

Properties of *Aspalathus linearis* extracts as sunscreen ingredients. A biological filtering system for skin photoprotection

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Abstract

The new horizons in the development of sunscreens are based on the discovery of novel active ingredients from natural sources. The objective of the work was to analyze the potential of a combination of *Aspalathus linearis* (AL) extracts in the enhancement of different biological protection factors of topical sunscreen formulations, as well as their influence in the photostability of standard organic UV filters under simulated solar UV radiation. Various sunscreen formulations were prepared incorporating different concentrations of both non-fermented and fermented AL extracts. The UV transmittance properties as well as different protection factors (solar protection factor, UVA protection factor, immune protection factor) were analyzed by *in vitro* techniques (ISO24443:2022) when formulated alone or in combination with the UVB and UVA standard UV filters Octyl-methoxy-cinnamate (OMC) and Butyl-Methoxy-dibenzoyl-methane (BMDMB). Additionally, the protective potential of the natural extracts against photodegradation was evaluated by applying increasing erythemal doses emitted by a solar UV simulator. Water containing AL extracts showed a significant UV absorbance with an absorption peak around 285 nm. In sunscreen galenic formulas, when combined with OMC and BMDMB increased up to 50% the protection factors of the sunscreen compared to standard filters alone. AL extracts were also able to protect against photodegradation of BMDMB and OMC under increasing radiation doses up to 14.4 standard erythemal doses. In conclusion, the topical application of both fermented and unfermented AL extracts could appear as excellent sunscreen agents, offering a double benefit increasing the protection values against different biological effects as well as protecting against photodegradation.

1 Introduction

Exposure to high levels of UV increases the risk of developing the three main forms of skin cancer [1]. In fact, squamous cell carcinomas and basal cell carcinomas develop almost exclusively on sun-exposed areas of the skin, and estimates suggest that approximately 65–90% of melanomas are also caused by exposure to UV rays. As the occurrence of several types of skin tumors appears to be related to sun exposure, their risk can be reduced by limiting exposure to sunlight, which is the main source of ultraviolet radiation [2, 3]. In this context, the use of topical sunscreens is considered the best preventive measure, especially for parts of the body that are directly exposed to solar radiation. Sunscreens effectively prevent sunburn and are one of the most common practices to prevent skin cancer [4]. In fact, clinical trials have found that sunscreens effectively reduce the incidence of actinic keratosis, squamous cell carcinoma precursors, and the number of moles (the most important precursors and risk factor for melanoma) [4–6].

To protect humans from sun damage, scientists have developed sunscreen formulations containing different filters [7, 8]. Filters are compounds able to absorb and/or reflect specific wavelengths, such as UVA (320–400 nm), UVB (280–320 nm), and visible radiation, thereby preventing the radiation from reaching and penetrating the skin surface and causing damage. As the UV part of the spectrum is considered to have the most deleterious effect in the skin, currently all sun protection offered by

photoprotective products is based either on curbing the generation of skin erythema (mainly due to the UVB band) or the biological effects dependent on the UVA part of the electromagnetic solar spectrum, such as the generation of permanent pigmentation [9, 10]. Nowadays, the high capacity of some filters to absorb UV rays is well demonstrated, and the success and differentiation of the various sunscreens rely on the molecules added to this already established formula. On the other hand, any photoprotector that comes on the market must meet the efficacy and safety requirements dictated by regulatory agencies [10], and the efficacy and safety of most artificial sunscreen components are hindered by their photostability, toxicity, and damage to marine ecosystems [11–13]. Thus, maintaining high efficacy with increasing photostability, along with insignificant toxicity, is an uncovered need of the field and one of the goals of these formulations.

In this scenario, the use of natural compounds in sunscreens is attracting considerable attention. Natural selection and evolution have ensured that plants and animals developed effective protective mechanisms against the harmful effects of UV radiation and the related oxidative stress [14, 15]. Thus, there are substances found in living organisms, for instance, naturally occurring plant products rich in polyphenols, with photoprotective capabilities. Some of those compounds exert their photoprotective activities through their antioxidant effects, controlling skin inflammation, alleviating skin barrier deterioration, and preventing UV-induced aging [14–16]. Hence, the incorporation of natural substances and ethnobotanical antioxidants into sunscreen formulas is on the rise.

Aspalathus linearis (Brum.f) Dahlg. (Fabaceae) (AL), is a fynbos shrub native to the Cape Floristic Region of South Africa [17]. While it is widely consumed globally as an herbal tea, it is increasingly being incorporated into skincare products. Two typical types of AL can be found, known as green and red. The green (unfermented form of AL, ALU hereinafter) has a chemical profile rich in chalcones, including the unique chalcone, aspalathin. The red type (fermented form of AL, ALF hereinafter) results from the oxidative/enzymatic fermentation of green AL form under certain conditions, which alters the plant's chemical profile and reduces the content of dihydrochalcone derivatives, especially aspalathin [18]. Therefore, ALU is preferred for isolating aspalathin and other polyphenolic compounds of interest, which are active ingredients in cosmeceutical products. Extracts from ALU exhibit potent antioxidant, anti-inflammatory, and antitumor properties, with evidence supporting their photoprotective effects on the skin [19–21]. Indeed, different studies demonstrated the protective effects of compounds such as linearthin, aspalathin, and nothofagin from *Aspalathus linearis* against UVB-induced oxidative stress and toxicity in human skin cells [22, 23]. In a recent study by our group, we explored the photoprotective properties of both ALF and ALU, assessed individually and combined. ALF showed higher photoprotective capabilities than ALU in terms of increasing human dermal fibroblast survival, with synergistic properties observed when both extracts were combined [24]. Therefore, AL extracts appear as attractive candidates for exploring their inclusion in topical and systemic photoprotective strategies. Since their role in photoprotection has been studied using cellular models, no references have been reported on their photoprotection capabilities in terms of radiation absorption properties. Plant-based compounds that possess antioxidant, anticarcinogenic, and anti-inflammatory effects, such as flavonoids or other polyphenols, usually can absorb UVA and UVB rays, which contribute to their

photoprotective effect [25, 26]. Apart from flavonoids, other natural products such as certain vegetable oils, carotenoids, stilbenes, and ferulic acid also have UV-absorbing properties [7]. These UV-absorbing properties support their use as UV blockers, and if those are combined with other cellular and antioxidant photoprotective activities, the cosmeceutical potential of the ingredient to be used in sunscreen formulations would be enviable. Therefore, analyzing the optical properties of *A. linearis*, which is enriched with those potentially absorbing substances, is of considerable scientific and practical interest.

Another potential photoprotective role of active compounds of plant extracts can be related to the stabilization of UV absorbing molecules. The photostability of a sunscreen formulation has a profound influence on its performance, as its effectiveness depends on its stable protection attributes for as long as possible on the skin. This is explicitly important considering that the UV filters can experience degradation when exposed to high doses of solar UV radiation. In Europe, the list of approved UV filters remains limited [27], and classically, most sunscreen formulas are based on combinations of UVB and UVA filters, such as octyl-methoxycinnamate (OMC) and butyl methoxy dibenzoyl methane (BMDBM), respectively. Both filters are still the basis of the vast majority of formulas, as they are very effective and inexpensive, but they have very limited photoinstability [28, 29]. Other UV filters, such as octocrylene, have been added to maintain the formula's photostability, but they have been shown to cause photosensitivity pathologies in many patients [30]. So, the search for safer and more effective new filters is still ongoing, and testing natural substances can be of central interest for their potential photostability role, due their radiation absorption properties as well as their antioxidant capabilities.

Therefore, the present study aims to explore the UV absorption properties of *Aspalathus linearis* extracts (fermented and unfermented), their synergistic effects when combined with organic sunscreens such as OMC and BMDBM, and their potential to enhance the photostability of these standard filters under high doses of solar simulated UV radiation.

2 Materials and methods

2.1 Natural Extracts

Unfermented and Fermented controlled hydrophilic extract from the leaves of *Aspalathus linearis* were obtained from lyophilized powder supplied by Rooibos Ltd (Western Cape, South Africa). The extract was stored at room temperature and shielded from light following the supplier's instructions. Stock solutions were prepared at a concentration of 100 mg/ml in distilled water, under agitation at 25–30°C.

2.2 Properties of AL analysis

First, in order to analyze the potential of AL as sunscreen, the 2 extracts (unfermented and fermented) were diluted in distilled water at a final concentration of 6.25 mg/ml and their absorbance in the UV - visible (250-750nm) were measured in quartz UV-transparent cuvette by means of the UV-visible spectrophotometer Shimadzu UV-1607 (Shimadzu Co. Kyoto, Japan).

2.3 Preparation of galenic formulations

A second step was the analysis of AL extracts included in galenic formulations similar to that of cosmetics purposes. All formulations were made using the simplest preparation base to maximize the observation of our compounds of interest. This preparation was a self-emulsifiable O/W “NeoPCL” base (20% w/w), propylene glycol (5% w/w), and distilled water to reach a total weight of 10 g of cream formula, including the extract weight. Each mixture was assayed in different variations: extracts only independently, extracts in combination, filters only or combinations of both extracts and filters. The different combinations are shown in Table 1. The combination of filters used to analyze the booster effect of the extracts was octyl methoxycinnamate for UVB region and butyl methoxydibenzoylmethane for UVA region.

Table 1

Different combinations of extracts and UV filters used for each extract sample in sunscreen.

Sample	Sample Concentration in formulation
ALF	ALF at (1%) and ALF at (2.5%)
ALU	ALU at (1%) and ALF at (2.5%)
ALU/ALF	ALU/ALF combination at (1%) and ALF at (2.5%)
ALF + Filters	ALF (1 and 2.5%) + OMC (4%) and BMDBM (2.5%)
ALU + Filters	ALU (1% and 2.5%) + OMC (4%) and BMDBM (2.5%)
ALU/ALF + Filters	ALU/ALF combination (1% and 2.5%) + OMC (4%) and BMDBM (2.5%)
Filters	OMC (4%) and BMDBM (2.5%)
Blank	Excipient (Neo PCL 20%+PPG 5%+H2O)

2.4 Determination of Protection Factors of sunscreen formulations

The *in vitro* protection factor of each formulation was determined by measuring the spectral transmittance and calculating the absorbance of the formulations across the UV and High Energy Visible Light (HEVL) range (290–450 nm) on PMMA plates (Schönberg, Hamburg, Germany). This process followed the protocol specified in ISO 24443:2022 for evaluating the solar protection factor of sunscreens [31].

This protocol specifies spreading 1.3 mg/cm² of the formulation over a 5x5 cm² PMMA plate. These plates simulate the relief of real skin surface, and are specific for this ISO protocol. After 15 minutes in the darkness where the emulsion was stabilized, the sample was measured in a Shimadzu UV 3101 PC spectrophotometer (Shimadzu, Japan), equipped with three photodetectors covering transmittance and spectral absorbance measurements from 190–3200 nm. The spectrophotometer also includes an

integrating sphere for diffuse transmittance and reflectance measurements (240–2600 nm), in accordance with ISO 24443:2022. The transmittance spectrum was analyzed at 1 nm intervals within the 290–450 nm range, using the spectral transmittance of a blank PMMA plate coated with glycerol.

Sun protection factor (SPF) or protection potential against skin erythema was calculated using the following formula:

$$SPF = \frac{\int_{290}^{400} (E_{\lambda} \times \varepsilon_{\lambda})}{\int_{290}^{400} (E_{\lambda} \times \varepsilon_{\lambda} \times T_{\lambda})}$$

Where: SPF = sun protection factor, E = spectral irradiance of solar simulator, ε = biological action spectrum of erythema (290-400nm), T = transmittance spectrum of the sample.

Following the same procedure as for the calculation of the SPF, UVA protection factor was also calculated by using the action spectrum of Persistent Pigment Darkening as shown in the ISO 24443:2021. In case of protection factors against photo immunosuppression (HIF), the sample transmittance in the UV region was pondered by the action spectra published for the human skin photo immunosuppression [32]. The action spectra data between 290–400 nm at intervals of 1 nm were calculated from cubic spline interpolation between the data points of the respective action spectrum employed to provide values of 1 nm increments, and the integral in the equation was replaced by a summation with increments of 1 nm step. Spline interpolation was made by the software Table Curve 2D 5.0. The error in the interpolation and summation in 1 nm step is estimated to be lower than 5%.

The final protection factor against the different biological effects of the UV radiation in skin was calculated from 3 samples per treatment (4 PMMA plates), in which 3 transmittance spectra were made, obtaining a total of 12 replicas. Following ISO guidelines, protection factors were calculated by the mean value of the 12 replicas, followed by the standard deviation. The confidence interval of 95% must be less than 17% of the mean value to achieve correct protection factors.

2.5 Analysis of the photostability of sunscreens

The photostability tests were assayed by irradiation of the plate's samples and consecutive measurements at the spectrophotometer to observe the reduction of absorbance. Irradiation was carried out employing a solar-simulating setup using an Oriel 1000 W Solar Simulator (Cleveland, USA) with Xenon Arc Lamp with continuous spectrum emission from 290-400nm. The solar simulation spectrum is achieved through the combination of dichroic filters (which eliminate the UVC, part of visible light, and infrared range above 1350 nm), interference filter UG11 (which eliminates total visible radiation), and Schott UG320 for selecting the final solar simulating spectra. Spectral characteristics as well as irradiance emitted were in accordance with ISO 24443:2022.

After initial absorbance spectrum of the samples, PMMA plates were situated under the solar simulator and exposed to an increasing dose of total UV corresponding to 0-3.6-7.2-10.8 and 14.4 standard

erythral doses (SEDs) where 1 SED corresponds to 10 mJ/cm² of UV erythral effective dose [33]. Selected doses were according to normal human skin exposure under sun, which is approximately the number of SEDs that one person would receive after 2 hours of exposure on a typical summer day in mean mediterranean latitudes.

2.6 Statistics

Calculated data on the Protection Factor for various UV-induced skin biological effects (erythema, PPD and HIF), as well as the critical wavelength, was determined based on UV transmittance measurements taken from three different locations on the PMMA plates. Statistics were based on 4 plates per sample (glycerol, base formulation with extract, base formulation with filters, and complete formula with both extract and filters), with each plate receiving 3 measurements from different areas, covering a total surface of 2.8 cm² per plate (complying with the ISO requirement of at least 2 cm² per plate). The final data represents the mean absorbance across different spectral bands, calculated from the 3 sub-replicates of the 4 plates per sample, accompanied by the standard deviation. Mean values are considered valid if the upper limit of the 95% confidence interval falls within 17% of the mean. Percentage of change of different combinations of AL with standard filters with respect to filters alone were compared by means of an One-way ANOVA. The significance level was $p < 0.05$. In case of the photostability experiment for different combinations of AL with UVB and UVA standard filters OMC and BMDBM, the changes in parameters from absorbance curves (mean UVB absorbance, UVA absorbance, as well as changes in biological protection factors) were expressed in terms of % of decay from the initial value. The best fitting model for the curves was polynomic second degree. To analyze the statistical significance of the fitted curves, a Student's t-test for the comparison of the slopes was used, accounting for the homogeneity of the variances. This homogeneity was calculated using the F test.

3 RESULTS

3.1 AL UV absorbance properties

The different concentrations of AL diluted in distilled water produced an increase of absorbance in the UV spectral region with a peak value around 285 that decreased gradually along the UV spectrum (Fig. 1). Due to a slight brownish color of the AL extracts in water, their absorbance in the visible region, up to 450 nm in the range of the High Energy Visible Light (short blue light wavelengths), is significant.

The fermented AL (ALF) showed significantly higher absorption properties in the whole spectrum than the unfermented extract. Finally, a previously reported specific combination of both compounds (ALU/ALF) led to an absorbance slightly but significantly lower than the fermented fraction, yet higher than the unfermented one (ALU). We found that all extracts tested showed very high absorbance in the part of the UV spectrum wavelengths, the part of the solar spectrum that generates both erythema (mainly UVB from 290-320nm) and skin pigmentation (mainly UVA 320-400nm).

3.2 AL booster effect when combined with standard UVB and UVA filters.

For the case of AL formulated as cosmetic sunscreens, their photoprotection potential was analyzed by calculating the spectral absorbance obtained from the transmittance in the region of 290–420 nm. With the obtained data, SPF, UVAPF, and HIF values were calculated. The spectral characteristics were analyzed at 2 different concentrations of the AL extracts (1% and 2.5% in galenic formula for topical application). The absorption spectrum and transmittance spectra of ALF and ALU at 1% is shown in Fig. 2A-B; and at final concentration of 2.5% in Figs. 2C and D. The sunscreen formulas with ALF and ALU, as well as the combination of both, showed a gradual increase in the absorbance along the UV spectrum with maximum values of 0.23 AU for ALF at a concentration of 1% (Fig. 2A), and 0.35 AU of ALF at 2.5% (Fig. 2C).

The combination of filters OMC at 4% and BMDBM at 2.5% increased absorbance up to maximum values of around 1.2 AU in the UVB region (due to OMC absorbance) and with a second peak of 1 AU around 365 nm (due to the absorbance of BMDBM). This combination of standard UVB and UVA filters leads to SPF of 15.43 ± 1.4 , 8.35 ± 1.05 UVAPF, 10.5 ± 1.15 for the human immunological protection factor (HIPF), as shown in Table 2. The addition of 1% of ALU slightly but significantly increased the protection factor of erythema (SPF) to 104%, relative to the value of standard filters alone (100%, Table 2). In contrast, adding 1% of ALF to the filters significantly increased SPF (up to 119%), as well as the other biological factors. When the specific combination of ALU/ALF was added to the filters, the values of protection factors importantly increased with respect to standard filters, although not as high as ALF alone. Interestingly, a role of AL extracts as a booster in photoprotection was clearly observed when combined with standard filters at concentrations similar to them (2.5% in formula). In this case, the protection factors importantly increased, reaching 128% for SPF, 118% UVAPF, and 120% HIPF, when the ALU extract was incorporated into the formula. When the addition of ALF extract was tested, the protection factors increased even more significantly, reaching 176% in the case of SPF, 150% corresponding to UVAPF values, and 156% for HIPF. The combination of ALU/ALF increased protection factors at intermediate levels, higher than ALU but lower than ALF (Table 2).

Table 2

Biological protection factors (SPF, HIPF, UVAPF) as well as the ratio of UVAPF/SPF of the different samples ALU, ALF, and ALU/ALF independly and when combined with solar filters Octyl Methoxy Cinnamate (4% and Butyl methoxy dibenzoyl methane 2.5%). A. linearis extracts were assayed at 1% and 2.5% in galenic formulation combinations. (= significant difference between AL combinations with filters with respect to filters alone (100%) at $p < 0.05$; one-way ANOVA)*

A. linearis 1% in galenic formula				
COMBINATIONS	SPF	HIPF	UVAPF	UVAPF/SPF
ALU	1.35 ± 0.22	1.26 ± 0.15	1.2 ± 0.10	0.89 ± 0.09
ALF	1.59 ± 0.29	1.45 ± 0.17	1.33 ± 0.09	0.90 ± 0.03
ALU/ALF	1.43 ± 0.06	1.35 ± 0.04	1.29 ± 0.03	0.83 ± 0.10
ALU + FILTERS	16.06 ± 2.7	10.63 ± 2.15	8.44 ± 1.83	0.52 ± 0.02
ALF + FILTERS	18.38 ± 5.4	11.88 ± 2.53	9.36 ± 1.66	0.5 ± 0.07
ALU/ALF + FILTERS	17.54 ± 6.2	11.73 ± 3.72	9.34 ± 2.85	0.53 ± 0.02
FILTERS	15.43 ± 1.4	10.5 ± 1.15	8.35 ± 1.05	0.54 ± 0.02
AVERAGE BOOST	SPF	HIPF	UVAPF	
% increment ALU + FILTERS	104.11*	101.28	101.19	
% increment ALF + FILTERS	119.13*	113.2*	112.16*	
% increment ALU/ALF + FILTERS	113.69*	111.72*	111.92*	
Filters	100	100	100	
A. linearis 2.5% in galenic formula				
COMBINATIONS	SPF	HIPF	UVAPF	UVAPF/SPF
ALU	1.53 ± 0.27	1.40 ± 0.18	1.30 ± 0.12	0.85 ± 0.10
ALF	1.95 ± 0.34	1.69 ± 0.20	1.51 ± 0.11	0.77 ± 0.12
ALU/ALF	1.67 ± 0.08	1.53 ± 0.05	1.44 ± 0.04	0.86 ± 0.04
ALU + FILTERS	19.76 ± 3.3	12.60 ± 2.57	9.86 ± 2.2	0.50 ± 0.02
ALF + FILTERS	27.24 ± 6.5	16.35 ± 3.02	12.53 ± 1.9	0.46 ± 0.08
ALU/ALF + FILTERS	22.46 ± 7.4	14.39 ± 4.4	11.27 ± 3.4	0.50 ± 0.02
FILTERS	15.43 ± 1.6	10.50 ± 1.37	8.34 ± 1.25	0.54 ± 0.02
AVERAGE BOOST	SPF	HIPF	UVAPF	
% increment ALU + FILTERS	128.08*	120*	118.16*	

A. linearis 1% in galenic formula				
COMBINATIONS	SPF	HIPF	UVAPF	UVAPF/SPF
% increment ALF + FILTERS	176.52*	155.75*	150.14*	
% increment ALU/ALF + FILTERS	145.57*	137.05*	135*	
Filters	100	100	100	

3.3 Photostability assay

Since the composition of AL extracts is rich in substances with antioxidant potential, we considered that AL extracts could play a dual role in photoprotection. On one hand, as a booster of standard filters, and on the other hand, photostabilizing filters that are known to be unstable. To address that hypothesis, PMMA plates with the different AL extracts at 2.5% combined with or without the same standard of OMC and BMDDBM filters, were exposed to increasing solar simulated UV doses corresponding to 0, 3.6, 7.2, 10.8, and 14.4 Standard Erythematous Doses (SEDs). In Fig. 3, the changes in the absorbance curves for the different samples after exposure to increasing doses of UV radiation are shown. Based on Fig. 3 absorbance curves, the different biological photoprotection factors were calculated, and their changes represented in Fig. 4. In the case of the AL extracts in the absence of the standard filters, both ALU, ALF, and their combination showed a very high photostability performance, with no changes in the absorption properties along the exposure to up to 14.4 SEDs (Fig. 3A, B, C and Fig. 4). In Table 3 the number of SEDs necessary for a decrease of the biological protection factors to 50% from the initial values were calculated using the regression fitting of the curves (second grade polynomic model) obtained in Fig. 4. No doses for biological factors 50% decay could be estimated when AL extracts were exposed in the absence of the filters, reflecting the high photostability of both AL extracts and their combination. In contrast, the exposure of the standard filters OMC and BMDDBM to the same increasing UV doses led to a gradual decrease in the absorbance properties (Fig. 3G). The regression models (Fig. 4) showed that exposure of 6.15 SEDs led to a 50% decrease of initial SPF; a decrease that was achieved with 9.4 SEDs for HIPF and 7.9 SEDs for UVAPF (Table 3). When ALU was added to the standard filters, the stability of the combination increased slightly but significantly (Fig. 3E and 4), and 7.9 SEDs were necessary for a 50% of SPF decay, as well as 12.1 and 9.8 SEDs for HIPF and UVAPF, respectively (Table 3). Higher photostability was offered by the addition of ALF, as can be observed in Fig. 3D and 4, increasing the SEDs necessary for 50% of SPF decay to 14.1 (Table 3). More than the maximal SEDs used in the experiment appear to be necessary for the 50% decay of HIPF and UVAPF (Table 3). The combination of both extracts also provided a significant photostability, close to that offered by the fermented extract (Fig. 3F, 4 and Table 3).

Table 3

Number of Standard Erythral Doses applied to the different samples containing ALU, ALF, and ALU/ALF independently and when combined with the solar filters Octyl Methoxy Cinnamate (4%) and Butyl methoxy dibenzoyl methane (2.5%) necessary for the 50% of decay of the different Biological protection factors (SPF, HIPF, UVAPF). Data are extracted from second degree polynomial fitting regression lines of the BPF decay curves of Fig. 4. (= significant difference between AL combinations with filters with respect to filters alone at $p < 0.05$; one-way ANOVA)*

Number of SEDs for 50% decay of BPFs			
COMBINATIONS	SPF	HIPF	UVAPF
ALU	> 14.4	> 14.4	> 14.4
ALF	> 14.4	> 14.4	> 14.4
ALU/ALF	> 14.4	> 14.4	> 14.4
ALU + FILTERS	7.9*	12.6*	9.8*
ALF + FILTERS	> 14.4*	> 14.4*	> 14.4*
ALU/ALF + FILTERS	10.5*	> 14.4*	> 14.4*
FILTERS	6.15	9.4	7.9

4 Discussion

The present study demonstrates that all *Aspalatus linearis* (AL) extracts tested, including fermented (ALF), non-fermented (ALU), and their combination (ALU/ALF), exhibit enviable optical properties supporting their consideration for the development of future photoprotective formulas.

Initially, a broad UV absorption spectrum increasing gradually from UVA up to UVB, with the highest absorption peak around 285 nm was observed for AL water extracts. This effect of natural extracts has also been reported in our previous work [12, 42], and could be due to the presence of UV-absorbing phytochemicals within the plant tissue [34, 35]. One of the most probable groups of compounds contributing to the observed effect is flavonoids. Flavonoids possess a typical UV-VIS spectra including two bands, corresponding with the absorption of the B ring (band I: 310–385 nm), and the A ring (band II 250–285 nm) [36]. They are demonstrated natural UV filters [15, 41], and some of them have been tested against keratinocyte damage in experimental settings [22]. The water extracts of AL contain high concentrations of different phenolic compounds known to absorb UV radiation, including 3 dihydrochalcones (aspalathin, nothofagin, and linearthin), and other flavonoids such as orientin, isoorientin, quercetin, and luteolin [34]. Given their structure, the presence of the C-glycoside dihydrochalcones could confer a high absorption peak at 286 nm, while the presence of rutin, quercetin, or other flavonols could explain the UVA peak at 353 nm. Interestingly, our data showed that the UV

absorbance of ALF was higher than that of ALU. It is well known that during the plant processing (known as fermentation), the dihydrochalcones are rapidly oxidized by nonenzymatic mechanisms to flavanones and polymeric brown products [37] [38, 39] [40], then noticeable browning takes place [18]. Therefore, the appearance of the oxidized forms from the pool of flavonoids and hydrochalcones may change the absorption properties of the extracts, increasing ALF UV absorbance. It is worth noticing that the autooxidation process decreases the antioxidant potential of the extracts, underlaying the lower antioxidant capability ascribed to ALF vs ALU [24, 41]. Thus, considering the important benefits attributed to antioxidant ingredients in sunscreens (ref), plus the recent synergistic biological photoprotective effect shown by a specific combination ALU/ALF (ref), we decided to include the spectral properties analysis of that combination. As expected, we found intermediate absorbance properties, lower than ALF but significantly higher than ALU, which would support the use ALU/ALF combination, to take advantage of maintaining high antioxidant capacities, optimal absorption properties, and the synergistic biological photoprotective effect.

We are in a new era, where we aim natural compound combination providing the sunscreen formula absorption properties as “real biological” filters for UVB and UVA bands of the solar spectrum. Walking towards the practical inclusion of AL extracts in commercial sunscreens, our next step was to assess the performance of the extracts when incorporated into real sunscreen formulas, tested their absorption properties following the international standards outlined in ISO 24443:2022, and calculated SPF, UVAPF, and HIF values. SPF and UVAPF are reference parameters commonly tested in sunscreens, and account for the specific spectrum of action for erythema and permanent pigmentation darkening, respectively. However, Damian et al. 2011, determined 2 peaks of UV radiation (310 and 370nm), key for immunosuppression, which is one of the most relevant factors in the steps previous to skin carcinogenesis [32]. Then, a biological protection factor against human photoimmunosuppression can be calculated (named as HIF). Since natural products included in cosmetics (e.g. *Polypodium leucotomos* extracts) have been reported as immunomodulators [42]; and AL extracts show a broadband absorption covering other biological factors beyond erythema and permanent pigmentation darkening, we decided to include HIF analysis in this study. AL extracts, individually and combined, showed a significant absorbance increment from UVA to UVB, leading to SPF values close to 2 at concentrations of 2.5%, reinforcing their properties as radiation filters. Few works in the literature have reported the absorption properties of phenolic compounds for photoprotection purposes. For instance, quercetin and especially rutin offer high UV absorption potential, reaching SPF values above 35 [49], and total polyphenols extracted from some plant leaves can achieve SPF values above 20 [50]. The traditional herbal formulation, Ubtan, based on different plant seeds (rich in flavonoids), can reach SPF values above 30 [51]. Although those works showed higher SPF values, it is worth mentioning they used methanol and other organic extractions, which yield much higher concentrations. Conversely, the water extractions used in this work are considerably less toxic, affordable, and environmentally sustainable, which represents a strong advantage for their industrial implementation. Additionally, those works did not include the extracts in galenic formulations or test the regulated standards for real sunscreens. Therefore, the results presented here demonstrate not just the enviable broadband absorbing properties of aqueous extracts

of AL, but also its performance in complex formulations analyzed under regulated standards, which strongly support and facilitate its practical inclusion as natural filters in current sunscreens.

Interestingly, the most surprising results were obtained when AL extracts were combined with other filters (OMC and BMDDBM). The addition of 2.5% ALF was able to increase the SPF of the reference filters from 15.43 to 27.24, an increase of 76.52%. Similarly, the UVAPF increased 50% and the HIPF increased 56%. ALU extract showed lower values than ALF, yet conferred a very important increase in all biological protection factors, and the combination ALU/ALF showed intermediate photoprotection factors closer to ALF (increment of 45,57% SPF, 35% UVAPF, and 37.5% HIPF). Given the potential adverse effects of certain organic filters on human health and the environment, the incorporation of natural products into cosmetic formulations is a growing trend, aiming to reduce reliance on high concentrations of synthetic UV filters [52]. In this scenario, this work demonstrates a booster effect on commonly used synthetic UV-filters when AL is included in the formula. This would allow reducing the amount of filters used, maintaining the efficacy of the sunscreen while improving safety and sustainability.

Finally, the additional role of the AL extracts as photostabilizers was demonstrated. It is well known that the combination of BMDDBM and OMC, with very extended use as UVB and UVA filters in commercial sunscreens, is photo-unstable. This process is initiated by BMDDBM, which after absorbing UVA radiation, reacts with OMC to form cycloaddition products [53]. To palliate this effect, other molecules are included in sunscreen formulations not just as UV filters, but as stabilizers. This is the case of bisethylhexyloxyphenol methoxyphenyltriazine (tinosorb S) [54], methylbenzylidene camphor [55], diethylhexyl 2,6-naphthalate [56], or octocrylene (ref). However, in the current scenario where synthetic filters are under scrutiny, octocrylene, due to its toxic potential in humans and animals, is currently being phased out by industry, justifying the need for alternatives [30]. As the light-induced degradation mechanism of BMDDBM and OMC proceeds through the initial formation of free radicals and singlet oxygen [57, 58], scavenging these reactive species by antioxidants has been proposed as an approach for photostabilization [59, 60]. In this context, triplet-triplet energy transfer is an important photostabilization mechanism for organic UV filters [54], and the stabilizing potential of flavones and flavonoids, including quercetin, has been described [61] [62]. In this study, both AL extracts, especially ALF and the combination ALU/ALF, were able to significantly prevent the absorbance decrease of the reference filters under UV radiation. This importantly reinforces their double role, not just as filtering ingredients but as alternative photostabilizers.

In conclusion, the evidence presented herein highlights the promising potential of natural extracts as components for advanced solar photoprotection. Up to now, none of the commercially available sunscreen molecules presents ideal behavior as a photoprotection ingredient, which implies very high absorption properties in the UV region, high photostability, and negligible toxicity or phototoxicity. AL extracts combination demonstrated in the present work that they can play this role, acting as biological filters and photostabilizers, allowing the development of future topical photoprotective agents based on natural sources, effective, and safer for human health and the environment, which in current times could potentially enhance user adherence to the recommended photoprotection protocol.

Declarations

Competing Interests

JA, PS and MVG received a grant for the present research project. S.G. has a consultant role for Cantabria Labs, ALS and LH. belong to the Innovation and Development Department at Cantabria Labs SA.

Author Contribution

J. A.: Writing – original draft, Conceptualization, Methodology, Investigation. P. S.: Writing- original draft, Investigation. A.L.S.; Methodology, Investigation, Review & editing. L. H.: Conceptualization, Review & editing. S. G.: Review & editing. M. V.G.: Review & editing.

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Data availability statement:

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figures

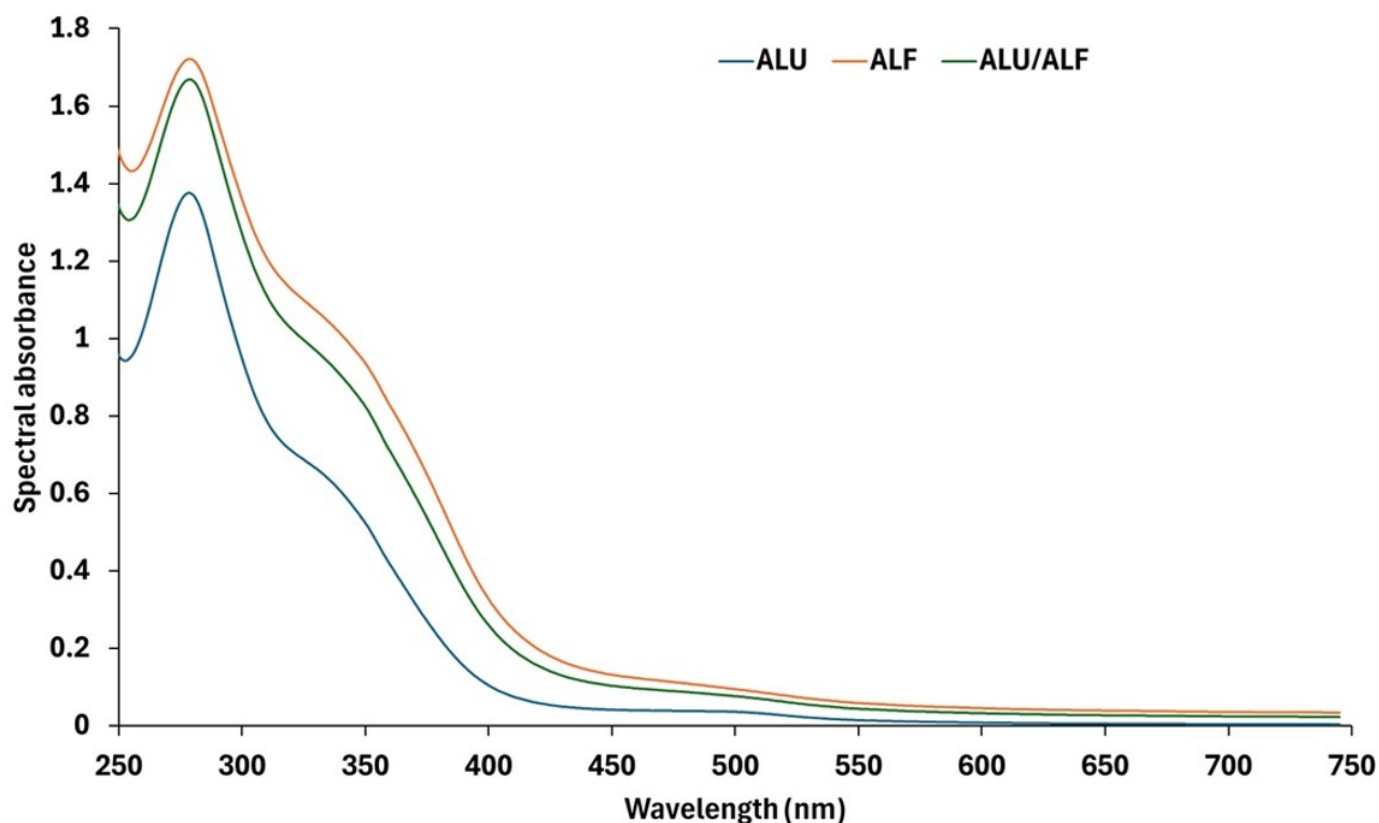


Figure 1

Absorbance spectrum of the *A. linearis* fermented (ALF), unfermented (ALU), and a specific combination of fermented and unfermented extracts of ALU/ALF in the UV and the visible spectrum range (250-750nm).

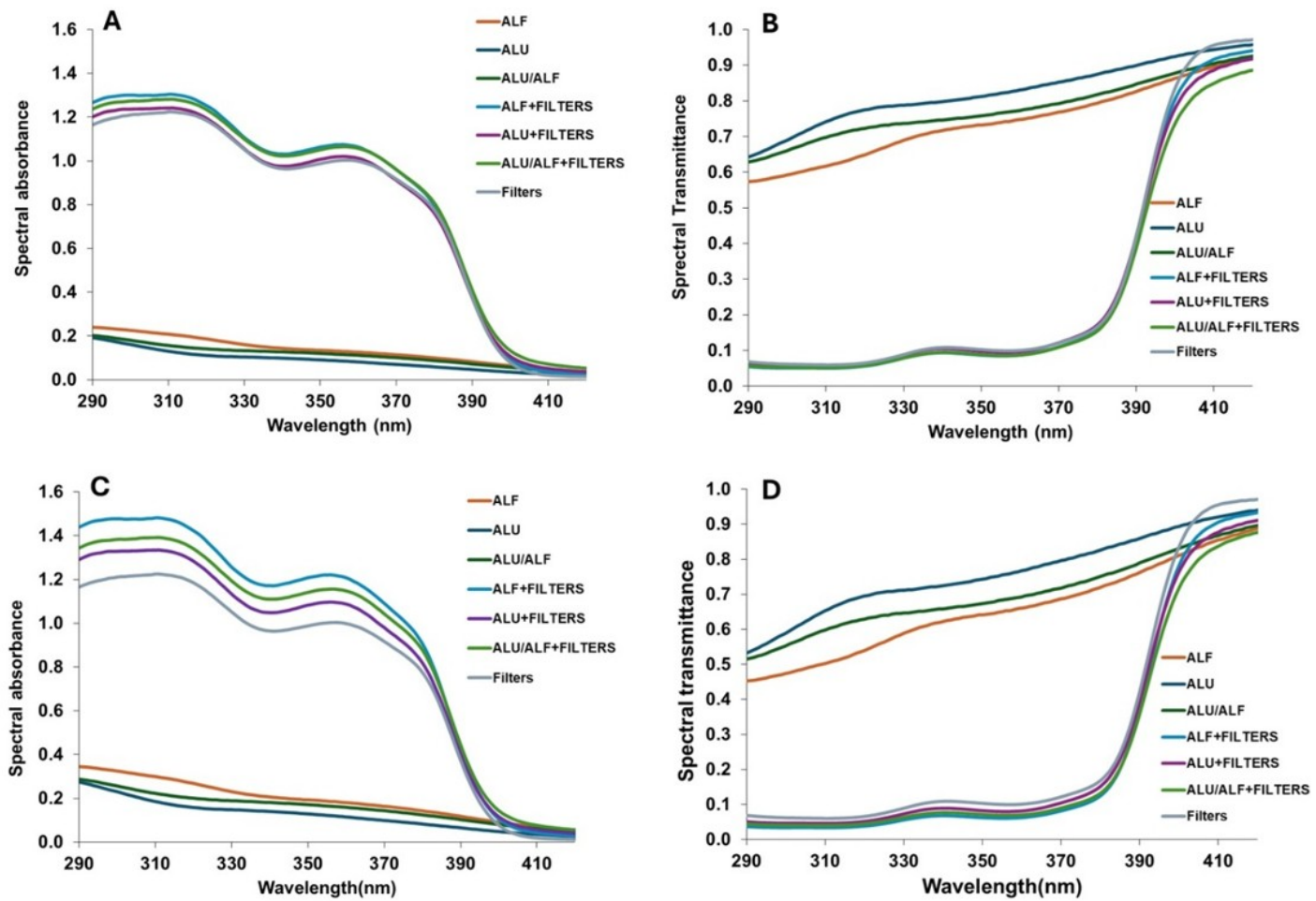


Figure 2

Spectral absorbance and transmittance of ALF, ALU, and ALU/ALF at 1% (A-B) or 2.5% (C-D), alone and when combined with the solar filters Octyl Methoxy Cinnamate (4%) and Butyl methoxy dibenzoyl methane (2.5%).

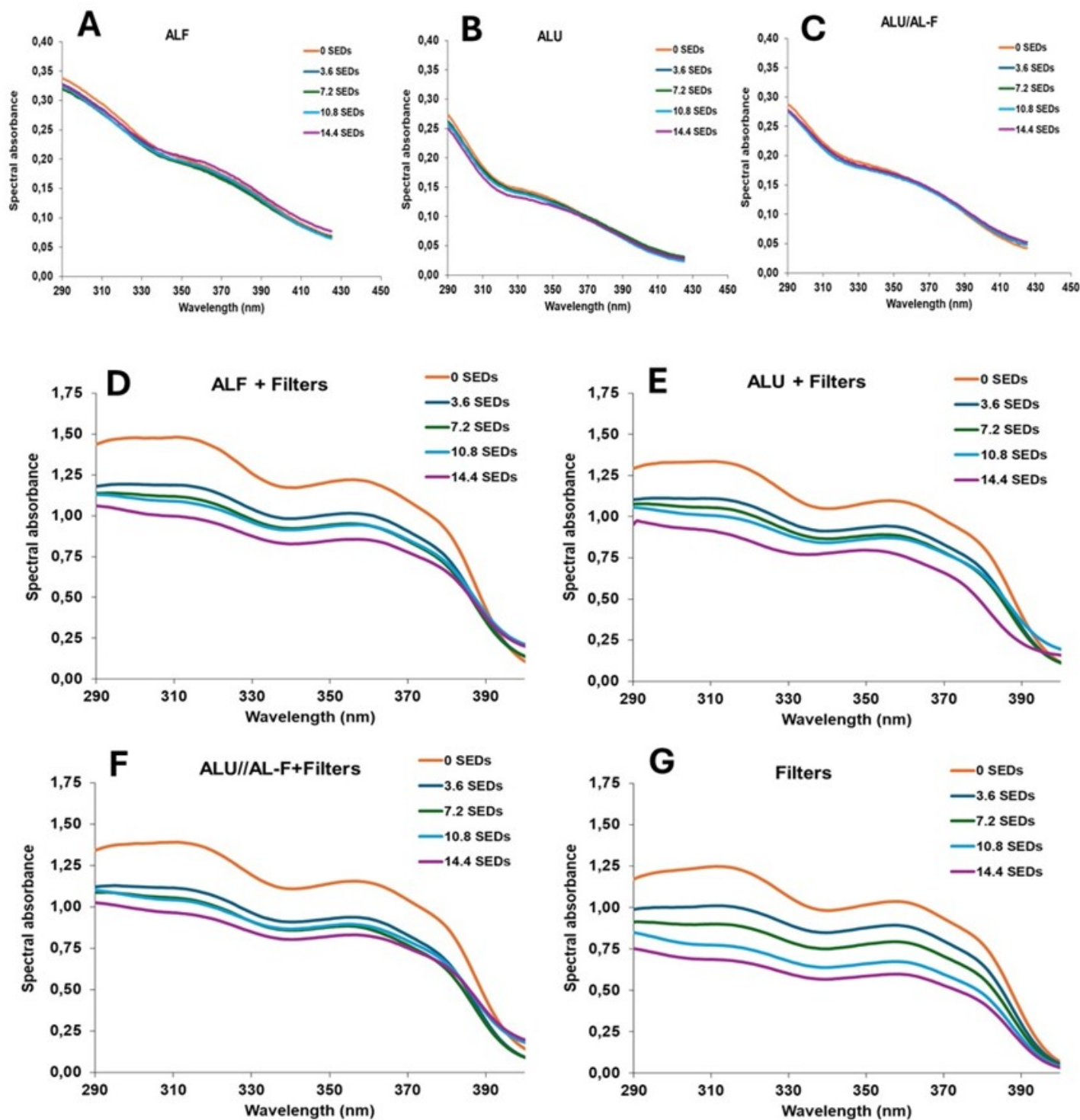


Figure 3

Photostability assays. PMMA plates with the different extracts and standard filters were exposed to increasing doses of solar simulated UVB +UVA from 0 to a dose corresponding to 14.2 Standard Erythemal Doses (1 SED= 10 mJ/cm²). Then UVB and UVA absorbance means are represented. A-C) Spectral absorbance changes ALF, ALU, and ALU/ALF at 2.5% alone, and (D-G) when combined with OMC (4%) and BMDBM (2.5%).

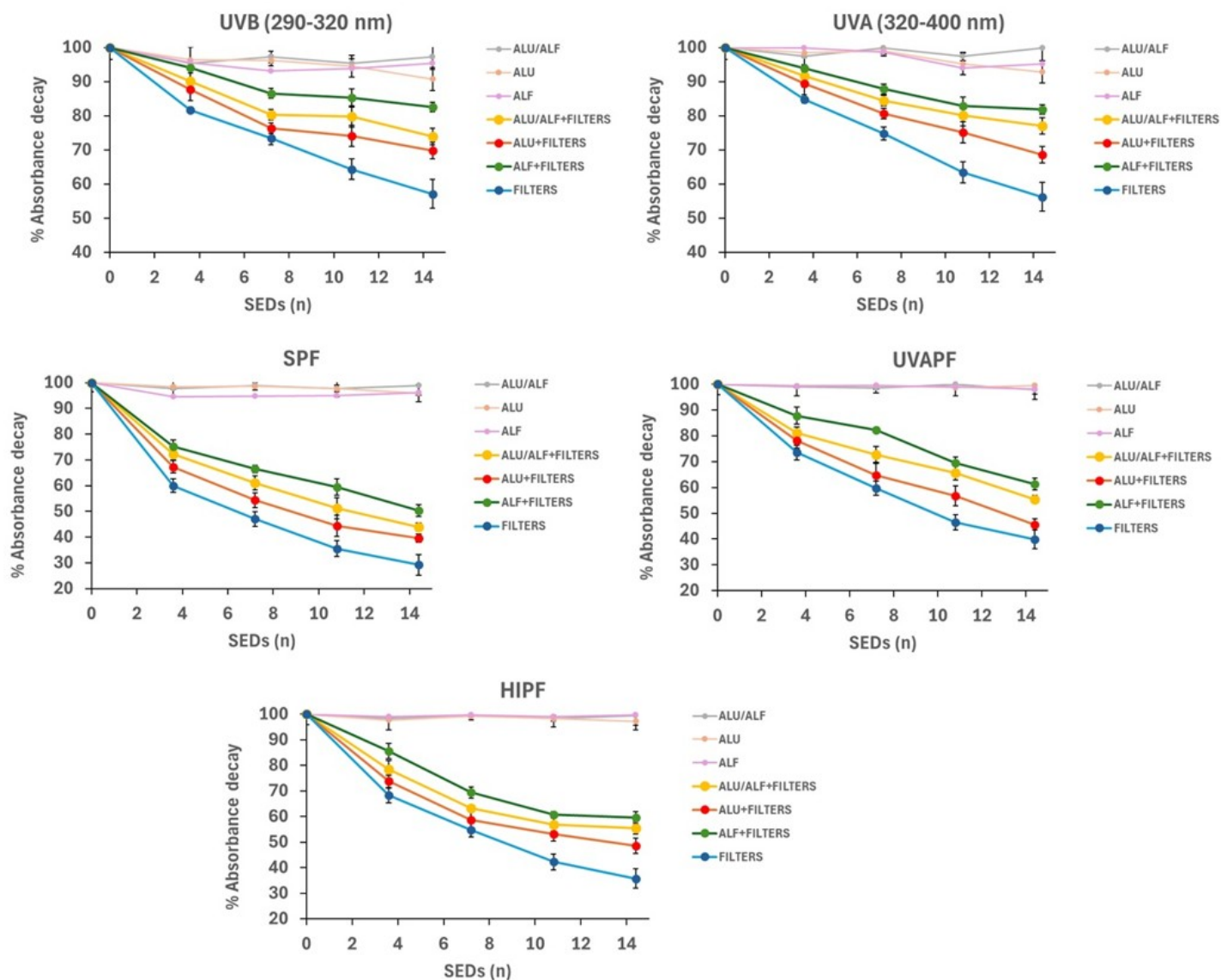


Figure 4

Photostability assays relative to photoprotection factors. Changes of the different biological photoprotection factors calculated from the absorbance curves of Figure 3, for each point of the curves of ALF, ALU and ALU/ALF at 2.5% alone as well as combined with OMC (4%) and BMDBM (2.5%). Results are expressed as mean percentage of absorption and photoprotection factors decay $\pm$ standard deviation.