

Conference paper

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In vitro photoprotection and antioxidant capacity of *Sphagnum meridense* extracts, a novel source of natural sunscreen from the mountains of Colombia

DOI 10.1515/pac-2015-0302

Abstract: Excessive ultraviolet radiation can cause skin cancer and related health problems in humans. Traditionally, organic and inorganic sunscreens have been used to minimize these effects. Besides, some phenolic compounds present in plants play an important role as photoprotectors. *Sphagnum meridense* (L), found in Colombia, is continuously exposed to sunlight on high mountain ecosystems. In this work, we evaluated the potential of *S. meridense* extracts to be applied as UVA-UVB filter in cosmetic formulations and its antioxidant capacity. The mixture acetone-37% hydrochloric acid (1%, v/v) showed the best polyphenol content and UVA-UVB absorption coefficient. These extracts also exhibited promissory UVAPF values, UVA/UVB ratio, critical wavelength (λ_c) and antioxidant capacity *in vitro*, comparable to that of conventional sunscreens.

Keywords: antioxidants; natural sunscreen; Photobiology-16; photoprotection; *Sphagnum meridense*; *Sphagnum* sp.; ultraviolet radiation.

Introduction

The ultraviolet radiation (UVR) of sun spectrum has a decisive role in several processes of the biosphere with beneficial effects in different fields; however, when safety limits are exceeded, the capacity for self-protection of some animal and plant species is destabilized, resulting in cell damage [1, 2]. Additionally, this radiation has a dual effect on plants; it may be beneficial or detrimental to the morphology and physiology of plants, modifying the content of the secondary metabolites and their metabolism [3, 4]. On the other hand, uncontrolled exposure to UV radiation can cause multiple dermal disorders such as skin cancer, erythema, melanoma, photoaging, etc. All of them may stimulate numerous processes associated to premature aging such as depigmentation, folds and distortions [5, 6].

Phenolic compounds are well known for its antioxidant potential and this has been correlated with a photoprotection effect, preventing damage associated with oxidative stress caused by exposure to UVR [7, 8].

Article note: A collection of invited papers based on presentations at the 16th International Congress on Photobiology (ICP-16), Córdoba, Argentina, 7–12 September 2014.

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Therefore, the use of natural extracts is an approach to reduce the UV-generated ROS-mediated photodamage, immune-suppression and skin cancer. In recent years, naturally occurring herbal compounds such as phenolic acids, flavonoids, and high molecular weight polyphenols have gained considerable attention as cutaneous protective agents [9]. Mellegård et al. [10] detected phenolic compounds in *Sphagnum papillosum* such as p-hydroxyacetophenone, p-hydroxybenzoic acid and p-coumaric acid with a demonstrated antibacterial activity. In addition, Rasmussen and Rudolph [11] have detected the presence of *trans*-sphagnum acid (p-hydroxy- β -[carboxymethyl]-cinnamic acid, as the main secondary metabolite in different *Sphagnum* species. Moreover, phytochemistry studies of different species of moss, such as *Sphagnum*, have described the presence of sphagnum acid, a derivative of cinnamic acid, which is chemically related with cinnamates that have been used in sunscreen formulations as organic solar filter [11–13]. According to our knowledge, studies about photoprotection potential and antioxidant capacity of *Sphagnum* spp. have not yet been reported yet. The aim of this research was to evaluate the photoprotective and antioxidant capacity of *Sphagnum meridense* extract, a native moss from high mountain ecosystems of Central and South America. Also, different extraction procedures were tested in order to evaluate their effects in the content of phenolic compounds of each extract [14–16].

Materials and methods

Chemicals and plant material

2,2-Diphenyl-2-picrylhydrazyl (DPPH) stable radical, gallic acid and polysorbate 80 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Ascorbic acid, butylated hydroxy anisole (BHA) and butylated hydroxy toluene (BHT) were acquired from Alfa Aesar (Ward Hill, MA, USA). Folin-Ciocalteu phenol reagent, methanol, ethanol, acetone, hydrochloric acid, acetic acid, sodium carbonate and hexane were obtained from Merck Chemical Supplies (Damstadt, Germany). Carbomer 940 (Carbopol 940), trietanolamine, propylene glycol and glycerin were obtained from LM. Chemicals (Medellín, Colombia). Plant material was collected in September 2013, in Llanos de Cuivá, Santa Rosa de Osos, Antioquia, Colombia at 2730 meters above sea level. (Geographic coordinates 6°49'50.6"N; 75°29'29.9"W). A voucher specimen (HUA190428) was deposited in the Herbarium of the University of Antioquia, Colombia.

Extraction procedure

Fresh vegetal material was dried at room temperature, protected from light. Then dry material (DM) was crushed using an electric grinder (IKA, A11 basic S1). 1.0 g of DM was subjected to extraction with 50 mL of solvent (methanol, ethanol or acetone) acidulated with 0.5 mL of hydrochloric acid or acetic acid at room temperature (ca. 25 °C) with magnetic stirring at different extraction times (Table 1). The crude extracts obtained were concentrated to dryness in a rotary evaporator (IKA, RV10 basic) at 40 ± 2 °C. Finally, dry extracts were re-dissolved in 25 mL of methanol and kept at -4 °C. All extracts and assays were done in triplicate.

Total phenolic contents

The total phenolic contents (TPC) of the samples were measured using a modified colorimetric Folin–Ciocalteu method [17]. Briefly, 100 μ L of extract solution and 525 μ L of deionized water were added to a test tube. Then 125 μ L of Folin–Ciocalteu reagent were added to the solution and allowed to react for 5 min. After that, 1250 μ L of 20 % sodium carbonate solution were added into test tubes and mixed. Then, the absorbance was read at 760 nm using an Evolution 60S spectrophotometer (Thermo Fisher Scientific, Inc., Shanghai, China). The results are expressed as milligrams of gallic acid equivalents per g of dry vegetal material (GAE/g DM), ($y = 0.123x - 2.908E-2$; $r = 0.9998$; where y = absorbance and x = concentration of gallic acid).

Table 1: Extraction conditions of phenolic compounds.

Assay	Solvent	Acid	Time (h)	Assay	Solvent	Acid	Time (h)
E1	Methanol	HCl	2 × 2 ^a	E16	Ethanol	AcOH	16
E2	Methanol	AcOH ^b	2 × 2	E17	Ethanol	HCl	20
E3	Methanol	HCl	4	E18	Ethanol	AcOH	20
E4	Methanol	AcOH	4	E19	Ethanol	HCl	24
E5	Methanol	HCl	16	E20	Ethanol	AcOH	24
E6	Methanol	AcOH	16	E21	Acetone	HCl	2 × 2
E7	Methanol	HCl	20	E22	Acetone	AcOH	2 × 2
E8	Methanol	AcOH	20	E23	Acetone	HCl	4
E9	Methanol	HCl	24	E24	Acetone	AcOH	4
E10	Methanol	AcOH	24	E25	Acetone	HCl	16
E11	Ethanol	HCl	2 × 2	E26	Acetone	AcOH	16
E12	Ethanol	AcOH	2 × 2	E27	Acetone	HCl	20
E13	Ethanol	HCl	4	E28	Acetone	AcOH	20
E14	Ethanol	AcOH	4	E29	Acetone	HCl	24
E15	Ethanol	HCl	16	E30	Acetone	AcOH	24

^a2 × 2: Two-time extraction, 2 h each. ^bAcOH: Acetic acid.

Antiradical capacity – DPPH assay

For each antioxidant extract, different concentrations were tested (expressed as mg of dry vegetal material/mmol DPPH). Extract solution in methanol (0.1 mL) was added to 1.9 mL of a 100 µmol/L methanol DPPH solution. The decrease in absorbance at room temperature was determined at 514 nm after 0, 5, 10 and 15 min, and every 15 min, and so forth, until the reaction reached a steady state or until absorbance declined <10% [18]. Antiradical capacity in a series of concentrations of extract was used to calculate the effective relative concentration (EC₅₀) at which 50% of DPPH had been removed and was expressed as mg of dry vegetal material /mmol DPPH radical, based on eq. 1:

$$EC_{50} = \text{concentration of sample at steady state} / \text{concentration of DPPH}_{t=0} \quad (1)$$

The exact initial concentration of DPPH (95.4 µmol/L) in the reaction system was calculated according to a calibration curve ($y = 1.146E-2x - 4.192E-3$; $r = 0.9999$; where y = absorbance and x = concentration of DPPH) at 514 nm. All experiments were performed in triplicate. All spectrophotometric data were acquired using a Thermo Scientific Evolution 60S UV-Visible spectrophotometer. Disposable cuvettes (1 cm × 1 cm × 4.5 cm) were used for visible absorbance measurements.

UVA-UVB absorption coefficient

Shortly, an adequate dilution of the extracts was added to a quartz cuvette (1.0 cm pathlength) and its absorption spectra (wavelengths 200–400 nm) were acquired using a Thermo Scientific Evolution 60S UV-Visible spectrophotometer against a blank containing methanol. The absorption coefficient (absorbance/mg DM/mL) was calculated at 290, 310, 340 and 380 nm. Benzophenone 3 (B3), butyl methoxydibenzoylmethane (BMDM) and ethylhexyl methoxycinnamate (EHMC) (at 0.017, 0.018 and 0.017 mg/mL, respectively) were used as conventional sunscreen standards.

In vitro determination of photoprotective capacity

According to the UVA-UVB absorption results, the *in vitro* photoprotective efficacy was assessed on the dry crude extract (E29) of *S. meridense*. The E29 extract was dissolved in a mixture of ethanol-propylene glycol-

water (40:30:30) with a final concentration of 50 % (w/w). After that, the solution was incorporated into a gel formulation (see Table 2) by the final concentration of 10 % (w/w). Then, sunscreen product was accurately applied (0.75 mg/cm²) to roughen polymethylmethacrylate PMMA plates (Helioplate HD6, Labsphere, Inc., North Sutton, NH, USA) and was distributed uniformly over the whole surface using a cot-coated finger. Finally, the film was left to equilibrate in a dark place under ambient temperature (25 ± 2 °C) for 15 min. UV transmission measurements (from 290 to 400 nm) were performed using a spectrophotometer equipped with an integrating sphere (UV Transmittance Analyzer UV-2000S, Labsphere, North Sutton, NH, USA). *In vitro* photoprotection efficacy was calculated according to the following parameters: UVB efficacy by estimating sun protection factor (SPF); UVA efficacy by UVAPF and UVA/UVB ratio; and critical wavelength (λ_c). Measurements were read six times for each sample [19–21].

Photostability of sunscreens

The photostability study of the extract E29, was performed using the method of Jarzycka et al. [22]. Plates (see above section) were irradiated for 2 h (every 20 min) with a solar simulator apparatus (Solarbox 1500e; Erichsen, Germany) equipped with a xenon arc lamp (1500 W) and special UV glass filters cutting off radiation below 290 nm. The light source emission was maintained at 650 W/m² in accordance with global solar spectral irradiance. Before and after irradiation, all characteristic parameters of photoprotection of the formulations (SPF, UVAPF, critical wavelength (λ_c) and UVA/UVB ratio) were measured *in vitro*. The degree of photostability was expressed as the percentage of effectiveness after exposure of both protection factors: the SPF *in vitro* (%SPF_{eff}) and the UVA-PF (%UVAPF_{eff}) and was calculated according to eqs. (2) and (3), respectively [23]. Three plates were prepared and the measurements were done nine times for each sample.

$$\%SPF_{\text{eff}} = [SPF_{\text{in vitro}} \text{ after irradiation} / SPF_{\text{in vitro}} \text{ before irradiation}] \times 100 \quad (2)$$

$$\%UVAPF_{\text{eff}} = [UVAPF_{\text{in vitro}} \text{ after irradiation} / UVAPF_{\text{in vitro}} \text{ before irradiation}] \times 100 \quad (3)$$

Statistical analysis

The results were expressed as the means ± SD. All data were analyzed by three-way analysis of variance (ANOVA) followed by Tukey tests, when appropriate, using R Development Core Team (2011), “R: A Language and Environment for Statistical Computing and Microsoft Excel”. *p*-Values < 0.05 (*p* < 0.05) were considered significant.

A factorial study of three factors A (solvent type), B (acid) and C (extraction time) was used to study the A, B, C (main effects), AB, AC, BC (interaction effects of two factors) and ABC (triple interaction) effects. Being *i*, *j*, and *k* the number of levels of the factors used, A, B and C, respectively; and *n* the number of replicates for each treatment. The type of solvent for each extraction was: with level *i* = 1 for methanol, *i* = 2 for ethanol and

Table 2: Composition (% w/w) of gel formulations used for evaluating the photoprotective capacity and photostability.

Ingredient	(%, w/w)
Carbomer 940	2
Trietanolamine	2
Glicerín	2
Polisorbate 80	0.5
50 % dried extract	20
Distilled water, qs	100

$i = 3$ for acetone. Whereas, type of acid, with levels $j = 1$ to hydrochloric acid and $j = 2$ to glacial acetic acid; and extraction time, with levels $k = 1-2 \times 2$ h, $k = 2-4$ h, $k = 3-16$ h $k = 4-20$ h and $k = 5-24$ h were considered.

Results and discussion

Extraction procedure effect on polyphenols content

According to analysis of variance for three-way factor interaction (solvent, acid and extraction time), these were critical with a significance level of 0.05 for all extraction process.

The highest total phenolic content was obtained using methanol and acetone solvents, both acidulated with HCl (Table 3). Moreover, the best extraction time was 16 and 24 h for methanol and acetone, respectively. In addition, the lowest quantity of polyphenols compounds was obtained with ethanol and did not show a significant difference regarding time extraction. This result is in accordance with that reported by Kajdžanoska et al. [24]. The authors compared the extraction yield using acetone and methanol as solvents, and the first one showed the maximum isolation percentage on phenolic compounds. Therefore, the extraction system,

Table 3: *In vitro* antioxidant capacity and total polyphenol contents.

Assay [‡]	% Yield [‡]	EC ₅₀ [‡]	TPC [‡]
E1	11.1±0.9	9.90±0.33	2.47±0.371 ^a
E2	5.9±0.3	128.29±54 ^a	1.36±0.09 ^e
E3	10.1±1.1	10.76±0.50 ^b	1.78±0.45 ^c
E4	5.5±0.2	116.53±26.1 ^a	1.58±0.08 ^c
E5	13.4±0.8	10.93±0.25 ^b	2.89±0.09 ^a
E6	5.8±0.2	97.75±15.20 ^a	0.89±0.12 ^f
E7	11.5±0.7	15.21±0.57	2.09±0.20 ^b
E8	6.4±0.4	115.64±4.05 ^a	1.12±0.17 ^{d,e}
E9	13.2±0.4	9.23±0.15	2.21±0.07 ^b
E10	7.2±0.8	46.07±2.73	1.10±0.120 ^{d,e}
E11	6.0±0.5	13.46±0.29 ^c	1.06±0.11 ^d
E12	3.2±0.2	121.12±28.36 ^f	0.59±0.05 ^h
E13	5.0±0.5	12.46±1.87 ^d	0.89±0.14 ^f
E14	3.3±0.4	153.86±16.4 ^e	0.64±0.03 ^h
E15	7.8±0.6	11.61±1.31 ^b	1.10±0.15 ^d
E16	3.5±0.6	118.91±1.25 ^f	0.61±0.10 ^h
E17	7.7±0.7	12.31±0.89 ^d	1.13±0.03 ^d
E18	3.4±0.2	170.98±12.3 ^e	0.68±0.05 ^{g,h}
E19	8.7±0.9	13.88±0.83 ^c	1.23±0.04 ^{d,e}
E20	4.1±0.4	94.63±7.61 ^a	0.8±0.06 ^g
E21	8.8±0.5	12.68±0.41 ^d	1.29±0.08 ^e
E22	2.5±0.3	175.22±63.5 ^g	0.29±0.04
E23	8.2±0.6	10.54±0.21 ^b	1.01±0.04 ^d
E24	2.7±0.2	153.04±42.2 ^g	0.26±0.03
E25	12.3±1.3	12.01±0.31 ^d	1.67±0.20 ^c
E26	2.7±0.3	94.20±8.60 ^g	0.43±0.04 ⁱ
E27	13.6±1.4	11.47±0.43 ^b	2.22±0.26 ^b
E28	3.5±0.5	72.55±1.70	0.41±0.02 ⁱ
E29	16.6±1.2	12.29±0.50 ^d	2.61±0.15 ^a
E30	4.1±0.2	64.92±0.85	0.60±0.06 ^g

Results are expressed as the mean value ± standard deviation ($n = 3$). ^{a-i}Values in the same column followed by different letters are significantly different at the 5% level. [‡]% on dry extract. [‡]EC₅₀, Efficient Concentration 50 (g dry extract/mmol DPPH). [‡]TPC, Total Phenolic Content (mg GAE/g DM). [‡]Experimental conditions, see Table 1.

acidulated with hydrochloric acid, exhibited the best extraction yield compared to those acidulated with acetic acid. Moreover, acetone would be an advantage over methanol due to its low impact on environmental and pharmaceutical or cosmeceutical applications.

These findings are comparable to those obtained by Li et al. [25], who reported that antioxidant capacity and total phenolic contents from infusions of 223 medicinal plants, were extremely dissimilar, ranging from 0.19 to 101.33 mg of GAE/g DM. Similar results were detected by Polonini et al. [26] on *Lippia* species. Because of the results obtained, the E29 extract was selected for assessing the *in vitro* photoprotective activity and photostability into a gel formulation.

Antioxidant capacity

The DPPH radical assay has been critically for the reaction mechanisms, since the quantitative responses are altered by many environmental factors and the radical site is highly hindered to be easily accessed by complex molecules [27]. Nevertheless, this assay is commonly used for fast screening of antioxidant capacity, because of its stability and ease of use. On the other hand, the UVA radiation induces photosensitivity reactions and dehydration causing a dehydrated and inelastic skin; also, it can produce reactive oxygen species (ROS), which can oxidize proteins, lipids and DNA bases, altered tumor suppressor genes such as p53 and, thus, cause cancer. Consequently, the use of antioxidants could be an effective approach to prevent symptoms related to photo-induced aging and skin cancer [28–30].

The *in vitro* EC₅₀ values obtained were very divergent among extracts (from 9.23 to 170.98 g of dry extract/mmol DPPH). Nevertheless, the extract obtained with HCl-acetone system was better than those acidulated with acetic acid; extract E29 showed the best TPC and an acceptable antioxidant capacity (2.61 mg GAE/g DM and 12.29 g of dry extract/mmol DPPH, respectively) (Table 3). These results were in good agreement with data reported on common essential oils (ranged from 10 to 650 g oil/mmol DPPH), all of them stated as a potent natural antioxidants [31]. Therefore, phenolic compounds detected on these plants could be, in some way, the responsible for scavenging free radical activity and partially contributing to the photoprotection efficacy against UVA-UVB radiation.

According to these findings, the level of lipid peroxidation was measured on extract E29, and was expressed as mmol malondialdehyde (MDA)/kg methyl linoleate (MeLo), using a molar extinction coefficient of 156 000/M cm [32]. As anticipated, the presence of the extract showed a decrease on lipid oxidation of methyl linoleate compared with the blank solution (12.10 and 14.0 mmol MDA/kg MeLo, respectively). Comparable results have been reported regarding the antioxidant methods and their reaction mechanisms based on proton/electron transfer (DPPH assay), as well as by inhibition of lipid peroxidation [33, 34]. Therefore, these outcomes are correlated to total phenolic content and strongly dependent on the method used. Herein, we have shown that the *in vitro* activity values obtained are in close agreement with each other; furthermore, these assays were appropriate for screening purposes.

UVA-UVB absorption coefficient

The absorbances of the treatments with acetic acid were significantly lower than those obtained with HCl acid (Fig. 1b, d, f and h). *p*-Value was 9.466e-12 for solvent:acid:time interaction and a *p*-value of <2.2e-16, 4.3e-11, and <2.2e-16, for solvent:acid, solvent:time and acid:time, respectively. Furthermore, extract obtained with methanol and acetone, both acidulated with HCl, showed the highest absorption coefficient. However, acetone, as the extraction solvent, was highly dependent on time extraction (Fig. 1a, c, e, and g). In addition, a significant correlation between TPC and absorption coefficient values at 290, 310, 340 and 380 nm (R^2 0.9691, 0.9761, 0.9743 and 0.9353, respectively) was observed.

The *S. meridense* extracts exhibited a significant absorbance in the UVA and UVB range comparable to that obtained with conventional filters. This result has been correlated with the presence of polyphenols compounds in the extracts that have demonstrated good absorption of UV radiation [35]. The UV spectra of

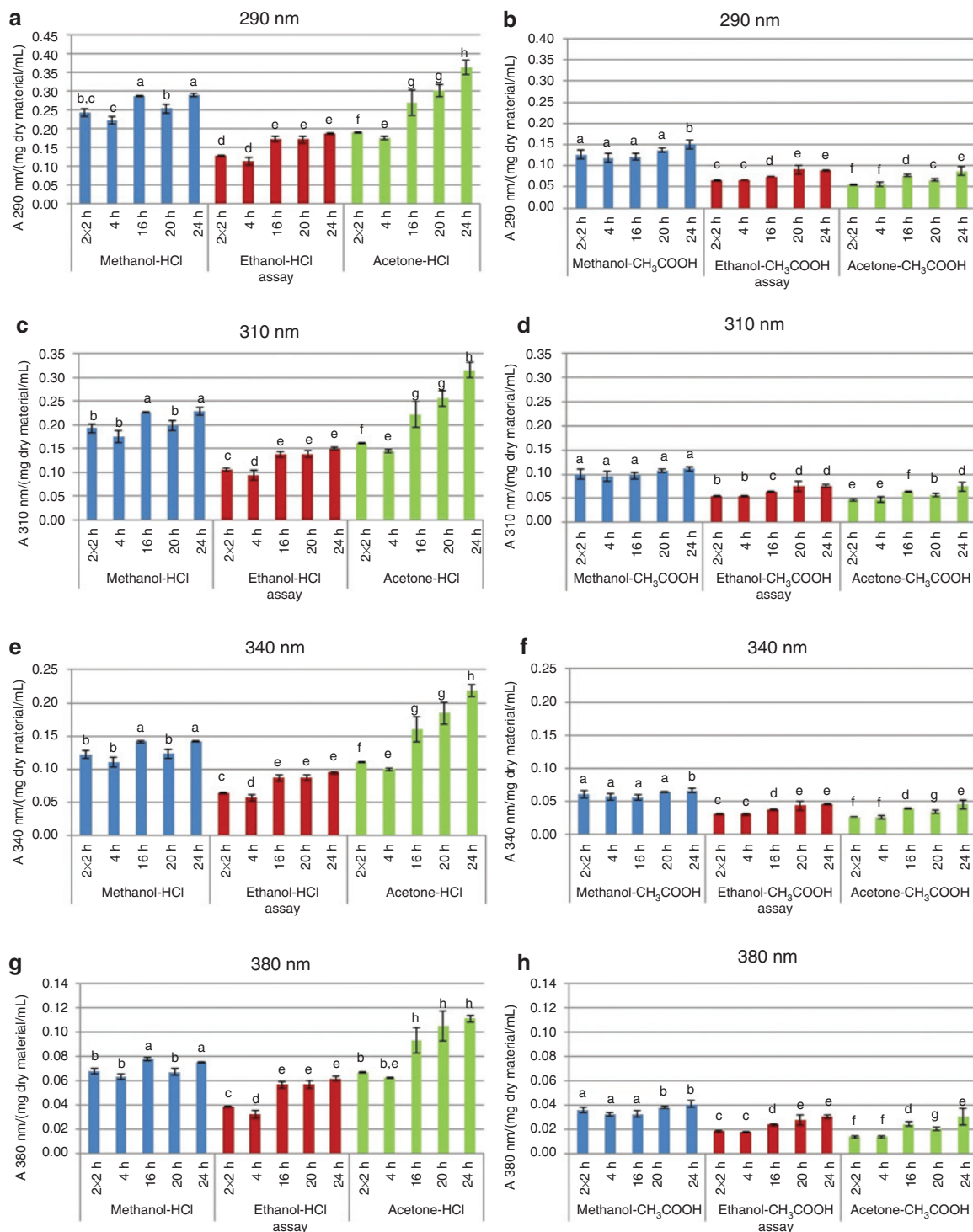


Fig. 1: Solvents, time extraction and acid type interaction on UVA-UVB absorption coefficient. Data are the means of three replicates with standard deviation shown by vertical bars. Bars topped by different letters are significantly different at the 5% level.

the extract obtained by acetone-HCl (E29) showed a relative maximum absorbance in the range 290–330 nm. However, a particular characteristic observed was the stable absorbance at high wavelengths (360–400 nm) for all extracts with respect to the standards. The spectrum of a mixture of three UV conventional sunscreens (B3, BMDM, EHMC) dissolved in methanol and *S. meridense* extracts are shown in Fig. 2. As estimated, the above results will possibly be associate to the intrinsic presence of polyphenols in the extracts, which have

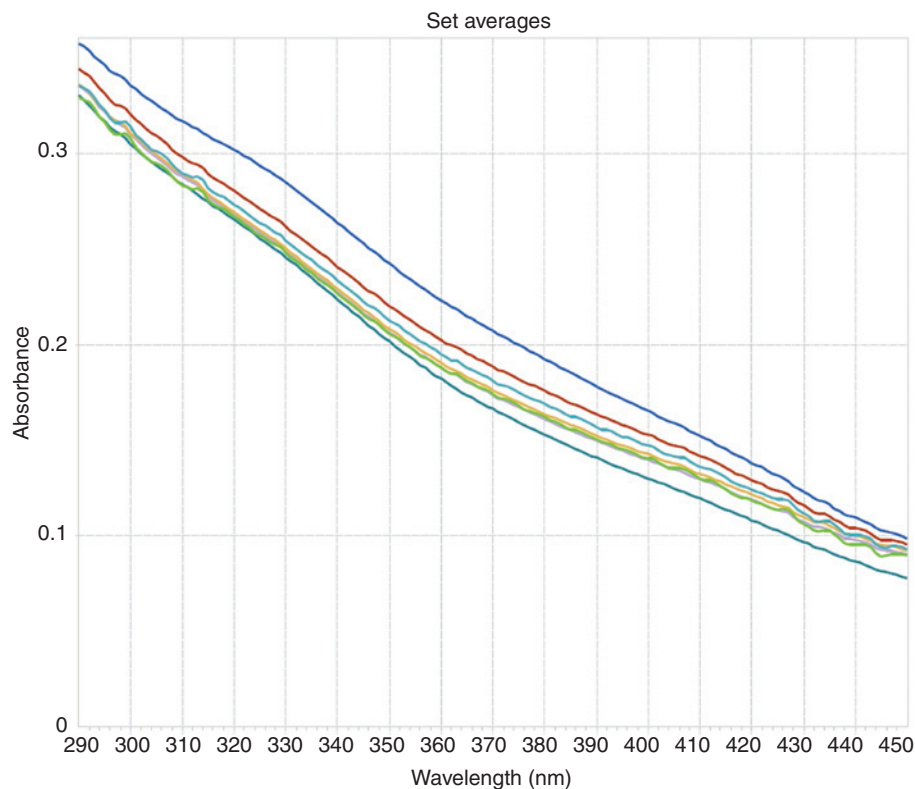


Fig. 3: UVA-UVB photostability profiles of *S. meridense* gel formulation (at 10 % w/w dry extract). The curves represent the average of three PMMA plates and the measurements were done nine times for each plate.

reduce their photoprotective efficacy. Furthermore, it has been found that these organic filters and photolysis products could generate contact dermatitis and photocontact, respectively [37]. Therefore, it is crucial to assess the photostability of sunscreen products to ensure effectiveness and safety. The photostability of the E29 extract was measured using a light source emission maintained at 650 W/m^2 (global solar spectral irradiance at sea level) in accordance with the guidelines of the International Commission on Illumination.

Table 4 shows the percentage of effectiveness after exposure in terms of the UVAPF, UVA/UVB Ratio, λ_c and SPF, where decrease through the time was not significant. A sunscreen product is considered photostable when its $\%SPF_{\text{eff}}$ and $\%UVAPF_{\text{eff}}$ are at least 80 % [38]. Our findings shows that $\%SPF_{\text{eff}}$ and $\%UVAPF_{\text{eff}}$ were higher than 95 %. In addition, the UVAPF in this formulation remained unchanged and λ_c only changed one nanometer. Finally, the UVA-UVB photostability profiles of the *S. meridense* formulation are shown in Fig. 3. The spectrum shows a decreasing in absorbance; however, these variations are not significant and do not affect the SPF and UVAPF values. Moreover, because polyphenolic compounds have properties as antioxidants, photostabilizers and UV filters, the extract evaluated in this research could be improve in order to be suitable for use in photoprotection.

In conclusion, the *in vitro* test of the *S. meridense* extracts showed, that this matrix could be a potential ingredient in association with conventional organic and inorganic sunscreens for application in cosmetic suncare formulations. However, the extract must be improved in order to be successfully employed as photoprotective ingredient in topical cosmetic and health benefits. It should have an adequate broad spectrum of protection against UVA and UVB radiation, comparable to that obtained with conventional sunscreen compounds, in addition to its antioxidant capacity. Moreover, *S. meridense* could be an interesting matrix to study its *in vivo* photoprotection effect.

Acknowledgments: JC Mejía-Giraldo acknowledges doctoral fellowship granted by Colciencias (National Research Council). This work was supported by CODI-University of Antioquia (Project no. IN632CE).

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