolite MIX treated samples (circa 2-fold) confirming the anti-inflammatory properties of the metabolites MIX. As depicted in Figure 5B, a similar trend was noticed for NOX-4 expression. NOX-4 levels did not increase in the first 3 hours after UV-irradiation while its induction was significant after 24 hours, with a 1.72-fold upregulation in the irradiated samples. This increase was significantly (p<000.1) reduced in metabolite MIX treated co-culture models.

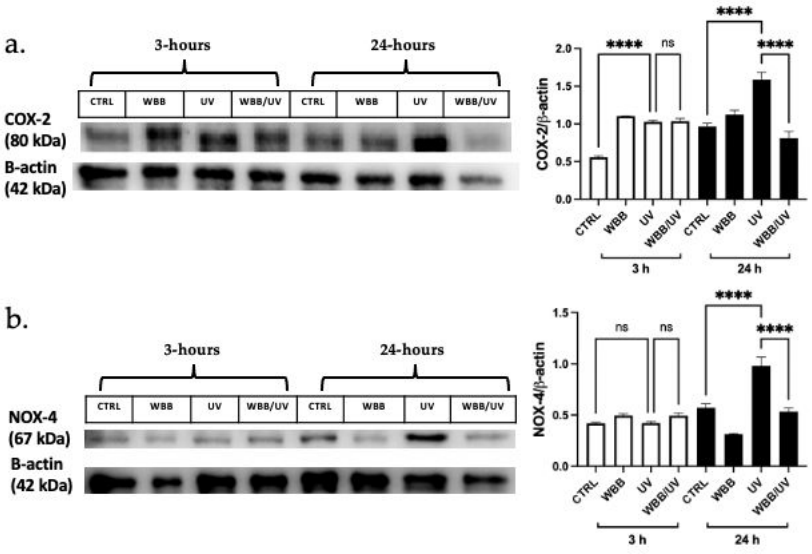


Figure 5. (A) Representative Western blot for COX-2 protein expression in skin biopsies exposed to UV light and co-cultured with HUVEC cells after indicated timepoints (B) Representative Western blot for NOX-4 protein expression in skin biopsies exposed to UV light and co-cultured with HUVEC cells after indicated timepoints. Band signals were quantified using the ImageJ software 1.53a (Java 1.8.0_172). * $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$; **** $p < 0.0001$

Metabolites MIX Promotes Skin Resilience

The ability of the metabolites MIX to protect against cutaneous degradation was evaluated through the measurements of involucrin, a crucial structural protein of the stratum corneum, and collagen type I, the predominant protein in the dermal extracellular matrix (ECM) involved in skin resilence.[46-47] Collagen type I was significantly protected from UV degradation after 24-hours with metabolites MIX treatment as depicted in Figure 6A. After 24-hours, UV caused a 1.47-fold decrease in cutaneous collagen type I ($p<0.0001$) and this effect was clearly rescued by the treatment with the metabolites MIX. The immunofluorescence assays in figure 6B confirmed the collagen type I trend, highlighting its distribution in the dermis. In addition, Figure 6C demonstrates the evident significant decrease of involucrin levels after exposure to UV light. Compared to control, UV-induced a 23.1% and 33.% degradation of involucrin after 3 and 24-hours respectively. Involucrin levels were conserved with metabolites MIX pre-treatment. Involucrin loss was perpetuated across timepoints as shown by the loss of fluorescence in UV-treated conditions.

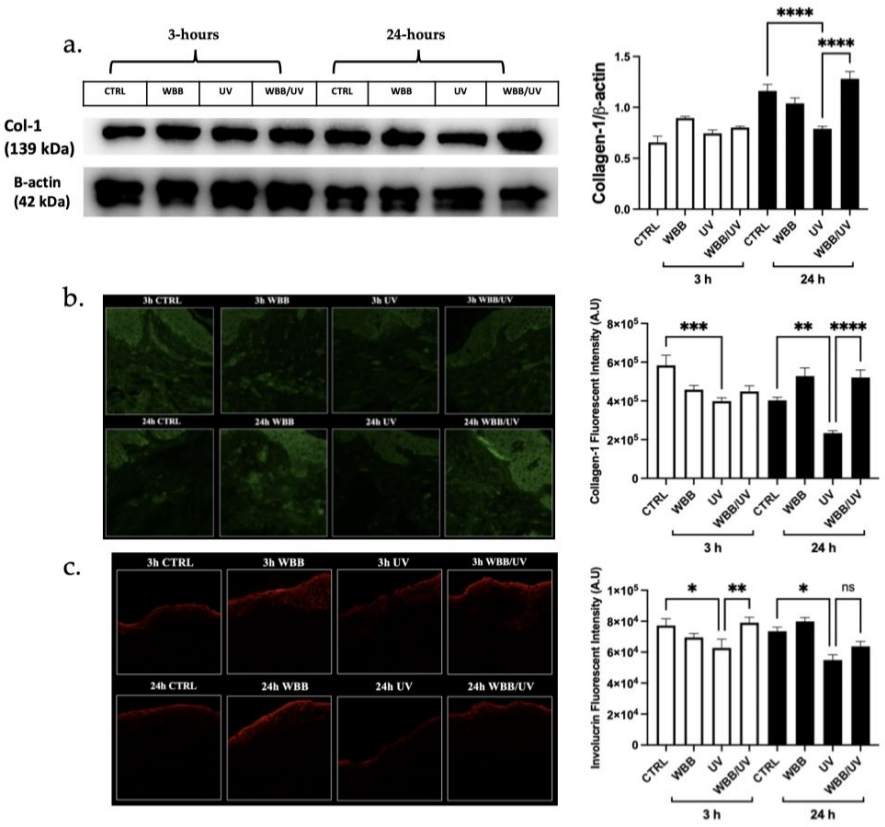


Figure 6. (A) Collagen type I protein expression in skin biopsies exposed to UV light and co-cultured with HUVEC cells after indicated timepoints via Western blot. (B) Collagen type I protein expression in skin biopsies exposed to UV light and co-cultured with HUVEC cells after indicated timepoints via immunofluorescence (C) Involucrin protein expression in skin biopsies exposed to UV light and co-cultured with HUVEC cells after indicated timepoints via immunofluorescence Images were taken at 40× magnification and the fluorescent signal was quantified using the ImageJ software 1.53a (Java 1.8.0_172). * $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$; **** $p < 0.0001$

Metabolites MIX Prevent UV-Induced Skin Redness

As demonstrated in Figure 7A, UV exposure induced a significant increase ($p<0.0001$) in cutaneous erythema after 3-hours (1.32-fold). Prescense of the metabolities MIX in the co-culture model reduced the overall redness in the explants by 1.15-fold ($p<0.05$). This effect was not evident after 24 hours, suggesting the increased redness is one of the earlies responses of the skin to UV exposure. The pre-treatment with the metabolites MIX was able to prevent the increased erythema maintaining the redness at the physiological levels.

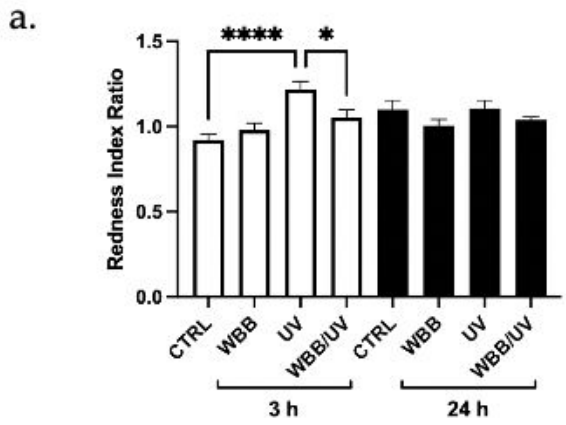


Figure 7. Erythema measured via DermaLab Probes in skin biopsies co-cultured with HUVEC cells and exposed to UV light collected after indicated timepoints. Redness index expressed as a ratio to initial measurement (before UV exposure). Statistical significant calculated with ordinary one-way ANOVA with subsequent Tukey multiple comparison for each variables tested; * $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$; **** $p < 0.0001$.

Metabolites MIX Prevent UV-Induced OxInflammatory Response in Endothelial Cells

As shown in Figure 8A, HUVEC cells co-cultured with UV-irradiated skin explants responded with a strong upregulation of HO-1 protein levels. A 95% increase of HO-1 expression was detected 24-hours after the irradiation of skin explants demonstrating the ability of cutaneous UV-exposure to modulate endothelial function. The presence of metabolites MIX was able to prevent the induction of HO-1 (Figure 8A).

To further assess the anti-inflammatory properties of the phenolic acids metabolites MIX, the release of pro-inflammatory cytokines IL-1β and IL-8 in the co-culture media was also

assessed. UV irradiation of skin explants stimulated the release of both IL-1β and IL-8, and this response was prevented by the metabolite MIX pre-treatment as shown in Figure 8B and 8C. UV exposure increased levels of IL-8 in co-culture media by 13% after 24-hours, the presence of the metabolite MIX significantly reduced IL-8 ($p<0.0001$). The release of IL-1β by UV exposure was even more evident when compared to IL-8 levels, with over 2 fold increase respect to the control. Also in this case, the metabolite MIX reduced the release of both inflammatory cytokines, IL-1β by 21-fold and IL-8 by over 30%.

Nitric oxides, which are oxidized to nitrites and nitrates, are potent vasodilators whose excess production is also involved in tissue damage and inflammation.[48-49] Significant increases in nitrates were detected in UV exposed co-culture model compared to untreated control. The increase of UV-induced nitrate release was also in this case significantly inhibited by the metabolite MIX pre-treatment at 24-hours' time point.

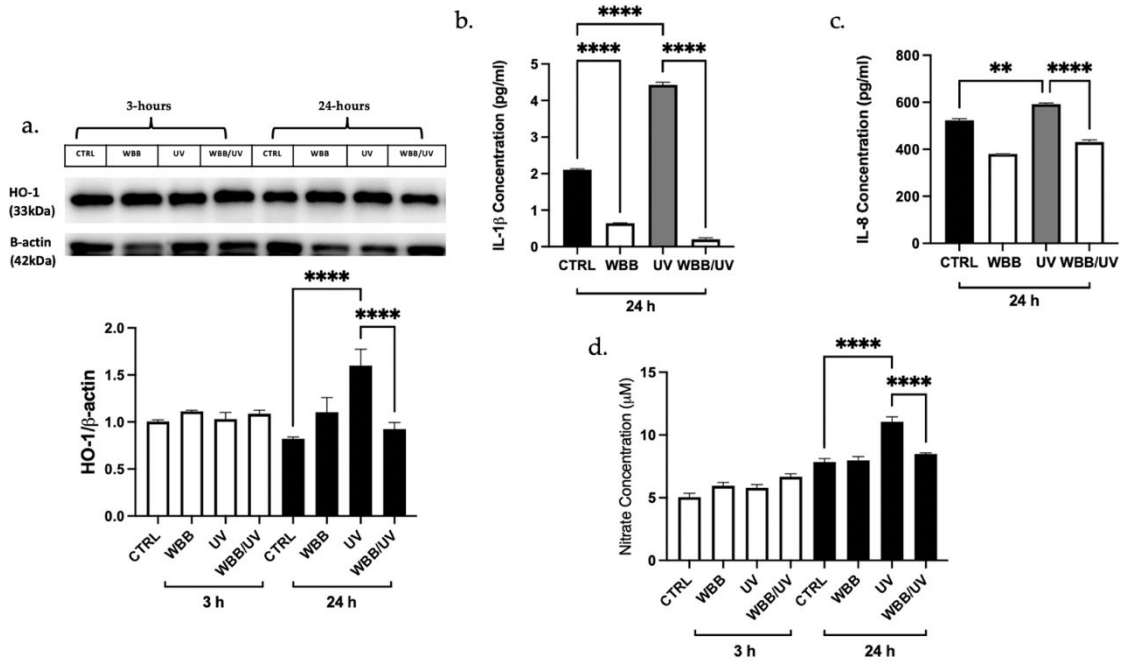


Figure 8. (a) HO-1 protein expression in HUVEC exposed to UV light and co-cultured with skin explants after indicated timepoints via Western blot. (b) Released levels of IL-1β (pg/ml) in co-culture medium collected after indicated timepoints via ELISA. (c) Released levels of IL-8 (pg/ml)

in co-culture medium collected after indicated timepoints via ELISA. (d) Nitrate concentration (μM) in co-culture medium collected after indicated timepoints via Nitric Oxide Assay. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$; **** $p < 0.0001$

Discussion

Chronic UV and other environmental stressor exposure to cutaneous structures have been shown to cause detrimental health outcomes such as the development of a variety skin cancers, photoaging, and immunosuppression.[50] Cutaneous health can be modulated and protected through topical application and dietary supplementation.[51] While topical applications are a good procedure to protect skin tissues from extrinsic aging, due to the lack of vascularization in the epidermal layer, dietary supplementation can be suggested to augment the beneficial effect of topical approaches.[52] Topical application of nutrients can be challenging due to the inherent barrier function of the skin, similarly, nutritional approaches must to account for gastrointestinal metabolism and the true absorption of their bioactives.[10-53]

The importance of diet in skin health is evidenced by numerous clinical manifestations of nutritional deficiencies and disorders such as phrynoderma in vitamin B-complex, and C deficiencies.[54] The combination of topical and dietary skincare optimizes antioxidant protection to provide the maximal decrease of environmental cutaneous degradation.[51-55]

The primary mechanism by which UV and other environmental stressors cause cutaneous damage is through the production of reactive oxygen species (ROS) which can lead to an inflammatory response and affect tissue homeostasis.[56]

The aim of this study was to investigate the potential for dietary wild blueberries and their gut-derived circulating metabolites to reduce cutaneous OxInflammation and subsequent structural degradation induced by UV exposure. For this purpose, a novel co-culture model of endothelial cells and skin explants was set up to better mimic the physiological delivery of metabolites from blood vessels to the skin. Particularly, this model allows for interactions between the dietary metabolites, endothelial cells, and skin explant augmenting our ability to survey the role of endothelial cells in UV induced cutaneous damage. Notably, the endothelial cells were co-cultured with the UV-irradiated explants never receiving *direct* UV exposure; this is significant in establishing a truly physiological model as only the skin receives direct UV irradiation. In addition, any modulation of endothelial function is due to crosstalk with skin explants through the release of signaling molecules. Our results confirmed the presence of this crosstalk as demonstrated through the significant release of inflammatory cytokines and increase of endothelial HO-1 protein levels.

Our first experiments tested for cytotoxic effects of the blueberry metabolite standards through MTT assay after 24 and 48-hours of blueberry metabolite MIX incubation. At a variety of concentrations, the metabolites demonstrated no cytotoxic effects in the endothelial cells, and to some extent stimulated endothelial proliferation. These effects could be due to the ability of polyphenols to reduce endothelial dysfunction through reducing ROS levels and increasing nitric oxides as demonstrated in HUVECs treated with boysenberry polyphenols.[57] Substantial evidence can be found demonstrating polyphenol modulation of endothelial function through reduction of proinflammatory cytokines and increase in endothelial nitric oxide synthase.[58-59]

These results allowed for the continued investigation of these metabolites through an LPS-inflammatory model and co-culture with skin explants. To demonstrate the anti-inflammatory ability of the metabolites MIX, endothelial cells were challenged with 1μg/ml LPS which promoted significant nuclear translocation of NFκB, a major proinflammatory transcription factor involved in stress response.[60] Our results suggest the ability of the metabolites to significantly suppress NFκB activation and demonstrates their anti-inflammatory ability. The ability of the metabolites at physiological concentrations to reduce endothelial dysfunction provides promising in vitro evidence to the reduction of inflammation through wild blueberry consumption. In a similar study, a blueberry metabolite mix of hydroxyhippuric acid, hippuric acid and other metabolites reduced palmitate, a proinflammatory lipid, induction of ROS and NFκB activation in human aortic endothelial cells.[61] Furthermore, LPS induced HUVEC dysfunction through NFκB activation and subsequent release of the cytokines IL-1β, TNF-a, and IL-6 has been previously demonstrated.[62-63-64] The anti-inflammatory ability of the metabolite MIX could be attributed to the reduction of ROS levels thus preventing NFκB activation and reducing the production of proinflammatory cytokines.

To further validate and evaluate the anti-oxinflammatory ability of the metabolites MIX, we performed a series of experiments using a co-culture of endothelial cells and skin explants. Skin explants were challenged with UV, at the dose that can be comparable of speding a day outdoor in sunny day [36], whose exposure is known causes the excessive production of ROS which oxidizes DNA damage and causes lipid peroxidation.[65-66] As a proof that our model system was appropriate, UV exposure was able to induced the formation of 8-OHdG, a product of DNA oxidation, and 4-HNE/PA, a noxious aldehyde end product of lipid peroxidation which subsequently acts as a secondary stimulator of inflammatory pathways.[67-68-69]

It should be mentioned that our assays did not measured free 4-HNE but the adducts that the proteins formed with this aldehydes, so it is possible that higher levels of 4-HNE has been formed and maybe not detected as not binded to proteins or because already

metabolized. These data confirm the potential of these metabolites to reduce ROS formation and subsequent damage of DNA and lipid peroxidation; it is unclear if this reduction can be attributed through the direct chemical scavenging of ROS from the polyphenolic compounds, the stimulation of cutaneous antioxidant defenses via NRF2 activation, or a combination of both. The free radical scavenging ability of hippuric acid, isoferulic acid, salicylic acid, benzoic acid, α-hydroxyhippuric acid have been extensively investigated.[70-71-72-73] NRF2 pathway was not investigated in the present study although several reports have suggested that polyphenolic compounds and their derivatives have the ability to modulate the NRF2 antioxidant response.[74-75] For example, treatment with a salicylic acid derivatives demonstrated successful modulation of NRF2 response genes and catalase activity in skin explants exposed to UV.[76] In addition, ferulic acid was able to increase the production of antioxidant response enzymes like superoxide dismutase and glutathionine peroxidase through NRF2 pathway activation.[77] Further investigation of these individual metabolite derivatives in cutaneous models are needed to illuminate their individual involvement in cutaneous NRF2 protection from UV irradiation.

Nonetheless, these data are confirmed by numerous results demonstrating the antioxidant ability of these polyphenolic compounds to influence skin redox status.[25-78] To investigate the ability of the metabolites to alter the production of ROS in the skin, the protein expression of NOX-4, one of several NADPH isoforms in the skin responsible for the direct formation of ROS, was surveyed.[79] The treatment with the metabolites MIX significantly reduced the NOX-4 overexpression induced with UV exposure suggesting reductions in the ROS forming capacity of the skin. The inhibition of NOX-4 by the polyphenolic metabolites MIX could play a critical role in the overall reduction of DNA oxidation and lipid peroxidation. A consequence of cellular ROS overproduction is the downstream activation of COX-2, a proinflammatory mediator, whose suppression directly inhibits UV-induced skin damage.[43] Metabolite treated models demonstrated significant reductions in COX-2 in irradiated skin explants 24-hours after exposure however, was unable to reduce acute upregulation at 3-hours. Through the reduction of COX-2, the metabolites demonstrate a significant ability to reduce the perpetuated cutaneous inflammatory status following UV exposure. The ability of the polyphenolic metabolites to reduce both COX-2 and

NOX-4 expression could be due to the modulation of ROS levels thus preventing the downstream activation of NOX-4/NFκB pathway; alternatively, this result could be attributed to NRF2 activation attenuating redox imbalance through direct suppression/inhibition of NFκB.[80-81] The ability of dietary supplementation to reduce UV-induced NOX-4 and COX-2 overexpression have been previous demonstrated.[82]

Involucrin is involved in cornified envelope formation whose crosslinking allows for incorporation of other epidermal structural precursors.[83] The ability of UV exposure to impair skin barrier function through the degradation of involucrin and other epidermal proteins is well demonstrated.[18-84] Moreover, the downregulation of involucrin is correlated with increased barrier dysfunction and demonstrated in numerous cutaneous ailments like atopic dermatitis.[84] UV irradiation was indeed able to significantly decrease cutaneous involucrin compared to control. The presence of metabolites significantly protected from rapid degradation of involucrin. Similar studies have demonstrated the ability of antioxidant compounds to protect involucrin degradation.[18-85-86] Furthermore, recent research has demonstrated the ability of natural compounds to stimulate involucrin production.[87]

Collagen type I is the predominant structural protein in the ECM which is responsible for skin resilience and strength.[88] A hallmark of the photoaging process is the degradation of dermal collagen through the activation of matrix metalloproteinases.[89] In this study, UV treated explants exhibited significant degradation of collagen type I. The metabolite treated models were able to inhibit the UV-induced degradation of collagen type I; this inhibition was particularly evident after 24-hours compared to 3-hours after UV exposure. Comparatively, metabolites demonstrate an improved ability to inhibit the degradation of collagen type I over involucrin. This is plausibly due to the dermis having greater access to dietary nutrients while the epidermis relies on diffusion through the cutaneous layers. Nonetheless, these data demonstrate the ability of polyphenolic metabolites to inhibit photoaging through protection of ECM degradation and preservation of the epidermal barrier structure. These results compare to in vitro and in vivo studies which demonstrate the ability of dietary antioxidants consumption and polyphenol treatments to improve collagen type 1 synthesis.[90-91]

HUVEC cells co-cultured with irradiated skin explants demonstrated a significant increase in HO-1 expression following UV exposure; HO-1 is mainly under the regulation of Nrf2 and primarily functions in heme catabolism providing anti-inflammatory and antioxidant effects.[92-93]

The upregulation of HO-1 in endothelial cells despite no direct exposure to UV demonstrates the crosstalk between the epidermis and endothelial cells. With this co-culture model, the communication between epidermal/dermal layers and endothelial cells can be further characterized with a variety of extrinsic and intrinsic stressors to better understand cutaneous physiology. With phenolic acid metabolites treatment, the production of endothelial HO-1 was significantly reduced suggesting the ability of metabolites to modulate perpetuated downstream anti-inflammatory signaling through the reduction of UV-induced inflammation. Our study demonstrates the upregulation of HO-1 in endothelial cells following co-culture with an irradiated skin explant. These data support a crosstalk between endothelial cells and skin plausibly through the release of inflammatory cytokines or other mediators which can then simulate endothelial HO-1 overexpression.

A consequence of UV irradiation of the skin is the cutaneous production of cytokines such as IL-1β and IL-8.[94-95] Cytokines are able to stimulate the surrounding cells and facilitate an immune response such as the recruitment of neutrophils and macrophages or stimulation of further inflammatory cytokines release.[96] In this study, we measured the release of both IL-1β and IL-8 to assess the ability of the metabolites to decrease the UV-induced activation of inflammatory pathways. UV exposure was indeed able to induce the both proinflammatory cytokines; moreover, the presence of the metabolites successfully inhibited the production of both cytokines particularly IL-1β. This confirms that in cutaneous UVB exposures, IL-8 is directly correlated with the overexpression of IL-1β.[97]

Nitric oxide (NO) acts a signaling molecule stimulating a variety of responses such as inflammation and tissue damage.[98] NO is key player in the regulation of immune responses; in low, normal physiological concentrations NO provides antioxidant benefits and inhibits cytokine synthesis, however, in large amounts, NO plays a pro-inflammatory role.[98-99] In the skin, NO is produced by numerous cells (keratinocytes, fibroblasts and endothelial cells) at a variety of concentrations and has been demonstrated to be inducible through UV exposure.[49-100] We found little variation in co-culture media NO levels 3-hours after exposure however, a significant increase was demonstrated 24-hours after exposure which was inhibited through metabolite MIX treatment. The reduction of NO overproduction and release suggests the ability of the metabolites to ameliorate UV-induced inflammation.

UV exposure has been demonstrated induce an immediate erythema response.[101-102] Following UV irradiation, the skin color probe revealed significant increases in redness after 3-hours; this demonstrates the cutaneous response to the UV exposure and the reliability of the model used in this study. The incidence of erythema was reduced in metabolite treated co-culture models further suggesting the abilities of the metabolites to act as UV antagonists.

Conclusion

Our study illuminates the ability of five metabolites typically found in circulation after blueberry consumption (hippuric acid, isoferulic acid, salicylic acid, benzoic acid, a-hydroxyhippuric acid) to protect from the UV induced oxinflammatory cutaneous responses and degradation of skin structural proteins. The suppression of 4-HNE, 8-OHdG, and NOX-4 formation exhibits the oxidative protection of the metabolites; furthermore, the reduction of COX-2 levels in skin explants and the lower release of proinflammatory cytokines IL-1β and IL-8 in co-culture media demonstrates the ability of the blueberries MIX to inhibit UV-induced inflammatory signalling. Blueberry dietary metabolites conserved epidermal and dermal proteins, involucrin and collagen type I, which were degraded by UV exposure. These results introduce a novel culture model of human skin explants co-cultured with endothelial cells to understand the ability of dietary metabolites and other bioactive compounds to modulate skin physiology. Moreover, this model allows for the investigation of cutaneous crosstalk with endothelial cells to illuminate how environmental oxidative stress to skin modulates endothelial function.

Author contributions:

Conceptualization: GV, MAL; Data Curation: JI, AB, FF, AP, AV, RS; Formal Analysis: JI, AB, FF, RS, AV, MI; Funding Acquisition: MAL, GV; Investigation: JI, AB, AP, FF; Methodology: JI, AB, RS, AV, MI; Resources: MAL, GV, AP, MI; Supervision: MAL, GV, AP; Validation: JI, AB, FF; Writing original draft: JI, FF; Writing-review editing: GV, MAL

Conflict of interest:

The authors have no conflict of interests related to this publication.

Aknowledgemnts

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