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From sugarcane to skin: Lignin as a multifunctional ingredient for cosmetic application

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ABSTRACT

Lignin has been suggested as a promising candidate for cosmetic applications due to its remarkable potential to absorb ultraviolet rays and distinctive antioxidant activity. This study aims at evaluating the performance of lignin from sugarcane bagasse (SCB) as natural UV blocker, antioxidant, and pigment. Lignin was extracted from SCB, characterized and incorporated into a blemish balm (BB) cream. The biological potential, concretely, *in vitro* and *in vivo* sun protection factor (SPF) and *in vitro* UVA-PF, and safety were assessed. A high-purity SCB lignin (>92 %) was obtained by a mild alkaline extraction process. The results of cytotoxicity, mutagenicity, skin sensitization and *in vivo* acute cutaneous irritation demonstrated that SCB lignin is safe for topical applications. Lignin showed capacity to scavenge both ABTS and DPPH radicals, which were preserved after its incorporation into the cosmetic formulation. Notable results were achieved in terms of *in vitro* and *in vivo* SPF of 9.5 ± 2.9 and 9.6 ± 0.8, respectively. Furthermore, the tested lignin-based BB cream revealed a broad-spectrum UV protection (critical wavelength of 378 ± 0.5 nm). These results suggest SCB lignin as multifunctional and safe ingredient for use in cosmetic products.

1. Introduction

Chronic exposure to ultraviolet (UV) light can cause deleterious effects on human skin. For this reason, daily use of sunscreen is highly recommended to reduce the incidence of skin cancer and premature skin aging, and the application of products containing one or more UV filters to achieve broad-spectrum protection is encouraged [1,2]. Sun protection is arguably one of the most important steps of skincare and, based on that awareness, nowadays many cosmetic products have UV filters in their composition, such as, blemish balm (BB) creams, foundations, and moisturizing creams. However, despite the beneficial effects of these products, the safety of some UV filters has been discussed [3,4]. UV filters are classified as inorganics and organics, depending on their mechanism of action: inorganic filters reflect and scatter the UV radiation, while organic filters absorb the UV radiation. Zinc oxide and titanium dioxide - two inorganic UV filters - are generally recognized as safe and effective (GRASE). Regarding the organic UV filters, some concerns linked to their safety have been reported [4]. Studies have revealed the detection of UV filters in human biological samples, such as in urine and blood (particularly, benzophenone and cinnamate derivatives [5]) and in marine organisms, where organic UV filters were found in wild mussels [6].

Human skin exposure to UV radiation results in a cascade of biochemical reactions, including depletion of antioxidant enzymes, thus causing a dramatic increase in the production of reactive oxygen species (ROS) associated to skin aging, pigment darkening and even skin cancer [7,8]. Therefore, compounds with the capacity to prevent or reduce ROS production and, simultaneously, protect from UV radiation are of prime interest for skincare products [9]. Clear, strong and well-documented data supports the potential of lignins from different biomasses as natural antioxidants and UV blockers [10–12].

Lignin is one of the most abundant aromatic polymer on earth, available in lignocellulosic biomass. It is composed of phenylpropane units, including *p*-coumaryl alcohol, coniferyl alcohol and sinapyl

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alcohol, forming a complex and heterogeneous structure [13]. The variety of functional groups in lignin structure provides a wide range of properties with application in different fields. Recent studies elucidate the importance of lignin for energy [14], materials [15,16], biomedical and biotechnological [17–19], and cosmetic applications. For instance, the high structural flexibility of lignin, its molecular structure and the presence of hydroxyl groups are interesting to produce thermoelectric and carbon-based materials [14]. For the biofuels field, lignin represents an opportunity due to its high energy density, related to its structure and the presence of aromatic units [14]. On the other hand, the incorporation of lignin in construction materials contribute to the improvement of mechanical, thermal and biodegradability properties [16]. The application of lignin as a carrier of active substances in biomedicine, for drug delivery systems and tissue regeneration, as well as for controlled release, is due to its low solubility, pH-responsive chemistry, UV-blocking effect and high stability [17]. In addition to that, lignin has attracted the interest due to its antioxidant and UV blocker properties [12,20–23]. The radical scavenging properties of lignin are mainly attributed to the existence of free phenolic hydroxyl groups and ortho-methoxyl substitution in aromatic rings in its structure [12,20,22,24], while the UV chromophoric groups (phenolics, hydroxyl groups, double bonds, and carbonyl groups) are responsible for its absorption capability in the UV/visible regions [23]. The application of lignin as sun blocker is considered advantageous not only for its ability to improve the SPF value in formulations, but also for conferring photostability to conventional UV filters [25].

Therefore, the aim of this study was to investigate the potential of lignin from sugarcane bagasse (SCB) as multifunctional ingredient in a cosmetic formulation, thus reducing formulation costs. To achieve this goal, lignin was firstly extracted from SCB using an alkaline pretreatment, followed by physico-chemical characterization. The safety assessment and biological potential (antioxidant and UV blocker) of the active ingredient (SCB lignin) was assessed. Taking advantage of the lignin color, a Blemish Balm (BB) cream was originally formulated, containing SCB lignin as multifunctional ingredient, acting as pigment, antioxidant (*in vitro* methods), and UV blocker (*in vitro* and *in vivo* methods).

2. Materials and methods

2.1. Materials

Sulfuric acid (95.0–98.0 %), 2,2-diphenyl-1-picrylhydrazyl (DPPH, 98 %), 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS, ≥98 %), potassium persulfate (≥99.0 %), butylated hydroxytoluene (BHT, ≥99 %), L-lysine, cinnamic aldehyde, and cysteine were obtained from Sigma-Aldrich. Sodium hydroxide pellets were purchased from Labchem. Polystyrene molecular weight standards were acquired from Agilent. *N*,*N*-Dimethylformamide (≥99.9 %), acetonitrile (≥99.9 %), formic acid (≥99.9 %) were purchased from VWR. Lithium chloride (≥99 %) was obtained from Merck. TrypLEX express enzyme Dulbecco's Modified Eagle Medium (DMEM, GlutaMAX™), fetal bovine serum, pen-strep and dimethyl sulfoxide (DMSO) were purchased from Gibco. Human keratinocyte cell line (HaCAT) and PrestoBlue cell viability reagent were obtained from Thermo Fisher Scientific. *Salmonella typhimurium* containing a deletion mutation for histidine (His), with the following mutations: the type frameshift (TA98), base-pair substitution (TA100) and reversion/transversion (TA102), 2-Aminoantracene, ampicillin were obtained from Moltox. Nutrient broth-2, and phosphate buffer were obtained from Oxoid. Liver homogenate, S9 (Aroclor 1254-induced Sprague Dawley rat liver) was purchased from Xenometrix. Trifluoroacetic acid, ethanol absolute (≥99.8 %), chloroform (≥99 %) and methanol (≥99.9 %) were purchased from Honeywell. Nitrobenzene (≥99 %) was obtained from Acros Organics. Glycerin and caprylic triglyceride were obtained from Guinama. SOLAGUM® AX was acquired from M.O.S.S.A. Dermofeel® PA-12, Phenethylacohol nat., Tego® Care PBS 6 MB were obtained from DS Produtos Químicos. Geleol MB was obtained from Gattefossé. Shea butter was purchased from Acofarma.

2.2. Extraction of lignin

SCB, composed of 26.4 ± 0.8 wt% lignin, 41.6 ± 2.2 wt% cellulose, 21.6 ± 0.3 wt% hemicellulose, and 3.0 ± 0.9 wt% ash, was mixed with 2 wt% sodium hydroxide solution in a liquid to solid (L/S) ratio of 15. The pretreatment was performed in a 3.75 L Parr Reactor (Model 4551) at 90 °C for 30 min. After the extraction, the solid (pretreated biomass) and liquid (alkaline liquor) phases were recovered by filtration. The alkaline liquor was acidified with 30 wt% sulfuric acid for lignin precipitation. The precipitate was recovered by vacuum filtration using a Whatman Grade qualitative 113 filter paper. The filter cake was washed with water. Then, the SCB lignin powder was obtained by spray dryer. For that, the cake at 5 wt% solids in deionized water was fed into a Buchi B-290 mini spray dryer with a height of 0.48 m. The settings were as follows: aspirator (main drying air flow) rate 27.5 m^3/h (65 %); inlet air temperature of 160 °C; liquid feed pump rate of 4 mL/min; atomisation air flow rate ~473–601 L/h; and the nozzle cleaner set to position 1. These operating conditions resulted in outlet air temperature of 85 °C.

2.3. Characterization

2.3.1. Color

Color evaluation of SCB lignin and cosmetic formulations (base and lignin-based) were performed according to the Commission Internationale de l'Eclairage (CIE) color scale (CIElab) on a portable reflection spectrophotometer (CR-410 Chroma Meter, Konica Minolta) with a specular component excluded mode. This was used to determine the appearance of an object's color by excluding any specular reflected light. The equipment was previously calibrated with a CR A44 Calibration Plate for blank calibration. The values of L* (lightness and darkness), a* (red and green) and b* (yellow and blue) were determined.

2.3.2. Chemical composition

For the determination of the acid insoluble and soluble lignin and total carbohydrates content, the samples were submitted to acid hydrolysis as described in literature [26]. Briefly, 3 mL of sulfuric acid 72 wt% was added to 0.3 g of sample. The mixture was placed into water bath at 30 °C for 1 h. Afterwards, it was incubated into an autoclave at 121 °C for 1 h, after being diluted with deionized water up to 4 %. The solution was cooled down and filtered through sintered glass filter crucibles previously calcined at 550 °C. The acid insoluble lignin was determined after drying the glass filter crucibles at 105 °C. After dilution of the filtrate samples, the acid soluble lignin was quantified by spectrophotometry at 205 nm (UV-1900, Shimadzu) employing the absorption coefficient of 110 dm^3/g/cm. Total carbohydrates content in the filtrate solution was quantified by high performance liquid chromatograph (HPLC) equipped with refractive index detector employing an Aminex HPX 87H column 300 × 7.8 mm (Bio-rad laboratories). The chromatograms were run in isocratic mode at 0.6 mL/min and 50 °C. The mobile phase employed was 5 mM sulfuric acid solution and the volume injection was 10 μL. The water content and inorganics were gravimetrically determined at 105 °C (Venti-Line) and 550 °C (Nabertherm), respectively, until constant weight. The crucibles were previously calcined overnight at 550 °C. All samples were analyzed at least in duplicate.

2.3.3. Gel permeation chromatography (GPC)

The molecular weight determination was performed on an Agilent 1260 Infinity II system equipped with DAD detector and quaternary pump. Two Agilent gel columns were placed in series: Oligopore column 300 × 7.5 mm with nominal particle size of 6 μm and Mesopore column 300 × 7.5 mm with nominal particle size of 3 μm that measure molecular

weights up to 4500 g/mol and 25,000 g/mol, respectively. A guard column Oligopore 50 × 7.5 mm was placed prior to the columns. Detection was performed at 268 nm and the volume of injection was 20 μL. GPC analysis was performed in isocratic mode employing dimethylformamide with 0.5 % *w/v* of lithium chloride [27]. Chromatographic analysis was carried out at 70 °C and flowrate of 0.8 mL/min. Calibration curve was performed with 10 polystyrene molecular weight standards ranging from 162 and 9570 g/mol. About 5 mg/mL of each standard was dissolved in mobile phase solvent and filtered through 0.45 μm syringe filter before injection. Samples were dissolved in the mobile phase solvent (4.5–6.5 mg/mL), stirred overnight, and filtered through 0.45 μm syringe filter before injection. Samples were prepared in duplicate.

2.3.4. Attenuated total reflectance - Fourier transform infrared spectroscopy (ATR-FTIR)

The main functional groups of the SCB lignin were assessed through the ATR-FTIR analysis (Perkin Elmer, USA). Spectrum was recorded between 550 and 4000 cm^{-1} with 16 scans and resolution of 4 cm^{-1}. The Perkin Elmer FTIR Software (Perkin Elmer, USA) was used for all spectral manipulation.

2.3.5. Alkaline nitrobenzene oxidation

SCB lignin was submitted to nitrobenzene oxidation as described in the literature with minor modifications [28]. About 40 mg of samples were dissolved in 2 M sodium hydroxide solution; afterwards, 0.45 mL of nitrobenzene were added, and the mixture heated up to 185 °C for 2.5 h. The nitrobenzene oxidation was performed at least in duplicate. To remove nitrobenzene residues, the oxidized solution was extracted with chloroform (1:1 v/v). Then, the phenolic monomers were recovered with chloroform (1:1 v/v), after acidification with sulfuric acid 9 M. The organic fractions containing the phenolic monomers were dried under nitrogen atmosphere and dissolved in ethanol absolute. The solutions were diluted with ethanol:water 20 % v/v and quantified by Ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-QTOF/MS) in the negative mode. Phenolic monomers were separated in a BRHSC18022100 intensity Solo 2 C18 (100 × 2.1 mm, 2.2 μm, Bruker) column, through a gradient composed by Water with 0.1 % formic acid (A) and acetonitrile with 0.1 % formic acid (B): 0 min: 100 % A, 10 min: 79 % A, 14 min: 73 % A, 18.30 min: 42 % A, 20–24 min: 0 % A, 24.10–26 min: 100 % A, at a flow rate of 0.250 mL/min. The parameters for the MS analysis were set using negative ionization mode with spectra acquired ranging from *m/z* 20 to 1000 in an Auto MS scan mode. The samples injection volume was 5 μL. The Extracted Ion Chromatogram (EIC), with the analysis of the MS fragmentation of the deprotonated molecular ions ($[M-H]^-$), as well as their exact molecular mass (QTOF), were used to identify phenolic monomers. Identification of compounds was performed using Metaboscape software.

2.4. Antioxidant activity

2.4.1. Sample preparation

The antioxidant activity was evaluated for the SCB lignin and cosmetic formulations (base and lignin-based). The soluble fraction of SCB lignin was prepared by dissolving 10 mg of each extract in 1 mL of methanol and sonicated for 15 min. Then, the sample was centrifuged at 5000 rpm for 5 min. The lignin soluble fraction (supernatant) was spectrophotometrically quantified at a wavelength of 280 nm using a UV visible spectrophotometer [26]. For cosmetic formulations, 20 mg of sample were dissolved in 1 mL of DMSO and sonicated for 15 min.

2.4.2. 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay

The antioxidant activity was assessed by the radical cation ABTS, according to the method described in the literature [29] with some modifications. $ABTS^{\bullet+}$ was generated through a chemical oxidation reaction with potassium persulfate (7 mM ABTS and 2.45 mM potassium persulfate) and maintained in the dark at room temperature for 16 h before using. The concentration of ABTS radical was adjusted with methanol to an initial absorbance of 0.700 (± 0.020) at 734 nm. This solution (200 μL) was mixed with 15 μL of sample or butylated hydroxytoluene (BHT) (reference antioxidant) or solvent (blank) in a 96-well plate. The mixture was incubated for 5 min at room temperature in the dark, and the absorbance at 734 nm was measured in a microplate spectrophotometer (Epoch, Agilent). The radical stock solution was freshly prepared, and all the analyses were performed in triplicate. The scavenging activity was calculated as % inhibition using the following Eq. (1):

$$\%Inhibition = \frac{(A_c - A_s)}{A_c} \times 100 \quad (1)$$

where, A_c is the absorbance of control and A_s is the absorbance of the sample. The radical scavenging ability of SCB lignin and cosmetic formulations was expressed as half-maximal inhibitory concentration (IC_{50}), which corresponds to the concentration required to decrease the free radical activity by 50 %, under the specific experimental conditions. For the lignin-based BB cream, the IC_{50} was calculated in function of the SCB lignin concentration.

2.4.3. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

The ability of the extracts to scavenge DPPH radical was determined by the method described in the literature [30] with minor modifications. For the DPPH assay, 175 μL of DPPH methanolic solution (600 μM) were added to 25 μL of each sample or BHT or solvent (blank). After 30 min in the dark at room temperature, the inhibition of the DPPH radical was measured at a wavelength of 515 nm. The antioxidant activity was expressed as a percentage DPPH scavenging using Eq. (1). All experiments were performed in triplicate. The radical scavenging ability of SCB lignin and cosmetic formulations was expressed as IC_{50}. For lignin-based BB cream, the IC_{50} was calculated in function of the SCB lignin concentration.

2.5. Safety assessment of lignin

2.5.1. Cytotoxicity

The cytotoxicity of SCB lignin was assessed in a human keratinocyte cell line - HaCAT - with PrestoBlue Fluorescence assay. Briefly, after reaching 80–90 % confluence, HaCAT cells were detached with TrypLEX express enzyme (1×) and seeded to a final density of 1×10^5 cells/mL in DMEM GlutaMAX™, supplemented with 10 % v/v fetal bovine serum and 1 % of penicillin-streptomycin (Pen-Strep 100 UI/mL and 10 mg/mL, respectively) in 96 wells plate. After 24 h at 37 °C, in a humidified atmosphere of 5 % CO_2, cells were exposed to six serial dilutions of soluble SCB lignin from 46.9 to 1500 μg/mL. SCB lignin stock solutions were prepared in DMSO, and diluted with DMEM prior to cells exposure, so that the maximum DMSO concentration did not exceed 1 %. Positive control of cells death was DMSO 10 %. After 24 h of exposure, the cells were incubated with PrestoBlue reagent. Cell viability was evaluated by fluorescence spectroscopy after 2 h of incubation at 37 °C, using a Synergy HT Multi-detection microplate reader (Biorek, Bad Friedrichshall) operated by GEN5™ software, with excitation and emission wavelengths of 560 and 590 nm, respectively. Cell viability was expressed as percentage relative to the untreated control cells (1 % DMSO).

2.5.2. Mutagenicity

To infer about the mutagenic potential of SCB lignin and their metabolites, the miniaturized Ames test in liquid 384 well microplate format was performed. Three standard strains of *Salmonella typhimurium* containing a deletion mutation for histidine were used, with the

following mutations: the type of frameshift (TA98), base-pair substitution (TA100) and reversion/transversion (TA102). Briefly, the strains were inoculated in nutrient broth-2 and incubated with 25 μg/mL of ampicillin for 10–12 h at 37 °C in an incubator shaker (Innova 44, New Brunswick) at 120 rpm until a density of 1–2 × 10^9 bacteria/mL or approximately 1.0–1.4, OD 650 nm. The mutagenic potential of samples was assessed directly and in the presence of metabolic activation, provided by a liver homogenate, S9. For that, five SCB lignin concentrations were prepared in DMSO, and added to the exposure medium (inoculum at 10 % v/v). DMSO was used as negative control, and for the positive controls without S9 activation, 2-nitrofluorene (100 μg/mL), 4-nitroquinoline-N-oxid and Mitomycin-C (50 μg/mL each) dissolved in DMSO were used for the strains TA98, 100 and 102, respectively. In the presence of S9, the 2-Aminoantracene (100 μg) dissolved in DMSO was used for all strains. After 90 min of incubation, the reversion indicator media was added and 50 μL of the homogenous mixture were transferred to a 384-well plate, sealed into a plastic bag and incubated at 37 °C. *Salmonella* his+ revertants were counted after approximately 24 h (TA102) and 48 h.

2.5.3. Skin sensitization

Skin sensitization potential was performed through Direct Reactivity Peptide Assay (DPRA), following OECD Test guideline No. 442C [31]. The DPRA refers to the molecular initiating event of the adverse outcome pathway, *i.e.*, the covalent interaction with proteins [32]. The SCB lignin was dissolved in acetonitrile:DMSO (2.3:0.7 v/v) at a concentration of 8 mg/mL and diluted up to 1, 2 and 4 mg/mL (tested concentrations). The cysteine model peptide stock solution was 0.667 mM in phosphate buffer (pH 7.5) and lysine model peptide stock solution was 0.667 mM in ammonium acetate buffer (pH 10.2). Diluted samples were incubated for 24 h, at room temperature, with cysteine or lysine peptides, at ratio of 1:10 or 1:50 (*v*/v), respectively. Cinnamic aldehyde 100 mM in acetonitrile was used as positive control. Analysis of the free peptides was performed by HPLC reverse-phase (Agilent 1260 Infinity II, Agilent Technologies) equipped with a diode array detector (Agilent 1260 DAD HS) and a Poroshell 120 EC C18 column (3.0 × 150 mm; 2.7 μm). The analysis was performed in gradient mode with mobile phase A (0.1 % trifluoroacetic acid in water) and phase B (0.085 % trifluoroacetic acid in acetonitrile) starting at time 0 with 10 % B, going to 25 % B in 15 min, 90 % B in 3 min, 10 % B in 1 min and kept 6 min at 10 % B. Detection was carried out at 220 nm, with a flow rate of 0.35 mL/min, column temperature of 30 °C and with sample injection volume of 5 μL. Peptides were quantified building calibration curves of cysteine and lysine ranging between 0.033 and 0.528 mM.

2.5.4. Patch test

The potential of SCB lignin to cause allergic skin inflammation was tested using the Patch test. The clinical investigation plan was consistent with the main principles of International Council for Harmonisation of technical requirements for pharmaceuticals for human use (ICH), Good Clinical Practice (GCP), Helsinki declaration and Portuguese legal requirements. The study was conducted in a double-blinded fashion, in 10 female/male subjects of ages between 18 and 65 years old, with Fitzpatrick skin phototype between I and IV and without skin diseases or other conditions that might interfere with the study. The SCB lignin at 5 % in petrolatum and two comparative products were applied under occlusion with an 8 mm Finn chambers® on Scanpor, using a hypoallergenic adhesive containing occlusion chambers. SCB lignin at 5 wt% in petrolatum and control products (water type II and sodium lauryl sulfate at 2 %) were applied in the referred occlusion chambers and the adhesive was maintained on the skin for 48 h. After 48 h, the adhesive was removed, and the skin sites were evaluated after 30 min, and potential skin reactions were recorded. The final skin evaluation was carried out 24 h after removal of the adhesive (patch) for the final evaluation of the skin. All the procedures were performed under dermatological control. For the analysis of the results, the scoring of the negative control (water type II) was deducted to the scoring of the test product and positive control (sodium lauryl sulfate at 2 %). Based on these deducted results, the calculation of the acute irritation index (M.I.I.) for the test product and positive control was performed. The M.I.I. is an index calculated according to the following Eq. (2)[2]:

$$\text{M.I.I.} = \frac{\sum \text{of the grade (erythema + oedema)}}{\text{Number of subjects}} \quad (2)$$

2.6. Proof-of-concept

2.6.1. Cosmetic formulations

A lignin-based BB cream formulation was designed based on a clean label concept and the composition is detailed in Table 1. Considering the poor solubility of lignin, a dispersion was performed to improve the incorporation of this ingredient into the formulation (Table 2).

Lignin-based BB cream and cream base were stabilized for 24 h. The evaluations performed included color and pH. The color was measured according to the procedure previously described for the SCB lignin in the Section 2.3.1. The pH was determined at 25 °C using a pH meter (Metler Toledo).

2.6.2. Microbiological safety

Lignin-based BB cream was microbiologically controlled according to the European Standard ISO 17516:2014 - Microbiological limits for cosmetics [33]. The product is considered microbiological safe if *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans* are absent in 1 g. Additionally, total aerobic mesophilic microorganisms (bacteria, yeast and mould) count should be $<10^3$ colony-forming units (CFU)/g.

2.6.3. In vitro and in vivo SPF

For the *in vitro* SPF assessment, the lignin-based BB cream was spread as a thin film on a suitable synthetic substrate and the UV absorbance measured through this film with an UV spectrophotometer (SPF-290AS, Solarlight®). The SPF was determined by first irradiating the product

Table 1
Composition of lignin-based BB cream.

Commercial name	INCI	Function	Wt%
Part A (aqueous phase)			
Deionized water	Aqua	Solvent	63.46
Glycerin	Glycerin	Humectant	5.00
SOLAGUM™ AX	*Acacia senegal* gum; xanthan gum	Thickening and stabilizing agent	0.80
Dermofeel® PA-12	Sodium phytate	Chelating agent	0.10
Phenethylalcohol nat.	Phenethyl alcohol	Preservative	0.90
Part B (oily phase)			
Tego® Care PBS 6 MB	Polyglyceryl-6 distearate; polyglyceryl-6 behenate	Emulsifier	3.00
Geleol MB	Glyceryl stearate	Co-emulsifier	2.00
Shea butter	*Butyrospermum parkii* butter	Emollient	1.50
Caprylic triglyceride	Caprylic/capric triglyceride	Emollient	5.00
Squalane	Squalane	Emollient	3.00
Dispersion	–	Lignin dispersion	12.44
Part C			
E-Leen 8	Capryl glycol; glycerin; aqua	Antimicrobial booster	0.80

[2] The classification of the products according to the obtained index is the following: M.I.I. ≤ 0.20 - non-irritating; 0.20 < M.I.I. < 0.50 - slightly irritating; 0.50 < M.I.I. ≤ 1 - moderately irritating; M.I.I. > 1 irritating.

Table 2
Composition of dispersion for lignin-based BB cream.

Ingredient	INCI	Function	Wt%
Applecare PDS 300	Polyhydroxystearic acid; caprylic/capric triglyceride; isostearic acid; lecithin; polyglyceryl-3 polyricinoleate	Dispersant agent	2.30
Vegetable alternative to silicone (VAS)	Hydrogenated ethylhexyl olivate; hydrogenated olive oil unsaponifiables	Wetting agent; emollient	57.50
SCB lignin[a]	–	Active ingredient	40.20

[a] For the cream base production, deionized water was included instead of SCB lignin.

with 4 Minimum Erythemal Doses (MEDs) followed by the scanning of the sample from 290 nm to 400 nm. During the scanning, the obtained data was accumulated and stored at intervals of 1 nm to determine the monochromatic protection factor (MPF) for each of the selected wavelengths. Then, the MPF was used to calculate the SPF value, using solar irradiance and erythemal constants. The used equipment for irradiation was a Solar Simulator PV Cell testing 16S-300-002 (Solarlight®). The 16S-300-002 used a 300-watt Xenon arc lamp with a continuous spectrum ranging from 290 to 400 nm. The Poly(MethylMethacrylate) (PMMA) plates used as substrate for the sunscreen testing was performed by applying glycerin or modified glycerin solution to the rough surface of the PMMA plate. Glycerin was spread over the plate with the fingertip and then transmission (between 290 nm to 400 nm) was measured against air (with no plate) as the reference light path. The SPF value of the lignin-based BB cream was compared to a standard product (reference sunscreen) with a known SPF (16.1 ± 2.4). The lignin-based BB cream or reference sunscreen application to the PMMA plate was performed according to the following steps: (1) sample (32.2–32.8 mg) was spread to the roughened surface of the PMMA plate (1.3 mg/cm^2); (2) the absorbance (A) was measured for each of the selected wavelengths (l), the MPF determined and used to calculate the SPF value, applying solar irradiance (S) and erythemal constants (E). The SPF values are expressed as mean ± standard deviation of 9 measurements at the 9 different locations. The SPF determination for each test product and the reference sunscreen was determined according to the following Eq. (3):

$$SPF_{vitro} = \frac{\int_{290nm}^{400\ nm} E(\lambda) \times S(\lambda)}{\int_{290nm}^{400\ nm} E(\lambda) \times S(\lambda) \Big/ MPF(\lambda)} = \frac{\int_{290nm}^{400\ nm} E(\lambda) \times S(\lambda)}{\int_{290nm}^{400\ nm} E(\lambda) \times S(\lambda) \Big/ 10^{A_\lambda}} \quad (3)$$

The *in vivo* SPF assessment of lignin-based BB cream was performed according to the ISO 24444:2019. Five healthy human volunteers were selected according to the inclusion criteria for the study. A Solar Simulator (Multiport Simulator model 601–300 W, Solar Light Company) with WG-320 and UG-11 filters was used to induce UV irradiation at four different sites on the skin. Before and after UV exposure of each test site, the UV irradiance was checked with a calibrated radiometer (PMA2103LLG SUV detector, Solar Light Company). During UV exposure of each test site, the UV-output was dosed using a dose control system (PMA 2100, Solar Light Company) unprotected skin (MEDu), skin protected with an SPF 15 reference standard (MEDp) and skin protected with the lignin-based BB cream (MEDp), where MED represents the lowest dose of UV needed to induce erythema after 20 ± 4 h, by the judgement of a trained evaluator. The samples were applied to the skin at a concentration of 2 mg/cm^2 ± 2.5 %. Results were calculated by the original values ($n = 5$) and expressed as mean ± standard deviation. The SPF was calculated using the following Eq. (4):

$$SPF_{In\ vivo} = \frac{MEDp\ protected\ skin}{MEDu\ unprotected\ skin} \quad (4)$$

2.6.4. UVA-PF and critical wavelength

The *in vitro* UVA-PF of lignin-based BB cream was assessed according to the ISO 24443:2012. Specifications are given to enable the determination of the spectral absorbance characteristics of UVA-PF protection in a reproducible manner. The substrate/plate was MOLDED PMMA plates (PolyMethylMethacrylate, PlexiglasTM) with one side of the substrate roughened. The sample was applied and distributed as homogeneous as possible on the PMMA plates. The sample was spotted evenly across the plate surface with a microsyringe. The principle of the analysis is a transmission measurement. The glycerin on the reference substrate served as a "blank" emulsion (placebo) which contained no light-absorbing or scattering compounds and reduced artificial scattering by the roughened, dry surface much the same as a placebo. The final UVA protection factor (UVA-PF), and the critical wavelength (defined as the wavelength at which the integral of the spectral absorbance curve reached 90 % of the integral from 290 to 400 nm) were calculated. The UVA-PF was calculated following Eq. (5).

$$UVA - PF = \frac{\int_{320\ nm}^{400\ nm} P(\lambda) \times I(\lambda) \times d\lambda}{\int_{320\ nm}^{400\ nm} P(\lambda) \times I(\lambda) \times 10^{-A_0(\lambda) \times C} \times d\lambda} \quad (5)$$

where, P(λ) is the PPD action spectrum, I(λ) is the spectral irradiance received from the UV source (UVA 320–400 nm for PPD testing), $A_0(\lambda)$ is the mean monochromatic absorbance before UV exposure, C is the coefficient of adjustment, and dλ is the wavelength step (1 nm).

The critical wavelength (λc) is defined as the wavelength where the area under the absorbance spectrum for the irradiated product from 290 nm to λc is 90 % of the integral of the absorbance spectrum from 290 to 400 nm and is calculated as follows (Eq. 6):

$$\int_{290}^{\lambda C} A(\lambda) d\lambda = 0.9 \int_{290}^{400} A(\lambda) d\lambda \quad (6)$$

dλ is the wavelength step (1 nm).

2.7. Statistical analysis

Data were presented as the mean ± standard deviation (SD) of at least two independent assays. Statistical evaluation of the data was performed using a one-way analysis of variance (ANOVA) test using GraphPad Prism version 8.0. Tukey's HSD test or Dunnet's multiple comparison test were carried out to compare the significance of the different groups and compared to the control, respectively. The statistical significance was recorded as the *p*-value $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, $****p \leq 0.0001$.

3. Results and discussion

3.1. SCB lignin characterization

SCB lignin was characterized in terms of color, chemical composition, weight average molecular weight, and number average molecular weight, functional groups and free phenolics. The color of SCB lignin was evaluated quantitatively using the International Commission on Illumination CIE L*a*b* color space. The coordinate L* is related to the brightness or darkness of the sample, white when 100 and 0 when black; +a* is a red shade and −a* is a green shade; +b* is a yellow shade and −b* is a blue shade [10]. SCB lignin resulted in a homogenous powder (Fig. 1) and light brown color (color coordinates L* = 60.3 ± 0.4, a* = 6.1 ± 0.4 and b* 19.1 ± 0.5), color properties compatible with BB cream application.

The milder conditions of SCB lignin extraction which included time, temperature and alkaline solution, probably contributed to the lighter color of lignin [26]. Additionally, precipitation conditions (30 wt% sulfuric acid) and drying process (spray drying) also influenced the color

Fig. 1. SCB lignin powder.

of the extract [34]. SCB lignin showed a purity of 91.9 ± 1.2 %, with low content of sugars (2.0 ± 0.1 %) and ash (2.2 ± 0.1 %). The obtained powder presented pleasant characteristics for a cosmetic application, considering the light brown color as pigment and sweet odor with woody notes.

The recovered lignin had a weight average molecular weight (M_W) and number average molecular weight (M_n) of 15,199 and 10,793 Da, respectively. A low polydispersity index (PDI, ratio of M_W to M_n) of 1.41 was obtained. In terms of functional groups, the ATR-FTIR analysis revealed a typical lignin spectrum (Fig. 2). A broad band at 3650–3300 cm^{-1} was attributed to the hydroxyl groups in phenolic and aliphatic structures [36]. The phenolic groups content affects the UV-shielding properties: higher phenolic groups content provides higher UV-shielding properties [35]. The region between 3000 cm^{-1} and 2830 cm^{-1} was attributed to C—H stretch in methyl and methylene groups and methoxyl groups [36]. In the carbonyl/carboxyl region medium band, an unconjugated C═O stretching (vibrations of unconjugated ketones, esters or carboxylic acids) at 1700 cm^{-1} was found [36]. The fingerprint region of lignin was located between 700 cm^{-1} and 1600 cm^{-1}. The band 1596 cm^{-1} was attributed to aromatic skeletal vibration characteristic of condensed guaiacyl units, while 1324 cm^{-1} was related to aryl ring breathing with C—O stretch characteristic for syringyl ring, plus condensed guaiacyl ring. The band 1216 cm^{-1} was attributed to guaiacyl units. The shoulder at 1266 cm^{-1} was relative to C—O stretching of guaiacyl ring. Other aromatic skeletal vibrations were identified in bands 1513 cm^{-1}, 1463 cm^{-1} and 1423 cm^{-1} [36]. For *p*-hydroxyphenyl (H) units, an absorption band was observed at 834 cm^{-1}, typical in annual plants [37,38]. The presence of *p*-coumaric esters were detected at 1170 cm^{-1} and 989 cm^{-1} [38–40].

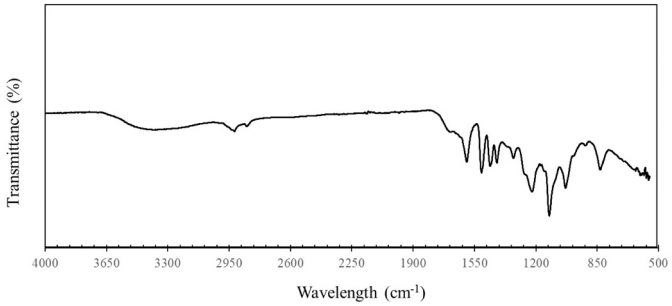

Fig. 2. ATR-FTIR spectra of sugarcane bagasse (SCB) lignin.

The basic structural information of lignin obtained by alkaline nitrobenzene oxidation (Table 3) confirmed the general conclusions from ATR-FTIR analysis. The identification of the aldehydes, syringaldehyde, 4-hydroxybenzaldehyde, and vanillin and the corresponding acids (syringic and vanillic acids) corroborated that SCB lignin is a *p*-hydroxyphenyl:guaiacyl:syringyl (H:G:S) type. The molar composition was 4.1:41.5:54.4 (H:G:S), being in accordance with the characterization reported by Río and co-workers for lignin from SCB [41]. Overall, the identified oxidative products are in line with previous studies on the structural elucidation of lignins from sugarcane [42].

3.2. Safety assessment

3.2.1. Cytotoxicity and mutagenicity

The safety assessment of SCB lignin was performed using keratinocytes (HaCat) at different concentrations (46.9–1500 μg/mL). As it can be observed from Fig. 3, SCB lignin presented a dose-dependent behaviour, with the highest tested concentrations (from 375 to 1500 μg/mL) revealing a significant cell viability decrease ($p < 0.001$) as compared to the control. Regarding the other tested concentrations, even though a decrease in cells viability has been observed, the percentage of viability was still 75 %, which is an acceptable value according to the Cytotoxicity Test ISO 10993-5. However, considering lignin topical application and possible exploitation in the field of cosmetics, the concentration 187.5 μg/mL can be considered safe. The obtained results agree with previous studies demonstrating that the cytotoxicity of lignin is only observed at higher concentrations [43,44].

The mutagenicity of SCB lignin was performed in *S. typhimurium* in the three tested strains without and with metabolic activation (S9+) (Fig. 4A and B, respectively). The tested SCB lignin concentrations (0.4 to 250 μg/mL) revealed no significative reversion ($p < 0.05$) as compared to the solvent (DMSO, non-treated bacteria), and no dose-dependent response for all the strains in both conditions (with and without metabolic activation). According to the results, SCB lignin did not induce mutagenicity for all the tested strains up to 250 μg/mL. In a previous study, several lignins from different sources (wood, corn and straw) were evaluated for the mutagenicity potential, the results without metabolic activation showed that the lignins were not toxic to the bacteria in concentrations varying from 125 to 500 μg/plate [45].

3.2.2. Skin sensitization

SCB lignin was no sensitizer based on cysteine and lysine reactivity at the concentrations of 1 and 2 mg/mL (Table 4). The higher concentration tested (4 mg/mL) was sensitizer, but with a low cysteine and lysine reactivity. To the best of our knowledge, this is the first time that lignin is tested against skin sensitization using the *in chemico* assay that models the first key event in the skin sensitization.

3.2.3. Patch test

Analyzing the M.I.I. calculated 24 h after patch removal, and according to the adopted scale, it can be concluded that the SCB lignin tested at 5 wt% in petrolatum, was non-irritating with an acute skin

Table 3
Oxidative products of sugarcane bagasse (SCB) lignin.

Oxidative products	Yield (wt%)
p-Hydroxybenzaldehyde	0.597 ± 0.005
Vanillin	7.04 ± 0.13
Vanillic acid	0.461 ± 0.04
Syringaldehyde	10.79 ± 1.05
Syringic acid	1.00 ± 0.04
Coumaric acid	0.752 ± 0.015
Ferulic acid	1.53 ± 0.04
Total yield	22.16 ± 1.28
H:G:S molar ratio (%)	4.1:41.5:54.4
S/G ratio	1.3

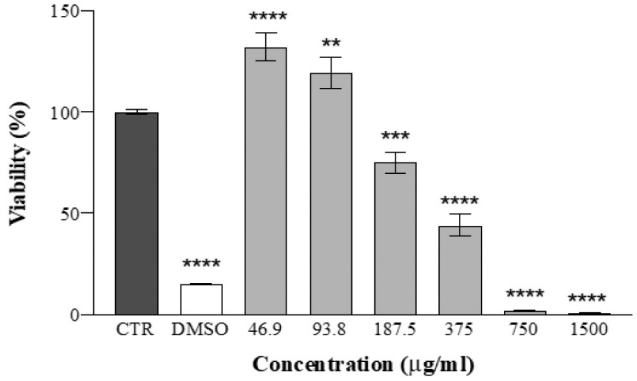

Fig. 3. HaCAT line cell viability after 24 h of incubation with serial dilutions of soluble sugarcane bagasse (SCB) lignin. Results are expressed as % of PrestoBlue reduction *versus* control (CTR, DMEM with 1 % DMSO). DMSO (10 %) represents the positive control. All the results are expressed as the mean ± SD of two independent assays, performed in quadruplicate. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ (ANOVA, Tukey HSD).

irritation index of 0.00. The positive control sodium lauryl sulfate, tested at 2 wt%, resulted in a 2.10 acute irritation index. These results reinforced the achievements of previous research on the eye and skin irritation potential of lignin from different sources, which demonstrated no irritation response, thus suggesting its opportunity for topical application [46,47].

3.3. Proof-of-concept

3.3.1. Cosmetic formulations

The BB cream formulation was originally prepared for the present study. The BB cream formulation with 5 wt% SCB lignin (lignin-based BB cream) and cream base were successfully prepared showing a smooth and homogeneous appearance. The cream base presented a white color, while the lignin-based BB cream showed a brown color consistent with a BB cream product (Fig. 5). The lightness (L*) was higher for the blank comparing to the lignin-based BB cream (Fig. 5). The pH of the cream base was neutral (7.89 ± 0.03) and the lignin-based BB cream was slightly acidic (5.15 ± 0.00) (Fig. 5). It means that lignin promoted a slight decrease in the original pH, fitting the ideal pH of the face skin care products. For further analysis, the pH of the blank cream was adjusted to 5.5.

3.3.2. Microbiological safety

The analyzed microbiological parameters (Table 5) showed that lignin-based BB cream was microbiologically safe for topical application and that it was produced under hygienic conditions.

3.3.3. Antioxidant activity

The antioxidant activity was evaluated for the SCB lignin and cosmetic formulations following two methods: ABTS and DPPH. Although the antioxidant activity of lignin has been widely evaluated through DPPH radical scavenging activity [48], the ABTS method should also be considered since it is usually used to screen complex phenolic mixtures [49], such as lignin. The obtained results suggested that lignin depicted a similar capacity to scavenge ABTS radical as compared to the reference standard BHT, which is commonly used in cosmetic products (Table 6). In the case of the DPPH method, the IC_{50} of lignin was slightly higher than the one exhibited by BHT. This can be related to the specificities of each method [50]: ABTS assay measures both hydrophilic and lipophilic antioxidant molecules, while the DPPH mainly measures lipophilic antioxidants [51]. For instance, other lignins from SCB extracted with different concentrations of sodium hydroxide solutions (1, 5 and 10 %) and precipitated with sulfuric acid until pH 2.0 exhibited high radical scavenging activity as compared to BHT, using

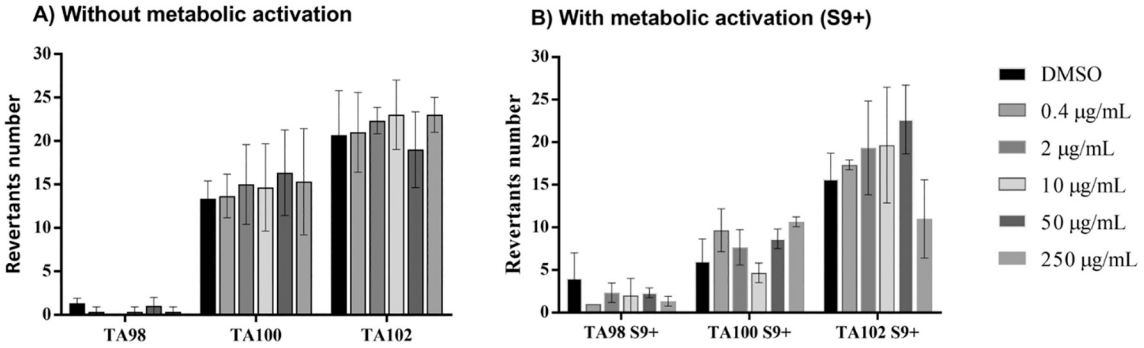

Fig. 4. Number of revertant *Salmonella typhimurium* individuals after exposure to different concentrations of sugarcane bagasse (SCB) lignin, A) without metabolic activation and B) with metabolic activation (S9+). Results are presented for the three mutant strains tested, TA98, 100 and 102, and expressed as the mean ± SD performed in triplicate.

Table 4
Cystein (Cys) and lysine (Lys) depletion (%), reactivity and prediction models for cinnamaldehyde (positive control) and sugarcane bagasse (SCB) lignin.

Sample	Concentration (mg/mL)	Cys % depletion	Cys and lys % depletion	Reactivity (Cys)	Reactivity class	DPRA prediction
Cinnamaldehyde (Positive control)	13.2	60.64 ± 1.08	60.8 ± 0.2	Moderate	High reactivity	Positive
SCB lignin	1	1.59 ± 0.60	0.8 ± 1.1	Minimal	Minimal	Negative
	2	4.77 ± 0.37	2.4 ± 3.4	Minimal	Minimal	Negative
	4	17.47 ± 0.04	11.0 ± 9.2	Low	Low	Positive

Reactivity to cys: Mean % Depletion ≤13.89 % (No or minimal reactivity); Positive: 13.89 % ≤ Mean % Depletion ≤23.09 % (low reactivity); 23.09 % ≤ Mean % Depletion ≤98.24 % (moderate reactivity);Mean % Depletion ≥98.24 (high reactivity).
Reactivity to cys + lys: 0 % ≤ Mean % Depletion ≤6.38 % (No or minimal reactivity); Positive: 6.38 % ≤ Mean % Depletion ≤22.62 % (low reactivity); 22.62 % ≤ Mean % Depletion ≤42.47 % (moderate reactivity); 42.47 % ≤ Mean % Depletion ≤100 % (high reactivity) A - Based on Cystein only prediction model; B - Based on mean of Cystein and Lysine prediction model. P- Positive; N - Negative.

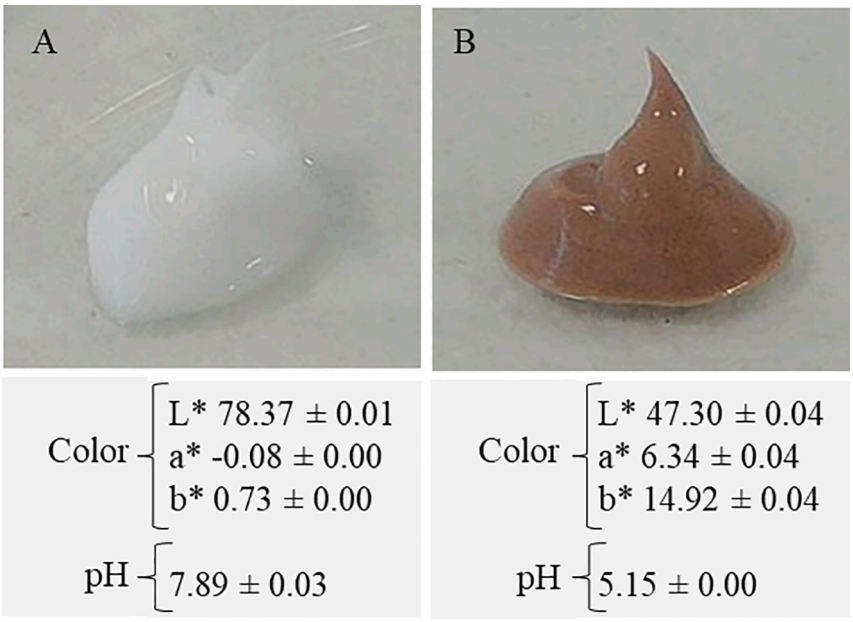

Fig. 5. Appearance, color coordinates (L*, a*, b*) and pH of cosmetic formulations. A) Cream base and B) lignin-based BB cream.

Table 5
Microbiological parameters of lignin-based BB cream.

Type of microorganisms	CFU/g
Moulds and yeasts	<10
Total viable aerobic count	<10
Escherichia coli	Absent
Pseudomonas aeruginosa	Absent
Candida albicans	Absent
Staphylococcus aureus	Absent

Table 6
Antioxidant activity of sugarcane bagasse (SCB) lignin and reference butylated hydroxytoluene (BHT).

Sample	IC_{50} (mg/mL)[a]	
	ABTS	DPPH
SCB lignin	0.201 ± 0.006	0.351 ± 0.006
BHT	0.288 ± 0.007	0.205 ± 0.005

[a] The IC_{50} values were given as the mean ± standard deviation of at least three individual determinations each performed in triplicate. ABTS - 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay; DPPH - 2,2-Diphenyl-1-picrylhydrazyl assay.

DPPH method [52,53]. The differences in the antioxidant activity of lignin are affected by several factors, such as the source, chemical structure, molecular weight, purity, and extraction method [54].

The obtained data on composition, molecular weight and functional groups of the present SCB lignin corroborate its antioxidant activity. The high purity and the low polydispersity positively contribute to the antioxidant properties [4]. The ATR-FTIR spectra presented the main functional groups, particularly phenolic and methoxyl, which are associated with the antioxidant activity [32,34]. Additionally, the nitrobenzene oxidation showed that SCB lignin has a syringyl-rich composition. In a study carried out by Guo et al. 2019, the antioxidant activity of different lignins (soda, kraft, DES, organosolv and hydrotrope) with similar total phenolic hydroxyl groups was tested [55]. Authors attributed the high radical scavenging to the presence of syringyl phenolic hydroxyl groups [55]. The free radical scavenging capacity of the lignin-based BB cream was also evaluated, and the results proved the ability of lignin to act as radical scavenger in formulation (0.535 ± 0.012 mg/mL for ABTS and 0.709 ± 0.110 mg/mL for DPPH) since in its absence (cream base) no antioxidant activity was observed.

3.3.4. *In vitro and in vivo SPF, in vitro UVA-PF and critical wavelength*

The *in vitro* and *in vivo* SPF, along with the *in vitro* UVA-PF and critical wavelength were evaluated for the lignin-based BB cream and the results are given in Table 7. Results demonstrated that the *in vitro* and *in vivo* SPF were identical (9.5 ± 2.9 and 9.6 ± 0.8, respectively). This is one of the most relevant findings and perhaps also the most significant, since the adopted *in vitro* method could efficiently predict the *in vivo* efficacy of the proposed lignin-based BB cream, in a safe, less time-consuming, and cost-effective way. As mentioned before, there is a body of evidence that suggests lignin has a potent UV-blocker agent. For instance, the study carried out by Ratanasumarn et al. where an oil-in-water sunscreen lotion containing 10 wt% of an alkaline SCB lignin extract achieved an *in vitro* SPF of 8.65 ± 0.21 [12]. In another research work, the *in vitro* SPF value of a sunscreen containing 10 wt% of organosolv lignin reached 7.57 ± 0.28 [56]. Lignin extracted from distinct biomass sources such as eucalyptus, bamboo, pine and corncob using different pretreatments was evaluated for their potential as UV blocker. Kraft lignin at 10 % in a baby lotion presented a SPF value of 6.56 [57]. A different research work reported that a lotion with an organosolv lignin submicrometer-based at 5 wt% resulted in SPF ranging from 2.80 to 3.53 [58]. Other example is a pure cream blended with 10 wt% of organosolv lignin with colloidal spheres of different sizes for which SPF values ranged from 7.57 to 15.03 [56]. Comparing to the literature, the performance of the present SCB lignin was superior, since only 5 wt% of SCB lignin was incorporated into the formulation. In this work, the broad-spectrum protection capacity of the isolated SCB lignin was also demonstrated by the critical wavelength value (378 ± 0.5 nm).

Table 7
In vitro and *in vivo* SPF, *in vitro* UVA-PF, and critical wavelength (C*w*) of lignin-based BB cream and reference sunscreen.

Sample	*In vitro* SPF	In vivo SPF	*In vitro* UVA-PF	Cw (nm)
Lignin-based BB cream	9.51 ± 2.92	9.6 ± 0.8	3.4 ± 0.2	378 ± 0.5
Reference sunscreen	16.1 ± 2.4	–	13.1 ± 0.8	–

According to Food and Drug Administration (FDA) guidelines, values of critical wavelength higher than 370 nm indicate a sunscreen with excellent broad-spectrum coverage for UVA protection [59]. As mentioned previously, the UV-absorbing property of lignin is due to its chemical structure with chromophores such as carbon-carbon double bonds, carbonyl groups, and aromatic systems [9].

4. Conclusions

In this work, the alkaline lignin from SCB was evaluated as a natural ingredient with multifunctional properties, acting as natural UV blocker, antioxidant agent and pigment. Results on cytotoxicity, mutagenicity, skin sensitization and acute skin irritation revealed that SCB lignin is safe for topical application. The bioactivity of SCB lignin and cosmetic formulation was confirmed following appropriate methodology and, in the case of the SPF test, the adopted *in vitro* methodology efficiently predicted the *in vivo* results. BB cream formulation containing 5 wt% lignin showed a broad-sprectrum UV protection. Based on our findings, we conclude that SCB lignin has a tremendous potential as a multifunctional candidate for color cosmetics.

CRediT authorship contribution statement

Filipa Antunes: Conceptualization, Methodology, Investigation, Writing – review & editing. **Inês F. Mota:** Conceptualization, Methodology, Investigation, Writing – review & editing. **Joana F. Fangueiro:** Conceptualization, Methodology, Investigation, Writing – review & editing. **Graciliana Lopes:** Conceptualization, Methodology, Investigation, Writing – review & editing. **Manuela Pintado:** Supervision. **Patrícia Santos Costa:** Conceptualization, Writing – review & editing, Supervision.

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