

Research Article

Antioxidant and Photoprotective Metabolites of *Bunodophoron melanocarpum*, A Lichen from the Andean Páramo

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Abstract

Background: Oxidative stress caused by exposure to ultraviolet radiation has been associated with dermal problems, including skin cancer. In this study, we determined the photoprotective and antioxidant activity of isolated metabolites from the lichen *Bunodophoron melanocarpum* (Sphaerophoraceae) to find new sunscreens prototypes.

Methods: The constituents of *B. melanocarpum* were isolated by phytochemical methods and their structures were determined by spectroscopy (IR, 1D and 2D NMR). Antioxidant activity was measured by scavenging DPPH free radicals (EC₅₀), ferric reducing power (FRP), and inhibition of lipid peroxidation (% ILP). The photoprotective capacity against ultraviolet (UVA and UVB) radiations was determined *in vitro* by calculating their sun protection factor (SPF), critical wavelength and UVA ratio and these values were compared against commercial sunscreens. The lipophilicity and possible skin penetration to the lipid-rich *stratum corneum* of the isolates, was determined by calculating their octanol/water partition coefficients (Log P) and Gibbs free energy of transfer ($\Delta_t G^0$).

Results: Sphaerophorin (1), evernicinic acid (2), sphaerophorol carboxylic acid (3) and friedelin (4) were isolated from *B. melanocarpum*. Orsellinic acid-type compounds 1 and 3 are dual agents with antioxidant capacity as free radical scavengers (EC₅₀ = 0.0857 and 0.1828 mol compound / mol DPPH•, respectively) and photoprotective properties particularly against UVB radiation (SPF 25.78 ± 0.53 and 22.00 ± 1.03, respectively). In addition, they had lipophilicity (Log P 7.07 ± 0.64 and 4.03 ± 0.32, respectively) and $\Delta_t G^0$ (-40.32 ± 3.67 and -22.97 ± 1.82 kJmol⁻¹, respectively) suitable to act on the skin

Conclusion: Sphaerophorin (1) and sphaerophorol carboxylic acid (3) are dual agents with antioxidant and UVB photoprotective properties and are also lipophilic substances that spontaneously would diffuse across the skin.

Introduction

Oxidative stress caused by exposure to ultraviolet radiation (UV-R) has been associated with dermal problems, including skin cancer and photoaging. Skin exposure to UV-R results in a direct damage to its DNA, peroxidation of its lipids, and in an alteration of its proteins.¹ The World Health Organization (WHO) has declared UV-R as a human carcinogen, causing both melanoma and non-melanoma skin cancer.² The use of substances with photoprotective properties (which protect the skin from the direct incidence of UV-R) along with antioxidants (which counteract the oxidative stress) in formulations intended for the skin, is beneficial to slow skin aging and to prevent skin cancer.¹ According to WHO, non-melanoma skin cancer ranked fifth worldwide whereas melanoma

represents less than 2 % of the total cases of skin cancer. However, melanoma causes the most deaths since it is the most aggressive type of cancer.³ Despite the use of sunscreen and antioxidant agents, the increase in the incidence of skin cancer demonstrates the inefficiency of these agents and emphasizes the need for more effective substances. Some sunscreens have the disadvantage of being photo-unstable, photo-allergic or not providing a uniform film of ingredients.⁴ Additionally, synthetic antioxidants such as butylhydroxytoluene, have been restricted given its hepatotoxicity and carcinogenicity.⁵ Finding alternative photoprotectors and antioxidants that are more effective, stable, potentially less toxic may have greater applicability in the dermo cosmetic field.

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Lichens are an original source of new biologically active chemical entities suitable for discovering alternative photoprotectors and antioxidants.⁶⁻⁸ Lichens are unique organisms resulting from the symbiotic association among a fungus, a yeast, and one or more autotrophic photosynthetic organisms (e.g., green alga, cyanobacterium). Lichens can thrive under extreme environments where other organisms could not survive. For example, lichens can thrive with virtually no nutrient supply in the outer space, high mountain ecosystems, areas with ozone layer deficiency, and polluted cities.^{9,10} This is possible thanks to complex mechanisms, such as the biosynthesis and accumulation of secondary metabolites, to protect themselves against external abiotic factors.^{10,11}

In this study, we report for the first time the isolation, as well as the photoprotective and antioxidant activities of metabolites from *Bunodophoron melanocarpum* (Sphaerophoraceae), to find new suitable sunscreens. This lichen grows at the Andean páramo of Sumapaz, a high mountain ecosystem (above 3500 m a.s.l.) restricted to the northwest corner of South America. In this place, lichens are subjected to extreme environmental conditions of temperature, relative humidity and UV-R, therefore, *B. melanocarpum* is expected to be adapted to UV-R through the biosynthesis of antioxidant and photoprotective agents. Previous chromatographic profiles of this species detected the presence of sphaerophorin¹² which in turn, showed a protective effect on DNA cleavage when it was induced by hydroxyl radicals.¹³ In addition, sphaerophorin inhibited the growth of melanoma cells inducing their apoptosis.¹³ In this study, the antioxidant activity of the extract and metabolites isolated from *B. melanocarpum* was tested by free radical scavenging, ferric reducing power, and inhibition of lipid peroxidation assays. Additionally, the *in vitro* photoprotective capacity, such as UVA and UVB absorbing molecules, was determined by calculating their sun protection factor (SPF), critical wavelength (λ_{crit}) and UVA ratio (UVA-r*). Since the isolated compounds could be used in the dermo-cosmetic field to protect the skin, their *n*-octanol-water partition coefficient (P) and their standard molar Gibbs free energy of transfer ($\Delta_t G^0$) were also calculated as a first approximation of their lipophilicity and their possible skin penetration to the lipid-rich *stratum corneum*.

Materials and Methods

Materials

Solvents: *n*-hexane, toluene, chloroform (CHCl₃), dichloromethane (CH₂Cl₂), ethyl acetate (EtOAc), acetone (Me₂CO), methanol (MeOH), ethanol (EtOH) and formic acid (HCOOH) were analytical reagent.

General experimental procedures

Melting points were determined on a Büchi SMP-20 apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer FTIR/FIR Paragom 500 Spectrum in KBr and UV spectra on a UV-1700 series spectrometer

(Shimadzu) in EtOH. NMR spectra were recorded on a Bruker Avance 400 at 400 MHz (¹H), or 100 MHz (¹³C), in CDCl₃ (99.8 atom % D) or (CD₃)₂O (99.8 atom % D) and TMS as internal standard. FIDs were processed by MestreNOVA® V6.0.2-5475 (trial version). EIMS was recorded on a gas chromatograph (Hewlett Packard® 6890) coupled to a mass spectrometer (Hewlett Packard® 5973) equipped with a ZB-5 capillary column (30 m, 0.25 mm I.D., 0.25 µm), ionization source 70 eV and helium as mobile phase (flow 1.1 mL / min). The temperature ramp started at 60.0 °C maintained for 1.0 min, then increased 7.4 °C / min to 310.0 °C keeping for 10.0 min. Mass spectrum was compared in the NITS database. Open column chromatography (CC) was carried out on Si-gel 60 (0.069-0.200 mm, Merck®) or Sephadex LH-20 (Sigma®). Thin layer chromatography (TLC) was performed on pre-coated aluminium backed Si-gel 60 F₂₅₄ TLC or HPTLC plates (Merck®) and spots were visualized under UV light or stained with H₂SO₄ solution (10 %) and heating (110.0 °C).

Lichen material

Bunodophoron melanocarpum Wedin (Sphaerophoraceae), was collected at páramo of Sumapaz, in Bogotá Colombia (04° 07.943' N; 074° 14.704' W; 3837 m a.s.l.). A voucher specimen (COL609188) is at the National Colombian Herbarium. The lichen was determined by R. Dávila (Fundación Nacional para el Estudio de la Biodiversidad Colombiana-FUNBIOCOL).

Extraction and isolation of lichen substances

Oven-dried (45 - 50 °C, 5 days) *B. melanocarpum* (200.0 g) was powdered on disc grinder and extracted with Me₂CO by ultrasound assisted maceration. The filtered was concentrated under vacuum to yield the dried extract. The extract (10.0 g) was subjected to open Si-gel CC (285.0 g) eluting with a gradient of *n*-hexane:toluene (1:0→0:1), toluene:CH₂Cl₂ (1:0→0:1), CH₂Cl₂:EtOAc (1:0→0:1), EtOAc:Me₂CO (1:0→0:1) and Me₂CO:MeOH (1:0→0:1) mixtures. Altogether, 75 fractions (100 mL) were collected and combined according to their TLC patterns to yield 12 primary fractions (F1-F12). By re-crystallization from *n*-hexane:CHCl₃ (3:1) mixture of fraction F5 (4.5 g), eluted with CH₂Cl₂:EtOAc (1:1) and EtOAc, a solid (2.4 g) was obtained, which was subjected to open Si-gel CC (53.0 g), eluting with a gradient of *n*-hexane:EtOAc (1:0→0:1), EtOAc:Me₂CO (1:0→0:1), Me₂CO:EtOH (1:0→0:1). Altogether, 177 (20 mL) fractions were collected and combined according to their TLC patterns to yield 13 secondary fractions (F5A-F5M). By re-crystallization from *n*-hexane:CHCl₃ (3:1) mixture of fraction F5E (622.7 mg) a solid (407.9 mg) was obtained, which was subjected to open Si-gel CC (10.2 g), eluting with *n*-hexane:CHCl₃ (9.5:0.5 and 9.0:1.0); *n*-hexane:CHCl₃:AcOH (9.0:0.9:0.1→7.7:2.1:0.2) and Me₂CO, resulting in 213 fractions (4 mL) collected in 8 combined fractions (F5E1-F5E8). By re-crystallization from CH₂Cl₂ of fraction

F5E3 (159.0 mg) sphaerophorin (**1**) (100.0 mg) was obtained as an amorphous white solid. Greater amount of **1** was obtained by re-crystallization from CH_2Cl_2 of mother liquors of F5 (2.0 g) combined with fractions F5D, F5F, F5E4 (1.5 g) which in turn were subjected to open Si-gel CC (90.0 g), eluting with *n*-hexane:EtOAc (9.6:0.4→9.5:0.5), *n*-hexane:EtOAc:HCOOH (9.75:0.25:0.05→8.0:2.0:0.2), *n*-hexane:EtOAc: CH_2Cl_2 :HCOOH (6.66:1.66:1.502:0.16→3.305:0.826:5.78:0.082), CH_2Cl_2 , EtOAc and Me_2CO obtaining 373 fractions (35 mL) that were collected in 26 combined fractions (Fm1-Fm26). Re-crystallization from CH_2Cl_2 of fraction F5m11 (104.4 mg) afforded more sphaerophorin (**1**) (60.0 mg). Through re-crystallization from CH_2Cl_2 of fraction F5m10 (89.9 mg), evernic acid (**2**) (60.0 mg) was obtained as white needles. Fraction F5m12 (398.5 mg) was subjected to size-exclusion CC (10.2 g) on Sephadex LH-20, eluting with CH_2Cl_2 : Me_2CO :MeOH (4:4:0.1) mixture resulting in 40 fractions (10 mL) collected in 7 combined fractions (F5m12S1- F5m12S7). Thru re-crystallization from CHCl_3 of fraction F-5m12S6 (15.8 mg), sphaerophorol carboxylic acid (**3**) (8.0 mg) was obtained as an amorphous reddish solid. Finally, fraction F2 (49.0 mg) was subjected to preparative Si-gel TLC (toluene: CHCl_3 ; 9:1) to give friedelin (**4**) as white crystals (10.0 mg).

Identification of the isolated compounds

The structures of the isolated compounds **1** to **3** were established by IR, 1D and 2D ^1H NMR and ^{13}C NMR spectral analyses comparing with those published in the literature. The structure of compound **4**, was established by ^1H NMR and Mass Spectrometry analyses comparing with those published in the literature.²²

Determination of antioxidant activity

Free radical scavenging activity

The potency (EC_{50}) of the extract of *B. melanocarpum* (**EBm**) and compounds **1** to **4** as free radical scavengers was determined at 25.0 °C using 1,1-diphenyl-2-picrylhydrazyl (DPPH•) as free radical according to Brand-Williams *et al.*¹⁴ methodology and some modifications as indicated by Rojas *et al.*¹⁵ Butylated hydroxytoluene (**BHT**) and ascorbic acid (**AA**) were the positive controls. EtOH solutions of DPPH• and extract or compounds were mixed in different ratios and their initial absorbance and its decrease was recorded (λ 515 nm, every 15 min) until a steady state was reached. The interval of ratios (mole of compound / mole DPPH•) to estimate the potency was: **1** (0.017 - 0.608); **2** (0.018 - 4.081); **3** (0.017 - 2.378); **4** (0.022 - 5.597); **AA** (0.066 - 0.446); **BHT** (0.041 - 0.462) and **EBm** (0.084 - 0.835) [mg **EBm**/mg DPPH•]. For each tested ratio, the reaction kinetics were plotted (% DPPH• remaining vs t). From these graphs, the percentage of DPPH• remaining at the steady state was determined and the values transferred onto another graph showing the percentage of residual DPPH• at the steady state as function of the concentration ratio (mol of compound/L / mol of DPPH•/L) or (mg of sample/L / mg of DPPH•/L). From this graph, EC_{50} was

calculated. It is defined as the amount of sample (extract, compound or positive control) necessary to decrease the initial DPPH• concentration by 50 % and it was expressed as mol of compound / mol of DPPH• or mg of sample / mg of DPPH•.^{14,15}

Reactivity of lichen compounds as free radical scavengers

The reactivity of **1** to **4** as free radical scavengers was determined at 25.0 °C by a kinetic study calculating the second-order rate constants (k_2 , $\text{M}^{-1}\times\text{s}^{-1}$) for the reaction between each compound (AH) and DPPH• in a ratio 8:1 to 24:1 $[\text{AH}]_0/[\text{DPPH}\cdot]_0$.¹⁵ Under the condition $[\text{DPPH}\cdot]_0 \ll [\text{AH}]_0$, the decrease of $[\text{DPPH}\cdot]$ followed a pseudo-first-order kinetics according to:

$$[\text{DPPH}\cdot] = [\text{DPPH}\cdot]_0 * e^{-k_{obs} * t} \quad \text{Eq. 1}$$

Graphics from experimental data of $\text{Ln} [\text{DPPH}\cdot]$ vs t were done with TableCurve 2D® program and from the slope of these graphics, k_{obs} (s^{-1}) were obtained. With these values and the initial concentration of each compound ($[\text{AH}]_0$), k_2 was calculated according to:

$$-\frac{d[\text{DPPH}\cdot]}{dt} = k_{obs} * [\text{DPPH}\cdot] = k_2 * [\text{AH}]_0 * [\text{DPPH}\cdot] \quad \text{Eq. 2}$$

Ferric reducing power

The ferric reducing power (FRP) of **EBm** and **1** to **4** was determined according to Oyaizu¹⁶ as is described by Rojas *et al.*¹⁵ The capability of antioxidants to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions was measured through the formation of Perl's Prussian blue complex. Samples and positive control (**BHT**) solutions (50 - 500 ppm) were prepared in EtOH. As the absorbance increases, a higher concentration of formed complex is measured which in turn, is indicative of a higher reductive power of the sample.

Inhibition of lipid peroxidation

The inhibition of lipid peroxidation by **EBm**, **1** to **4**, and positive control (**BHT**) was determined by the thiocyanate method¹⁷ with some modifications, using an emulsion of linoleic acid (0.02 M) in phosphate buffer (pH 7.0) as blank to measure maximum peroxidation. The samples were dissolved in EtOH (50 - 500 ppm). The formed peroxide (Fe^{3+} - thiocyanate complex) resulting of lipid peroxidation was determined by reading the absorbance (λ 500 nm) daily until one day-after linoleic acid reached its maximum value. The % of inhibition of lipid peroxidation (%ILP) was calculated according to:

$$\%ILP = \left[\frac{A_b - A_s}{A_b} \right] * 100 \quad \text{Eq. 3}$$

Where, A_b : blank absorbance; A_s : sample absorbance.

Determination of photoprotective activity (in vitro)

Sun protection factor (SPF) as a measure of photoprotective activity against UVB radiation

Sun Protection Factors (SPF) were determined by the *in vitro*

screening method of Mansur *et al.*¹⁸ as described by Rojas *et al.*¹⁵ Avobenzone (UVA filter), benzophenone-3 (UVA-UVB filter) and 2-phenyl-5-benzimidazolesulphonic acid (UVB filter) (Sigma Aldrich) were the positive controls. The samples were diluted in absolute EtOH (10, 50, 100 and 200 ppm) and a spectrophotometric scanning (290 - 400 nm, intervals of 1 nm), was performed in quartz cell (1 cm) using EtOH as blank. Calculation of SPF was obtained according to equation:¹⁸

$$SPF = CF \times \sum_{290}^{320} EE(\lambda) \times I(\lambda) \times Abs(\lambda) \quad \text{Eq.4}$$

Where, EE (λ): erythral effect spectrum; I (λ): solar intensity spectrum; Abs (λ): absorbance of sunscreen product; CF: correction factor (= 10). Values of EE x I are constants.¹⁹

According to Food and Drug Administration (FDA),²⁰ depending on SPF values, sunscreens show different level of photoprotection against UVB radiation: 2 to 15 (low); 15 to 30 (medium), 30 to 50 (high); > 50 (highest).

Determination of critical wavelength (λ_{crit}) as a measure of photoprotective activity against UVA radiation

From the absorbance data (at 200 ppm) of the previous determination, the critical wavelength (λ_{crit}) was calculated according to:

$$\int_{290 \text{ nm}}^{\text{crit}} Abs(\lambda) d\lambda = 0.9 \times \int_{290 \text{ nm}}^{400 \text{ nm}} Abs(\lambda) d\lambda \quad \text{Eq.5}$$

Where, Abs: sample absorbance. The area under the absorbance curve (AUC) at range 290 - 400 nm was set as 100 % and λ_{crit} was calculated as the wavelength at which 90 % of the AUC was reached.

According to FDA, depending on λ_{crit} values, sunscreens show different level of photoprotection against UVA: 0 ($\lambda_{crit} < 325$ nm); 1 (325 $\lambda_{crit} < 335$ nm); 2 (335 $\lambda_{crit} < 350$ nm); 3 (350 $\lambda_{crit} < 370$ nm); 4 (370 nm $< \lambda_{crit}$). A higher number on this scale, a higher ability to protect against UVA.

Determination of UVA ratio (UVA-r*) as a measure of photoprotective activity against UVA and UVB radiations

From the absorbance data (at 200 ppm) of the previous determination, the UVA-r* factor was calculated according to Springsteen *et al.*²¹ using equation:

$$UVA \text{ ratio} = \frac{\alpha_{UVA}}{\alpha_{UVB}} = \frac{\int_{320 \text{ nm}}^{400 \text{ nm}} Abs(\lambda) d\lambda}{\int_{290 \text{ nm}}^{320 \text{ nm}} Abs(\lambda) d\lambda} \quad \text{Eq. (6)}$$

According to UV-r* values, sunscreens can be claimed as having UVA protection according to: 0.0 < 0.2, "too low"; 0.2 < 0.4, "moderate"; 0.4 < 0.6, "good"; 0.6 < 0.8, "superior" and 0.8 \geq , "maximum".

Calculation of partition coefficient and standard molar Gibbs free energy of transfer as a measure of lipophilicity and skin penetration to lipid-rich stratum corneum

The partition coefficient (P) expressed as Log P of **1** to **4** and control sunscreens were calculated using molinspiration® interactive Log P calculator (<http://www.molinspiration.com/cgi-in/properties>), Marvin Sketch® calculator plugins for structure property prediction and calculation (Marvin 2016; ChemAxon) and ChemDraw® (trial versions) software. The standard molar Gibbs free energies of transfer from water to *n*-octanol were calculated according to:

$$\Delta_t G^\circ = -RT \ln P \quad \text{Eq. 7}$$

Where, $\Delta_t G^\circ$: Standard molar Gibbs free energy of transfer (at 298.15 K); R: ideal molar gas constant (8.314 J mol⁻¹ K⁻¹); T: absolute temperature in kelvin (298.15 K); P: partition coefficient.

Statistical analysis

Data were analysed by one-way analysis of variance (ANOVA) followed by Tukey's test ($p < 0.05$). Values are expressed as mean \pm standard deviation.

Results

Isolation of lichen substances

Acetone extract of *B. melanocarpum* was fractionated and separated by a combination of column chromatography, preparative TLC and re-crystallization methods to afford sphaerophorin (**1**), everninic acid (**2**), sphaerophorol carboxylic acid (**3**) and friedelin (**4**) (Figure 1). Their structures were established by IR, 1D and 2D ¹H NMR and ¹³C NMR spectral analyses comparing with those published in the literature.²² To our knowledge, orsellinic acid-type compounds **2** and **3** along with triterpene **4** are reported here for the first time for this species. The spectral data of **1** to **4** are presented below and their spectra are on supplementary data.

Spectral data for isolated compounds 1 to 4

Compound **1**; sphaerophorin (**1**), amorphous white solid (160 mg, 0.08 %) ; mp 133-134 °C; IR (KBr) ν_{max} 3422, 3186, 2920, 2851, 1655, 1609, 1578, 1315, 1238, 1207, 1142 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ ppm 0.90 (3H, *t*, *J* = 6.3 Hz, CH₃-7"), 1.41-1.30 (8H, *m*, 4x-CH₂-, H-3" to H-6"), 1.64 (2H, *q*, *J* = 7.3 Hz, -CH₂-2"), 2.66 (3H, *s*, -CH₃-8), 3.03 (2H, *t*, *J* = 7.7 Hz, -CH₂-1"), 3.86 (3H, *s*, -OCH₃), 6.41 (2H, *s*, H-3, H-5), 6.68 (1H, *s*, H-5'), 6.78 (1H, *s*, H-3'), 11.40 (1H, *s*, -OH); ¹³C NMR (101 MHz, CDCl₃) δ ppm 104.2 (C-1), 166.5 (C-2), 98.9 (C-3), 164.9 (C-4), 112.0 (C-5), 143.5 (C-6), 169.5 (C-7), 24.6 (C-8), 108.6 (C-1'), 155.0 (C-2'), 109.0 (C-3'), 165.2 (C-4'), 116.3 (C-5'), 150.0 (C-6'), 174.9 (C-7'), 36.6 (C-1"), 31.8 (C-2"), 29.7 (C-3"), 29.1 (C-4"), 31.7 (C-5"), 22.6 (C-6"), 14.1 (C-7"), 55.4 (CH₃-O).

Compound **2**; everninic acid (**2**), white needles (60.0 mg, 0.03 %) ; m.p. 168 °C; IR (KBr) ν_{max} 3402, 2986, 1620, 1458, 1366, 1265, 1204, 1157, 1034; ¹H NMR (400 MHz,

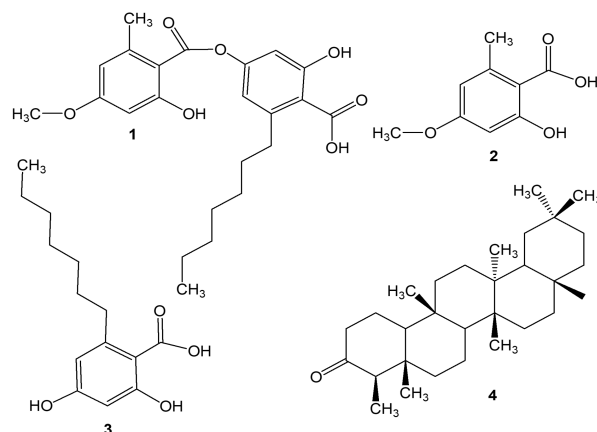


Figure 1. Isolated compounds from *Bunodophoron melanocarpum*. Sphaerophorin (**1**), everninic acid (**2**), sphaerophorol carboxylic acid (**3**) and friedelin (**4**).

$\text{Me}_2\text{CO}-d_6$) δ ppm 2.56 (3H, s, CH_3 -8), 3.83 (3H, s, $-\text{OCH}_3$), 6.32 (1H, s, H-3), 6.36 (1H, s, H-5); ^{13}C NMR (101 MHz, $\text{Me}_2\text{CO}-d_6$) δ ppm 104.7 (C-1), 166.4 (C-2), 98.6 (C-3), 164.3 (C-4), 110.6 (C-5), 143.6 (C-6), 173.3 (C-7), 23.4 (C-8), 54.8 (CH_3 -O).

Compound **3**; sphaerophorol carboxylic acid (**3**), amorphous reddish solid (8.0 mg, 0.004 %); m.p. 109 °C; ^1H NMR (400 MHz, $\text{Me}_2\text{CO}-d_6$) δ ppm 0.89 (3H, t, $J = 6.9$, CH_3 -7'), 1.30-1.35 (8H, m, 4x- CH_2 -, H-3' to H-6'), 1.59 (2H, q, $J = 7.5$, $-\text{CH}_2$ -2'), 2.94 (2H, t, $J = 7.8$, $-\text{CH}_2$ -1'), 6.24 (1H, s, H-3), 6.32 (1H, s, H-5); ^{13}C NMR (101 MHz, $\text{Me}_2\text{CO}-d_6$) δ ppm 103.4 (C-1), 166.1 (C-2), 100.7 (C-3), 162.4 (C-4), 110.7 (C-5), 149.0 (C-6), 173.0 (C-7), 36.3 (C-1'), 31.9 (C-2'), 29.3 (C-3'), 29.2 (C-4'), 31.7 (C-5'), 22.4 (C-6'), 13.4 (C-7').

Compound **4**; friedelin (**4**), white crystals (10.0 mg, 0.005 %); m.p. 190 °C; IR (KBr) ν_{max} 2932, 2870, 1713, 1458, 1389, 1188; ^1H NMR (400 MHz, CDCl_3) δ ppm 0.74 (3H, s), 0.89 (3H, s), 0.91 (3H, m), 0.97 (3H, s), 1.02 (3H, s), 1.03 (3H, s), 1.07 (3H, s), 1.20 (3H, s), 1.76-1.35 (21H, m), 1.98 (1H, m), 2.39 (2H, m), 2.43 (1H, m); EIMS m/z (% rel. int.): 426

(27), 411 (19), 341 (24), 302 (12), 287 (14), 273 (58), 259 (11), 246 (38), 231 (41), 218 (40), 205 (54), 191 (43), 177 (42), 163 (50), 150 (32), 137 (37), 123 (87), 109 (85), 95 (100), 82 (74), 69 (86), 55 (56), 41 (28), 28 (6).

Orsellinic acid-type compounds **2** and **3** could be considered as a possible artefact of the depside sphaerophorin (**1**) as a result of its hydrolysis. To confirm this, a fresh extract was analysed by TLC using **1**, **2** and **3** as standards (Figure 2). Due to the extract presented the presence of standards, it was concluded that **2** and **3** are natural substances, and it is possible that are biogenetic precursors of **1**.¹¹

Antioxidant activity

Free radical scavenging activity

The potency of **EBm** and isolated compounds **1** to **4** as free radical scavengers was determined in terms of EC_{50} expressed as mg sample / mg DPPH• or mol compound / mol DPPH• (Table 1). **EBm** ($\text{EC}_{50} = 0.2430$ mg **EBm** / mg DPPH•) was less potent than positive controls **BHT** and **AA** ($\text{EC}_{50} = 0.0752$ and 0.1118 mg control / mg DPPH•,

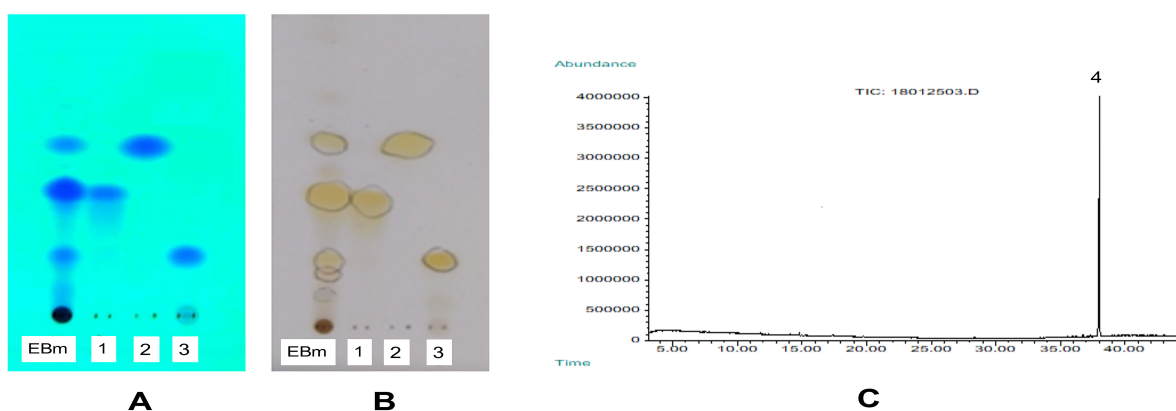


Figure 2. Chromatographic pattern of acetone extract and isolated compounds from *Bunodophoron melanocarpum*. **EBm**: extract, **1**: sphaerophorin, **2**: everninic acid, **3**: sphaerophorol carboxylic acid. TLC (Si-gel 60; *n*-Hex:EtOAc:HCOOH (34:4:1)). **A**: visualized under UV254 light. **B**: stained with H_2SO_4 (10 %) and heating (110 °C). **C**: GC of friedelin (**4**) (ZB-5 capillary column (30 m, 0.25 mm I.D., 0.25 μm), helium (1.1 mL / min); T = 60.0 °C (1.0 min) to 310 °C (7.4 °C / min and was maintained by 10.0 min).

Table 1. Free radical scavenging activity and ferric reducing power of extract of *Bunodophoron melanocarpum* and compounds.

Sample	Free radical scavenging activity			Ferric reducing power (FRP±SD) ^a			
	Potency (EC ₅₀)	Reactivity (k ₂ ±SD)	M ⁻¹ s ⁻¹	Absorbance			
	mg sample / mg DPPH [•]	mol sample / mol DPPH [•]		50 ppm	100 ppm	200 ppm	500 ppm
AA	0.1118	0.2503	22.50 ¹⁵ ± 0.70	ND	ND	ND	ND
BHT	0.0752	0.1345	5.64 ¹⁵ ± 0.12	0.3755± 0.0035	0.5430± 0.0014	0.7420± 0.0325	1.3870± 0.0085
EBm	0.2430	ND	ND	0.0393± 0.0006	0.0490± 0.0035	0.0815± 0.0049	0.1830± 0.0020
1	0.0905	0.0857	0.0188± 0.0028	0.0300± 0.0017	0.0390± 0.0071	0.0623± 0.0015	0.1810± 0.0035
2	0.0953	0.2064	0.0250± 0.0052	0.0263± 0.0046	0.0260± 0.0046	0.0247± 0.0038	0.0283± 0.0035
3	0.1169	0.1828	0.0485± 0.0040	0.0393± 0.0045	0.0442± 0.0059	0.0467± 0.0031	0.0575± 0.0053
4	3.4013	3.1431	0.0035± 0.0004	0.0247± 0.0040	0.0240± 0.0010	0.0283± 0.0015	0.0270± 0.0035

EC₅₀: fifty effective concentration. k₂: second-order rate constant. FRP: ferric reducing power. ^aEach value represents the mean of two or three independent experiments ± standard deviation (sd). ND: Not determined.

respectively) but it was more potent than other lichen extracts (EC₅₀ ca. 1) that have been a source of potent antioxidants.^{15,23,24} Sphaerophorin (**1**), everninic (**2**) and sphaerophorol carboxylic (**3**) acids (EC₅₀ = 0.0905, 0.0953 and 0.1169 mg compound / mg DPPH[•], respectively) were more active than the extract, whereas the triterpene friedelin (**4**) was inactive (EC₅₀ = 3.4013 mg compound / mg DPPH[•]) as free radical scavenger.

Comparing the potency of **1** to **3** vs **AA** and **BHT** (EC₅₀ = 0.2503, 0.1345 mol control / mol DPPH[•], respectively), **1** (EC₅₀ = 0.0857 mol compound / mol DPPH[•]) was the most potent antioxidant since it had the lowest EC₅₀ value.

The reactivity of **1** to **4** and positive controls as free radical scavengers was established based on their second-order rate constants (k₂, M⁻¹×s⁻¹) at 25.0 °C (Table 1), a higher value of k₂ was indicative of a higher reactivity. Compounds **1** to **3** had lower k₂ values than those presented by controls **AA** and **BHT** (*p* < 0.05) being less reactive.

Ferric reducing power

As it can be seen in Table 1, the positive control **BHT**, **EBm**, sphaerophorin (**1**) and sphaerophorol carboxylic acid (**3**) showed a depending on the concentration ferric

reducing power (FRP), reaching their highest values at 500 ppm. **BHT** was the most potent ferric reducing agent (*p* < 0.05) followed by **EBm**, **1** and **3**, whereas **2** and **4** were the least active along the evaluated concentration range.

Inhibition of lipid peroxidation

The percentage of inhibition of lipid peroxidation (% ILP) of **EBm** and compounds **1** to **4** comparing to **BHT** was determined measuring of the amount of peroxide produced during initial stages of oxidation (24 and 48 h at 37.0 °C) (Table 2). A maximum % ILP caused by samples was reached at 24 h. **BHT** and **4** were the most active substances in a range of 50 to 200 ppm (*p* < 0.05).

Photoprotective activity (in vitro)

Sun protection factor as a measure of photoprotective activity against UVB radiation

The results of the determination of the sun protection factor (SPF) are shown on Table 3. The extract (**EBm**), compounds **1** to **4** and controls avobenzone (**AVO**), benzophenone-3 (**BZ-3**) and 2-phenyl-5-benzimidazolesulphonic acid (**PBSA**) showed a SPF concentration-dependent. **PBSA** showed the highest SPF

Table 2. Inhibition of lipid peroxidation of extract of *Bunodophoron melanocarpum* and compounds.

Sample	Inhibition of lipid peroxidation (24 hours) (% ILP ± sd) ^a			
	50 ppm	100 ppm	200 ppm	500 ppm
BHT	45.11 ± 2.21	47.84 ± 2.15	45.64 ± 1.90	47.11 ± 2.65
EBm	32.18 ± 3.57	35.49 ± 0.67	40.38 ± 2.68	53.93 ± 0.02
1	26.18 ± 2.23	31.70 ± 5.13	34.70 ± 2.23	39.12 ± 2.23
2	38.64 ± 2.01	38.33 ± 3.35	37.85 ± 3.12	39.59 ± 3.79
3	31.07 ± 0.67	32.33 ± 5.13	40.38 ± 2.68	47.79 ± 0.67
4	47.00 ± 0.45	52.37 ± 4.02	57.26 ± 2.45	48.26 ± 2.23

^aEach value represents the mean of two or three independent experiments ± standard deviation (sd).

Table 3. Photoprotective activity and dermal permeability of extract of *Bunodophoron melanocarpum* and compounds.

Sample	Photoprotective activity						Dermal permeability	
	Sun protection factor UVB (SPF ± sd)				Sun protection factor UVA ^a		Partition coefficient (P)	Gibbs Free energy of transfer
	10 ppm	50 ppm	100 ppm	200 ppm	λ_{crit} (nm) ± sd	UVA-r* ± sd	Log P ± sd	$\Delta_i G \pm sd$ (kJ mol ⁻¹)
AVO	3.22± 0.08	13.10± 1.28	25.18± 1.32	32.73± 0.47	378.83± 1.47	2.241± 0.070	4.66±0.03	-26.60±0.017
BZ-3	2.95±0.02	17.84±0.81	24.05±1.14	28.55±0.21	354.00±0.82	1.515±0.025	3.48±0.15	- 19.85±0.87
PBSA	10.07±0.15	34.39±3.66	35.27±2.18	39.40±0.09	328.77±0.61	0.463±0.016	0.69±0.26	- 3.96±1.48
EBm	1.76±0.14	9.69±0.08	19.70±0.35	24.80±0.72	330.08±0.38	0.354±0.007	ND	ND
1	3.09±0.36	14.54±0.15	24.44±1.99	25.78±0.53	328.92±0.97	0.381±0.006	7.07±0.64	-40.32±3.67
2	2.24±0.23	11.46±0.09	12.66±0.14	23.90±0.80	315.58±0.58	0.021±0.005	1.71±0.21	-9.76±1.21
3	1.84±0.03	7.81±0.06	15.20±0.11	22.00±1.03	335.25±0.65	0.154±0.001	4.03±0.32	-22.97±1.82
4	0.30±0.04	0.34±0.01	0.47±0.05	0.66±0.02	ND	ND	7.87±0.36	-44.89±2.06

^aCritical wavelength and UVA ratio (UVA-r*) were determined at concentration of 200 ppm. Each value represents the mean of two or three independent experiments ± standard deviation (sd), respectively. ND: Not determined.

values according to UVB photoprotective properties. On the other hand, **BZ-3** showed intermediate SPF values due to its UVA-UVB photoprotective capacity followed by **AVO** (UVA sunscreen).

At low concentrations (10 ppm) the extract, compounds **1** to **3** and controls showed “low” UVB photo-protection (SPF: 2 to 15) whereas for **4** was “null” (SPF < 2). Likewise, increasing the concentration of sample, their UVB photoprotective properties increased. Sphaerophorin (**1**) was the most active substance against UVB radiation along all the evaluated concentrations. Particularly, at 200 ppm, the extract (24.80 ± 0.72), **1** (25.78 ± 0.53), evernic acid (**2**) (23.90 ± 0.80), sphaerophorol carboxylic acid (**3**) (22.00 ± 1.03) and benzophenone-3 (**BZ-3**) (28.55 ± 0.21) showed “medium” UVB photoprotective capacity (SPF: between 15 and 30) whereas for friedelin (**4**) was also “null” (SPF < 2).

Critical wavelength as a measure of photoprotective activity against UVA radiation

The λ_{crit} of the extract (**EBm**), compounds **1** to **3** and controls **AVO**, **BZ-3** and **PBSA** are shown on Table 3. **AVO** (378.83 ± 1.47 nm) was classified in level 4 with the “highest” coverage against UVA whereas **BZ-3** (354.00 ± 0.82 nm) had level 3 or “high” coverage in addition to its UVB protective capacity. **PBSA** (328.77 ± 0.61 nm) had level 1 or “low” coverage against UVA radiation. Evernic acid (**2**) (315.58 ± 0.58 nm) had level 0 or “null” protection against UVA radiation, **EBm** (330.08 ± 0.38 nm) and sphaerophorin (**1**) (328.92 ± 0.97 nm) showed level 1 or “low” whereas sphaerophorol carboxylic acid (**3**) (335.25 ± 0.65 nm) was cataloged at level 2, with “medium” protection against such radiation.

UVA ratio (UVA-r*) as a measure of photoprotective activity against UVA radiation

The UVA-r* of the extract, compounds **1** to **3** and controls

AVO, **BZ-3** and **PBSA** are shown on Table 3. The substances showed different degree of UVA photoprotection, **AVO** presented the highest UVA-r* (2.241 ± 0.070) followed by **BZ-3** (1.515 ± 0.025) whereas **EBm** and **1** (UVA-r* 0.354 ± 0.007 and 0.381 ± 0.006, respectively) and, **2** and **3** (UVA-r* 0.021 ± 0.005 and 0.154 ± 0.001, respectively) showed “moderate” and “too low” protection, respectively against such radiation.

Lipophilicity and skin penetration to lipid-rich stratum corneum of 1 to 4

The partition coefficient (P) expressed as Log P, of **1** to **3** and controls **AVO**, **BZ-3** and **PBSA**, along with their standard molar Gibbs free energies of transfer are shown on Table 3. **PBSA** and compound **2** showed the lowest Log P and $\Delta_i G^0$ values whereas **1** and **4** had the highest values.

Discussion

Solar radiation is vital for the development of life on Earth and provides health benefits. However, it also has deleterious effects particularly on human skin, mainly related to its UV component, which cumulative exposure leads to oxidative stress that eventually causes photo-premature aging and skin cancer.^{1,2} The use of photoprotective substances along with antioxidants in formulations for the skin, is beneficial to slow skin aging and to prevent skin cancer.^{1,2} The incidence of skin cancer is a public health problem.³ Therefore, it is necessary to find alternative sunscreens, preferably from natural origin, with better absorption in the UVA-UVB spectral range, more stable, less toxic and more friendly to the environment than the ones of synthetic origin. In this regard, lichen substances represent an alternative source of compounds for the prevention of skin problems caused by UV-R. Aromatics, mycosporines, melanins, and scytonemins of lichen origin act as UV-filters because they absorb and dissipate such radiation.^{7,8,25}

They also counteract the oxidative stress due to their antioxidant properties.⁶

B. melanocarpum is a lichen found at the páramo ecosystem under life-threatening environmental circumstances such as intense UV-R, indicating its capacity for biosynthesizing effective molecules to defend itself against environmental stress. In this study, the antioxidant and photoprotective properties of *B. melanocarpum* extract (**EBm**) and its isolated metabolites were determined in the pursuit of antioxidant and sunscreen prototypes.

EBm was less potent than positive controls (**BHT** and **AA**) to act as free radical scavenger but, it was more potent than other lichen extracts that have been a source of potent antioxidants.^{15,23,24} To our knowledge, evernicic acid (**2**), sphaerophorol carboxylic acid (**3**) and friedelin (**4**) are reported here for the first time for this species along with sphaerophorin (**1**) reported previously.¹² The orsellinic acid-type isolated compounds **1**, **2** and **3** were better free radical scavengers than **EBm**; therefore, they can be considered as its active constituents. Comparing to positive controls, compounds **2** and **3** were more active than **AA** but less active than **BHT** whereas sphaerophorin was more active. Similarly, to our findings, previous reports have demonstrated **1** scavenges hydroxyl and anion superoxide free radicals.¹³

Comparing the structure of **1** vs **2** and **3** (Figure 1), **1** is a depside formed by orsellinic acid-type entities **2** and **3**. Thus, the presence of these two pharmacophore groups in **1**, could let it form phenoxy free radicals more easily, explaining why **1** would be more potent than **2** and **3**. Although compounds **2** and **3** have an orcinol-like pattern, **3** was more active since its two hydroxyl groups are free and this facilitates the formation of resonance stabilized phenoxy free radicals, opposed to **2**, where one of them is etherified.^{15,23,24}

Brand-Williams *et al.*¹⁴ classified **BHT** as an antioxidant with a slow kinetic behaviour (spending *ca.* 24 h to achieve its maximum activity), and **AA** is one of fast kinetic (spending *ca.* 5 min), therefore, **BHT** is used to preserve formulations against oxidative damage for long time.¹⁴ Since isolated compounds **1** to **3** had lower k_2 values than controls ($p < 0.05$), they were less reactive and showed a slow kinetic behaviour (spending *ca.* 24 h to achieve their maximum activity). Comparing the reactivity (k_2) and potency (EC_{50}) of **1** to **3** with positive controls, compound **1** was more potent but less reactive. Consequently, it would exert an antioxidant effect for a longer time than the controls. In addition, **2** and **3** were more potent than **AA** but less reactive. Hence, they can also be considered as long-lasting antioxidants. Comparing the reactivity and potency of **2** vs **3**, the latter was more reactive and potent ($p < 0.05$) as a consequence to its structural differences already discussed.^{15,23,24}

According to previous studies using DPPH• model, **4** did not scavenge free radicals.²⁶ This is because it lacks a pharmacophoric entity (double or triple bond or a phenolic) capable of scavenge free radicals.¹⁴

The ferric reducing power (FRP) was performed to measure the capability of **EBm**, as well as the capability of **1** to **4** to donate electrons with the aim of preventing or terminating chain reactions of oxidative processes.¹⁶ **BHT** was the most potent ferric reducing agent followed by **EBm**, **1** and **3**. The results of **1** and **3** are consistent with their free radical scavenging activity. Additionally, evernicic acid (**2**) and friedelin (**4**), did not show significant differences ($p > 0.05$) in their FRP and they were inactive along the evaluated concentration range. Sunil *et al.*,²⁷ affirm **4** is a ferric reducing agent in the model used in the current study. Considering that the reduction of ferric ions involves their chelation by phenolic groups,¹⁷ **4** would be unable to reduce such ions because lacks such groups. Likewise, the low FRP of **2** vs **3** can be explained by the structural differences already discussed.^{15,23}

Considering that the exposure of skin to UV-R causes peroxidation in its lipids, the ability of **EBm** and **1** to **4** to avoid the peroxidation of linoleic acid was measured. **EBm**, **1** and **3** showed a depending on the concentration % ILP, reaching their maximum effect at 500 ppm (the highest tested concentration). **BHT**, **2** and **4** showed a non-depending on concentration behaviour, reaching their maximum % ILP at low concentrations (50 ppm). **BHT** and **4** were the most active compounds to inhibit lipid peroxidation (Table 2). In addition, compounds **1**, **2** and **3** (100 to 500 ppm) did not show significant differences on their % ILP ($p > 0.05$) therefore, they had similar inhibitory capability and they were less active than **BHT** and **4**. Consistent with the mechanism to inhibit lipid peroxidation, **BHT** and **4** would transfer hydrogen atoms to linoleic acid peroxy radicals to stabilize them and prevent its oxidative damage. This process could be favoured by the lipophilic nature of these compounds.¹⁷

The photoprotective activity of the extract and isolates of *B. melanocarpum* was evaluated determining their sun protection factor (SPF), critical wavelength (λ_{crit}) and UVA ratio (UVA-r*). The SPF is obtained from the ratio between the minimum amount of energy needed to produce minimally detectable erythema in skin with and without photoprotection.¹⁸ In this study, the SPF was determined *in vitro* by the Mansur *et al.*,¹⁸ spectrophotometric method. This method correlates with *in vivo* tests because it relates to the absorbance of a substance with the erythemogenic effect caused by UVB radiation (λ 290 - 320 nm). Therefore, SPF is a measure of photoprotective activity against UVB radiation.

FDA has set a strong emphasis on developing sunscreens with UVA photoprotection because this radiation (λ 320-400 nm) is associated with skin cancer and photoaging.²⁰ Substances that protect against UVA and UVB radiations are considered as broad spectrum and are preferable as sunscreens. FDA has adopted the pass/fail *in vitro* test critical wavelength (λ_{crit}) as a measure of UVA photoprotective capability. Sunscreens showing a $\lambda_{crit} \geq 370$ nm and a SPF higher than 15 can be considered as a broad spectrum.²⁰ In addition, λ_{crit} can be complemented

with UVA ratio (UVA- r^*) to determine the efficiency as a substance to protect against UVA radiation.²¹

Comparing photoprotective properties of **EBm** and compounds **1** to **4** to controls **PBSA**, **BZ-3** and **AVO** (at 200 ppm); **PBSA** showed the highest SPF value according to UVB photoprotective properties. In turn, **AVO** showed the highest λ_{crit} and UVA- r^* values according to UVA photoprotective properties, whereas **BZ-3**, showed intermediate values on those parameters due to its UVA and UVB photoprotective capacity.

The extract (**EBm**), and compounds **1** to **3**, showed “medium” UVB photoprotective capacity (SPF: between 15 and 30) whereas **4** showed “null” photoprotective capacity. The critical wavelength values of **EBm**, and **1** to **3** evidenced their low level of photoprotection against UVA radiation which was confirmed by their UVA ratios as “moderate” or “too low” protection against UVA radiation. According to FDA,²⁰ the extract and isolates from *B. melanocarpum* can not be considered as broad spectrum photoprotectors since none of them reached a critical wavelength value greater than 370 nm although they showed a SPF value > 15.²⁰

Friedelin (**4**) did not show photoprotective properties because it lacks a chromophore in its structure (Figure 1). As far as we know, the photoprotective properties of **1** to **3** are here reported for the first time.

Considering that the compounds **1** to **3** should be added as ingredients in sunscreen formulations to protect skin surface from UV-R, photoprotective agents should have enough lipophilicity to penetrate the lipid-rich *stratum corneum*, where their effects are desired.²⁸ In this study, the partition coefficient (P) expressed as Log P, of **1** to **3** and controls **AVO**, **BZ-3** and **PBSA**, along with their standard molar Gibbs free energies of transfer ($\Delta_t G^0$), were calculated as a measure of their lipophilicity and permeability through lipidic membranes by a passive diffusion mechanism (Table 3). Lipophilic antioxidants and photoprotective agents having a Log P above 2, with a molecular weight below 600 Da and negative value of $\Delta_t G^0$, would be expected to diffuse into the *stratum corneum* where a reservoir could be established.²⁸ Based on this criteria, **PBSA** and compound **2** would be hydrophilic substances unable of passively diffuse across biological membranes, although they have showed adequate photoprotective or antioxidant properties. In contrast, controls **AVO**, **BZ-3** and the antioxidant and photoprotective compounds **1** and **3** are lipophilic substances that spontaneously would diffuse across the skin surface.

Conclusion

Lichen compounds sphaerophorin (**1**) and sphaerophorol carboxylic acid (**3**) isolated from *B. melanocarpum* are dual agents with antioxidant and UVB photoprotective properties. Additionally, they are lipophilic substances that penetrate to some extent into the lipid-rich *stratum corneum*. Further studies are in progress with these compounds to evaluate their cytotoxicity and photostability.

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Author Contributions

JJA: Isolated the compounds and acquired antioxidant and photoprotective data, NAV and JLR: Conceived the study, designed the experiments, carried out data interpretation, and wrote the manuscript. All authors read and gave approval of the final manuscript.

Ethics Issues

B. melanocarpum was collected following the technical guidelines of the Contract for Access to Genetic Resources and their Derived Products No. 121 (January 22, 2016) given by the Colombian Ministry of Environment and Sustainable Development.

Conflict of Interest

The authors have declared no conflict of interest.

Supplementary Data

Supporting information contains spectral data of **1** to **4** which is available on the journal's web site along with the published article.

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