A multi-functional and ecofriendly ingredient to fight against photo-aging

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Abstract

Sunrays may cause photo-aging which consists in biological, and clinical skin alterations. Ultra-violet (UV) traditional filters and chemicals are widely used in cosmetic products as a preventive protection. However, they tend to have side effects on health and their impact on environment is now condemned.

In order to meet the demand for new, natural, ecofriendly photoprotecting ingredients, an original *Entada phaseoloides* seeds extract (EPSE) was designed. Using a patented extraction process, the concentration and stability of several bioactive constituents such as entadamide A, phaseoloidin and saponins were maximized.

EPSE provides the skin a strong protection against photoaging as it reduces UVB-induced DNA alteration and inflammation while reducing the blue light-induced MMP-1 production. The high concentration in phaseoloidin provides the active ingredient with strong anti-oxidative properties, while entadamide A is responsible for preventing the isomerization of trans-urocanic acid (UCA) into the immunosuppressive cis-UCA. Interestingly, EPSE is also able to reduce melanogenesis by inhibiting tyrosinase activity in melanocytes.

Taken together, these results show that EPSE is a cosmetic active ingredient with strong antioxidant and photoprotective abilities. This activity is due to a well-reasoned extraction process that specifically targeted bioactive metabolites such as entadamide A and phaseoloidin hence leading to a reliable and multifunctional extract. Among the biological activities of EPSE, one of the most interesting could be its ability to modulate melanogenesis for preserving a bright and even skin tone while providing an efficient photoprotection. This may prove very interesting for some markets such as Asia.

Key words: Photoprotection, *Entada phaseoloides*, entadamide A, phaseoloidin, UVB, blue light

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1) Introduction

Aging is a complex, multifactorial process that involves two independent mechanisms: intrinsic aging (genetically programmed) and extrinsic aging (induced by external stresses such as sun exposure, tobacco, environmental pollution, stress, ...). Photoaging, aging induced by sunlight, is believed to play a major role in the aging of exposed parts of the body. Repeated exposure to sun radiations accelerates most of the changes that occur with aging, but also produces

some characteristic clinical features, including deep wrinkling, leathery appearance, and pigmentary disorders (sallowness, lentigines formation) [1].
Ultraviolet (UV)-B (290–315 nm) and -A (315–400 nm) radiations are key-players since they are highly energetic. However, other radiations such as visible light (400-700 nm) or infrared (IR) radiations (700-4000 nm), primarily from the sun but also from artificial sources, have been shown to be harmful to the skin [2,3]. They likely are actively participating to photoaging.

UV exposure can cause direct effects on skin cells. Indeed, as a chromophore, DNA absorbs UVB which may result in lesions such as pyrimidines (6-4) pyrimidone (6-4PPs) or cyclobutane thymine dimers (CPDs) with multiple biological consequences such as apoptosis, mutagenesis, immunosuppressive signalling… [4,5]. They can also cause indirect noxious effects that are all mediated by free radicals such as reactive oxygen species (ROS) [5]. These cumulative effects often lead to the apparition of sunburn cells, a type of apoptotic cells typically observed in the epidermis after an exposure to UV, when DNA is too severely damaged [6].

By stimulating both NFKB and AP1 pathways in keratinocytes, UV induce the production of pro-inflammatory cytokines such as interleukin-1, (IL-1), IL-6, IL-8… and of immunosuppressive cytokines such as tumor necrosis factor alpha (TNF-α) and IL-10 [7-10].

The skin's main defence against UV is urocanic acid (UCA) and melanin, two molecules capable of absorbing UV, that play the role of "natural sunscreens".

Trans-UCA is produced in the upper layers of the epidermis. However, upon UV absorption *trans*-UCA is isomerized into *cis*-UCA that mediates immunosuppression through a complex signalling cascade involving ROS, inflammatory mediators, and galectin-7 [11,12]. This β-galactoside-binding lectin naturally expressed in the epidermis is overexpressed after UVB irradiation and participates to the apoptotic process setup in sunburn keratinocytes [13]. Besides, it was shown that UVB-induced galectin-7 overexpression leads to a down-regulation of T cells functions, thus pointing to its involvement in immunosuppression. Interestingly, *cis*-UCA alone induces galectin-7 overexpression, which strongly suggests that galectin-7 participates to the immunosuppressive signaling of *cis*-UCA [12].

Melanin is another important defensive mechanism of the skin against sunrays. It serves as a natural absorbent of free radical as well as a physical barrier to UV and visible radiation [14]. Although most of the melanin is accumulated in keratinocytes, the melanogenesis process actually takes place in melanocytes and depends on two key-rate limiting enzymes: tyrosinase and dopachrome tautomerase [15]. However, it was shown that melanin could be responsible for causing DNA alteration several hours after an exposure to UV [16]. Indeed, both UVA and UVB produce reactive oxygen and nitrogen species, which together create a higher-energy form of melanin (chemoexcitation process). This new form of melanin contains the energy of a UV photon and later transfers this energy to DNA (chemiluminescence process) that may cause a delayed mutation: a "dark CPD" [16].

As the most energetic and therefore most noxious sunrays for the skin, UVB and UVA were the main targets for photoprotection. However, it was more recently shown that IR-A and visible light (especially blue light) were responsible for producing ROS and altering the extracellular matrix (ECM) by stimulating matrix metalloproteinase-1 (MMP-1) expression [2,17]. As a result, it became obvious that the usual sun filters did not provide an effective large spectrum photoprotection [3]. Furthermore, sun filters were also condemned as an environmental threat.

In order to further protect the skin from photoaging, and for answering the market's need for natural, eco-friendly cosmetic products, Exsymol designed an original plant extract with an accurately defined content in photoprotective metabolites and antioxidants.

Entada phaseoloides is a widespread plant found in tropical regions (Asia, Southern Africa, Australia, …). It has been used for long in folk medicine, and multiple medicinal properties have been reported, including anti-inflammation, analgesic activity, anti-pyretic, anti-arthritis, anti-diabetic, … [18]. It produces huge seed pods holding large disk-shaped seeds (Figure 1A) that are especially rich in bioactive constituents such as entadamide A, phaseoloidin and saponins.

Entadamide A (Figure 1B) is a methylthiopropenoic acid conjugates (MTPC) that readily absorbs UVB and releases the absorbed photons energy by a "non-sacrificial" absorption mechanism of *trans* to/from *cis* isomerisation (Figure 4A). This accounts for entadamide A high UVB-absorbing efficacy (ε(290nm) = 9900 L/mol/cm), and also avoids photo-degradation and formation of potentially harmful by-products.
The seeds contain several antioxidant phenolic compounds [19], and especially phaseoloidin (Figure 1C), a stable phenolic glycoside that is by far the most abundant (>10% of the seeds' dry mass).

Figure 1 – ESPE is an optimized extract rich in bioactive molecules.
(A) Pictures of *Entada phaseoloides* and its seeds. Molecular structure of entadamide A (B) and phaseoloidin (C).

Interestingly, entadamide A was also reported to be a 5-lipoxygenase inhibitor [20], and several constituents of the seeds, including saponins, have proven anti-inflammatory activities [21].

All these constituents may provide benefits that are key for fighting photoaging.

A patented extraction process based on endogen hydrolases (glycosidases) was developed in order to maximize the concentration and the stability of the aforementioned molecules of interest in our *Entada phaseoloides* seeds extract or EPSE (publication No WO2019197548) [22].

As a result, EPSE contains more than 0.25% of entadamide A, 3% of phaseoloidin and 1.5-2.0% of saponins.

2) Aim of this study

Here, we present the efficacy of an original, and patented extract obtained from the seeds of *Entada phaseoloides* (EPSE) to protect the skin from the noxious effects of sunrays and artificial irradiations. To that end, we evaluated the impact of UV and blue light exposure in the presence or in the absence of EPSE on several key features, namely genotoxicity, pro-inflammation, immunosuppression and melanogenesis.

3) Material & Methods

Antioxidant activity *(DPPH and ABTS assay)*

1,1-diphenyl-2-picrylhydrazyl or DPPH (Sigma-Aldrich) is a free radical stable at room temperature that absorbs light at 517 nm (red color). Scavenging of DPPH radical modifies its absorbing properties (it switches to yellow). Monitoring of the optical density (OD) variations enables a precise quantification of the scavenging effect of tested products.
Various concentrations of EPSE (0.27 to 8.11 g/L) were mixed with DPPH in ethanol at 200 µM and left to stand during 30 min.
The optical density (OD) of the solution was then measured at 517 nm with a spectrophotometer (MicroQuant, BioTek Instrument INC).

Oxidation of ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt) with potassium persulfate (Sigma-Aldrich) generates ABTS+°, a rather stable cationic peroxyl radical with light-absorption maxima at 414, 645, 734 and 815 nm (blue/green color). Scavenging of ABTS+° by hydrogen-donating antioxidants (typically plant-derived polyphenols) modifies its absorption spectra. Monitoring of the optical density (OD) variations enables a precise quantification of the scavenging effect.
ABTS+° was diluted to obtain a solution that absorbs with an O.D. 0.7 at 734 nm. Various concentrations of EPSE (0.01 to 0.13 g/L) and concentrations of phaseoloidin (0.3 to 6.4 mg/L) were mixed with ABTS+° during 6 min.
The optical density of the solution was then measured at 734 nm with a spectrophotometer (MicroQuant, BioTek Instrument INC).

For both assays, the percentage of radical scavenging activity was expressed in percentage of the reagent and allows to determine the concentration of tested product inducing 50% of scavenging activity.

Cell culture

Normal Human Fibroblast culture*:* NHDF isolated from breast tissue (17-year-old patient) were seeded at D-3 in 24-well plates at the density of 15 000 cells/cm^2 in Dulbecco's modified Eagle Medium (DMEM 4.5 g/l glucose (Gibco) supplemented with 10% FCS (Biowest), 1% of glutamax (Gibco) and 1% of an antibiotic mix (penicillin-streptomycin-gentamicin) and incubated overnight at 37°C, 10% CO_2. At Day-1, cells were treated or not with different concentrations of EPSE during 24 hours (preventive mode). A D0, treatments were renewed (dilutions prepared in PBS) and cells were exposed to 30 J/cm^2 blue light (Waldman, fluocompact lamp Lighttech LTC 36W/2G11 CL 380-470 nm, irradiance: 13 mW/cm^2). After irradiation, cells were incubated for 24 hours with or without treatment at 37°C and 10% CO_2 (curative mode). N-acetyl-cysteine or NAC (Sigma-Aldrich) was used as reference antioxidant.

B16 Melanocyte culture: B16 Murine Melanocyte cell line obtained from ATCC were seeded in 6-well plates at the density of 40 000 cells/well in DMEM 4.5 g/l glucose (Dutscher) supplemented with 10% FCS, 2% of glutamax and 1% of an antibiotic mix (penicillin-streptomycin) and incubated overnight at 37°C, 5% CO2. After 24 h of culture, cells were treated or not with EPSE and kojic acid for 48 hours without renewal. For the study of dark CPD, cells were seeded on 8 wells glass slides (Falcon) at the density of 40 000 cells/well. At Day 1, the cells were irradiated in 400 µL of DPBS with calibrated solar box (Sunset CPS+, Xenon Lamp) at 1.8 J/cm². After the irradiation, EPSE (0.01% - 0.05%) prepared in culture media was applied for 2 h and maintained in the culture conditions. At the end of this period cells were fixed with acetic acid/alcohol (1/3) and dried at room temperature.

3D Skin Tissue culture

Reconstructed Human Epidermis: Skinethic RHE (0.5 cm^2) were obtained from Episkin laboratory (Lyon, France) at day 17 of culture. From reception, RHE were placed in specific maintenance medium (Episkin) at 37°C, 5% CO_2. After 24 hours, 100 µl of EPSE diluted at 1.5% and 2.5% in PBS were topically applied at the surface of the RHE before irradiation. Epidermis were then UVB-irradiated at 300 mJ/cm^2 (Waldmann lamp, 0.73 mW/cm^2 of irradiance).
Immediately after irradiation, treatments were renewed, and RHE were incubated for 5 hours at 37°C, 5% CO_2. At the end of this period, RHE were then divided in two equal parts and processed in paraffin or prepared for cryosections. Each condition was done in triplicate.
Culture supernatant were harvested and conserved at -80°C until cytokine dosage.

Human skin Explant culture: Human skin explants were obtained from breast skin of 42 and 56 years-old Caucasian female donors who underwent plastic surgery. Punch biopsies of 10 mm diameter were cultured at 37°C in a humidified atmosphere containing 5% of CO2 in DMEM culture medium with 4.5 g/l glucose, supplemented and containing antibiotics (penicillin-streptomycin-amphotericin). Skin explants were topically treated for 24 hours with or without EPSE at 2.5 and 5% in PBS (daily application 20 µl/punch biopsy) before the irradiation, using a calibrated solar light box (Sunset CPS+, Xenon Lamp) at 39 J/cm^2, and 24 hours after. At the end of this period, punch biopsies were then divided in two equal parts and processed in paraffin or prepared for cryosections. Each condition was done in triplicate.

Microscopic Acquisition: The slides were observed using an epifluorescence microscope (BX60, Olympus) at a 20x magnification. Microphotographs were taken with a DP72 camera (Olympus) with 3 pictures / section. Photos were analyzed with the Cell F software (Olympus).

DNA damage detection (CPD quantification and Sun Burn Cells counting) and pro-inflammatory cytokine dosage

CPD quantification: On frozen RHE sections, the number of CPD positive cells was assessed by immunofluorescence using an anti-thymine dimer antibody (Sigma) revealed by a corresponding Alexa Fluor® 488-coupled secondary antibody (Invitrogen). Nuclei were counterstained with 4',6-diamidino-2-phénylindole (DAPI) probe (Roth). For each section, the analysis was done on three microphotographs. DAPI positive nuclei and CPD positive cells were manually counted in the epidermis. The results are expressed as the ratio of CPD positive cells on total cells for each condition and expressed in percent of the irradiated UVB condition.

Sun Burn Cells quantification: Formalin-fixed paraffin-embedded sections of RHE were stained with Haematoxylin eosin standard protocol. The sunburn cells appeared as eosinophilic cells with pycnotic nucleus in the supra-basal layers. For each section, the analysis was done on three microphotographs. The results are expressed as the number of sunburn cells present in the epidermis.

IL-6, IL-8, TNF-α and Galectin-7 quantification: On RHE culture supernatant, the quantification of IL-6, IL-8 and TNF-α secretion were performed with an ELISA test using a commercially available kit from R&D Systems (Human IL6 Quantikine ELISA kit, Human IL8 Quantikine ELISA kit, Human TNFa Quantikine ELISA kit, Human galectin-7 Duoset ELISA kit respectively). Dosages were realized according to the manufacturer's instructions.

MMP-1 expression

On NHDF culture media, MMP-1 quantification was performed by ELISA (Human MMP-1 ELISA kit, Abcam) according to manufacturer's instructions. Results were normalized for 1 million cells and expressed in percentage of the control 100%.

In tubo urocanic acid isomerisation under UVB exposure

Control solution contains *trans*-UCA (Fisher-Bioblock ACROS) at the concentration of 1 mM in citrate buffer (Sigma Aldrich) 0.01 M at pH5.5.
Test solution contains EPSE (diluted at 2%, 3% and 5% in the same citrate buffer) and *trans*-UCA 1 mM.
Solutions with and without EPSE were irradiated with UVB (Oven LabOsi) at room temperature in Petri dishes. Irradiation doses were 4, 8, 16, 32, 64, 128, 256 and 1000 mJ/cm^2.
After irradiation, aliquots of each condition were stored at +4°C in the dark. Each condition was performed in triplicate.
Each aliquot was analyzed by High Performance Liquid Chromatography HPLC (ProStar chromatograph, Varian).
HPLC analysis of the mixture post-irradiation enables to quantify the amount of *cis*-UCA produced (by trans-UCA measure) and the protective effect of EPSE was expressed in percentage of trans-UCA isomerisation compared to the control 100%.

Absorbance UV

UV/Visible spectra of EPSE and *trans*-UCA in aqueous solutions were performed and processed from a Lambda 25 UV/Vis Spectrophotometer (Perkin Elmer).

Melanin quantification

After trypsinization, the melanocytes were harvested and centrifuged at 900 rpm for 5 minutes. The pellet was resuspended in 100 ml of 1N sodium hydroxide (Carlo Erba) and heated at 60°C until complete dilution of the melanin contained in the melanocytes. The solution obtained was transferred to a 96-wells plate and the optical density measurement was performed using a plate reader spectrophotometer (MicroQuant, BioTek Instruments INC) at a wavelength of 405 nm. The melanin quantity for each condition was expressed in percent of the control condition.

Tyrosinase activity

After trypsinization, the melanocytes were harvested and lysed at 4°C in RIPA lysis buffer (VWR) in the presence of 0.5 M EDTA (GBiosciences) and antiprotease (GBiosciences) and then centrifuged at 900 rpm for 5 minutes. The supernatant was harvested and stored at 4°C. In a 96-wells plates, 20 µl of 3,4-Dihydroxy-L-phenylalanine or L-DOPA (Sigma) solution at 1.972 g/L in phosphate buffer 50 mM and 70 µl of each cell lysate were introduced for each condition. After the addition of the cell lysates and 90 minutes of incubation at 25°C, the measurement of the optical density was performed at 475 nm using a spectrophotometer (ELX808, BioTek Instrument INC). The tyrosinase activity for each condition was expressed in percent of the control condition.

Dark CPD

On fixed melanocytes, the number of CPD positive cells was assessed by immunofluorescence using an anti-thymine dimer antibody (Sigma) revealed by a

corresponding Alexa Fluor® 488-coupled secondary antibody (Invitrogen). Nuclei were counterstained with DAPI probe. For each section, the analysis was done on three microphotographs. DAPI positive nuclei and CPD positive cells were manually counted. The results are expressed as the ratio of CPD positive cells on total cells for each condition and expressed in percent of the irradiated UVB condition.

4) Results

4.1) Antioxidant activity of EPSE

In order to assess EPSE antioxidant capacity, we performed DPPH and ABTS assays (Table 1).

EPSE is endowed with a significant antioxidant activity with an IC_{50} = 0.9 +/- 0.1 g/L and 0.073 +/- 0.028 g/L for DPPH and ABTS assays respectively.
For comparison, the reference scavenger ascorbic acid at 1% has an IC_{50} = 0.42 g/L and 0.43 g/L.

	DPPH	ABTS
EPSE	IC_{50} = 0.84‰	IC_{50} = 0.074‰
Vit C (1%)	IC_{50} = 0.42‰	IC_{50} = 0.43‰

Table 1 – ESPE is a strong antioxidant.

Phaseoloidin presents a similar antioxidant capacity (DPPH assay, data not shown). This suggests that the EPSE antioxidative capacity is mostly due to its high concentration in phaseolodin that accounts for 3% of the extract.

4.2) Genoprotection and cell death

UV cause direct and ROS-mediated indirect DNA alterations such as pyrimidines (6-4) pyrimidone (6- 4PP), cyclobutane pyrimidine dimers (CPD), 8 oxo-guanine (8 oxoG) [5].

In order to assess the ability of EPSE to prevent DNA alteration and the resulting cell death, reconstructed epidermis were exposed to UVB in the presence or in the absence of EPSE (applied during irradiation for assessing the direct DNA alterations, and for an additional 5 hours for assessing the indirect DNA alterations). The number of resulting CPD was measured and the presence of sunburn cells (SBC) with pyknotic nucleus and the morphological aspect of the HRE was assessed by histological analysis.

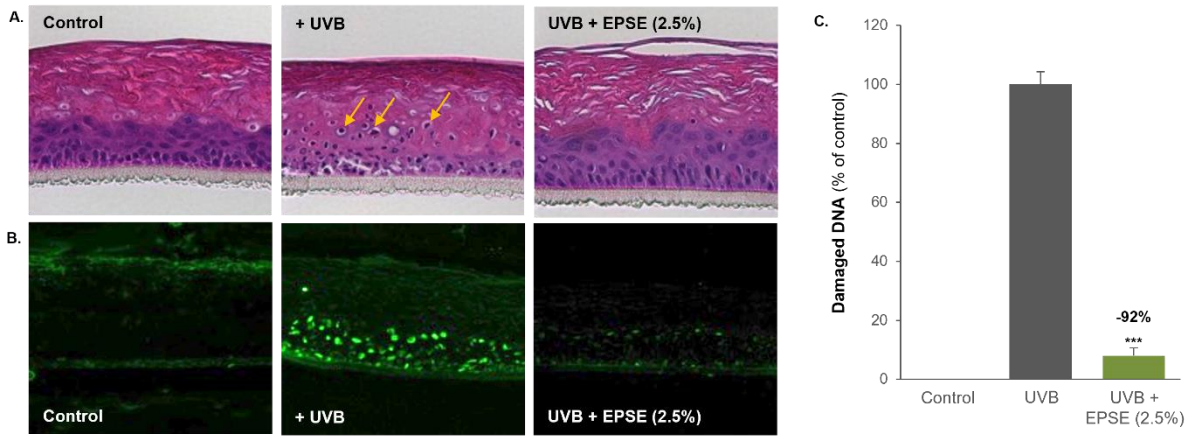

Figure 2 – EPSE prevents UVB-induced DNA alterations and cell death.
Microphotographs of HRE exposed to UVB (300 mJ/cm^2) in the presence or in the absence of EPSE (2.5%). (A) Morphological analysis. Sunburn cells are highlighted by the yellow arrows. (B) CPD were observed (in green) and (C) quantified.

After UVB exposure, RHE demonstrated a damaged morphology compared to control condition. Keratinocytes at the basal layers showed an impaired cohesion with the acetate cellulose filters. In the upper cell layers the keratinocytes presented vacuoles and SBC (Figure 2A).

The treatment with EPSE (2.5%) applied only during irradiation led to a 92% reduction of the number of UVB-induced DNA damages (Figure 2B, 2C).
In an alternative procedure, EPSE (2.5%) was applied during the irradiation and for 5 hours thereafter so that the antioxidant effect of EPSE can participate to the protection against indirect UV damage. We observed similar results with a 85% reduction in the number of CPD (data not shown).

Taken together, these results confirm that the active ingredient reduces cell death by preventing DNA damage in UVB-irradiated RHE.

Since UV may also induce a pro-inflammatory response in keratinocytes [7-10], we investigated the anti-inflammatory capacity of EPSE.

4.3) Anti-inflammation

Inflammation is a protective reaction that aims at neutralizing potentially harmful external elements of the body such as viruses or bacteria. However, an exposure to sunlight and especially UVB may cause an inflammatory response even in the absence of pathogens. Also known as erythema or sunburn, this reaction is characterized by redness, skin dehydration, painful oversensitivity, desquamation… [23].
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In order to assess EPSE ability to reduce UV-induced inflammation, RHE were exposed to UVB (300 mJ/cm^2) in the presence or in the absence of EPSE (1.5%). The secretion of several proinflammatory cytokines (IL-6, IL-8 and TNF-α) was measured using ELISA assays.

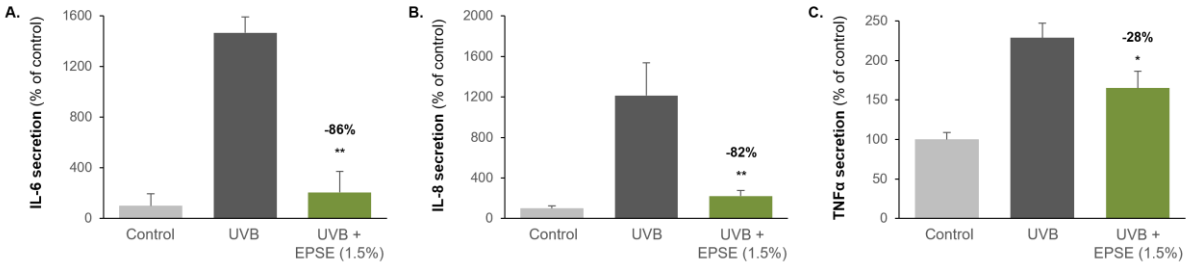

Figure 3 – EPSE has anti-inflammatory properties.
Quantification of IL-6 (A), IL-8 (B) and TNFα (C) secreted by HRE exposed to UVB (300 mJ/cm^2) in the presence or in the absence of ESPE (1.5%).

The treatment with EPSE (1.5%) decreases the UVB-induced secretion of IL6 by 86% hence completely negating the noxious effect of the UVB exposure (Figure 3A).
The treatment of HRE with EPSE (1.5%) led to a 82% and 28% decrease in the UV-induced secretion of IL8 and TNF-α respectively (Figure 3B, 3C).

Taken together, these results show that a topical treatment of EPSE led to a strong anti-inflammatory response after an UV exposure.

4.4) Immunosuppression

Several UV-induced mechanisms such as DNA damages, the release of pro-inflammatory cytokines and the isomerisation of *trans*-urocanic acid (UCA) into *cis*-UCA (Figure 4A), have all been reported to cause an immunosuppression in skin [9,12,24].

As a result, and besides the anti-inflammatory effects of EPSE against UVB, we also investigated the anti-immunosuppressive capacities of the ingredient.

EPSE is rich in entadamide A that is capable of absorbing UV in the same wavelength as trans-UCA (284 nm, Figure 4B) thus suggesting that EPSE could reduce the UV-induced isomerization of UCA. So, in order to assess this feature, *trans*-UCA was exposed to UVB in the presence or in the absence of EPSE. The formation of the immunosuppressive *cis*-UCA was monitored by HPLC.

EPSE limits UV-induced *cis*-UCA formation, indicating that UVB absorption by entadamide A partially avoids *trans*-UCA photo isomerization (Figure 4C). Thus, EPSE can limit UVB-induced immunosuppression.

Galectin-7 is described as being involved in UV-induced immunosuppression [12]. So, in order to assess the ability of EPSE to prevent the UV-induced galectin-7 overexpression, HRE were exposed to UVB (300 mJ/cm^2) in the presence or in the absence of EPSE and galectin-7 secretion was measured using an ELISA assay.

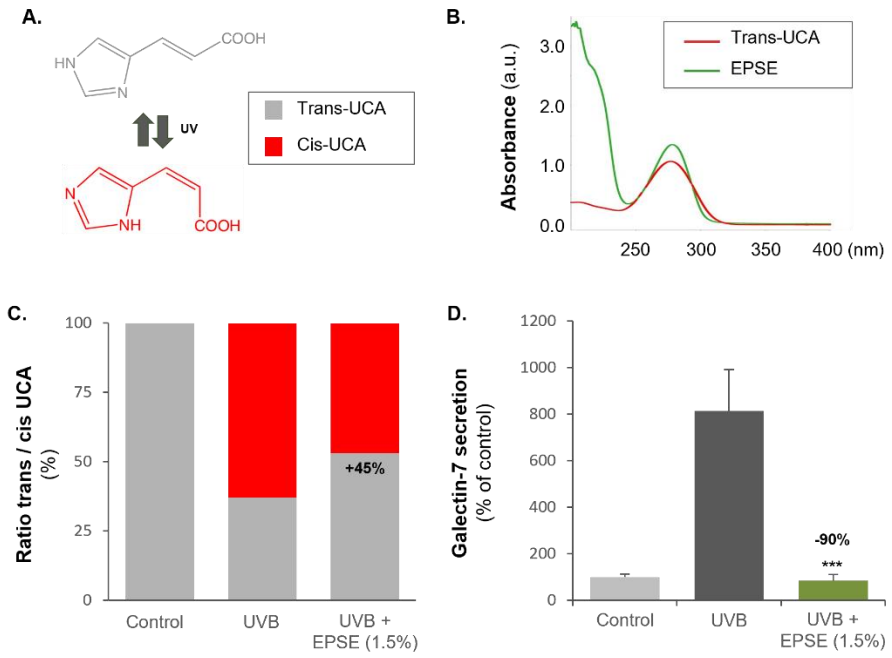

Figure 4 – EPSE has an anti-immunosuppressive activity by preventing UCA isomerisation. (A) UV-induced isomerization of trans-UCA into the immunosuppressive cis-UCA. (B) Ultraviolet absorption spectra of EPSE and trans-UCA. (C) Ratio of trans / cis UCA after an exposure to UVB (300 mJ/cm^2) in the presence or in the absence of EPSE (1.5%). (D) Quantification of galectin-7 secretion by HRE exposed to UVB in the presence or in the absence of EPSE (1.5%).

The topical treatment with EPSE (2.5%) led to a strong decrease (-90%) of galectin-7 expression (Figure 4D).

This observation is consistent with the previous result and, taken together, they show that EPSE is capable of preventing UV-induced immunosuppression.

UCA and melanin are the two main defensive mechanisms of the skin against UVB. And while UV-exposed UCA may cause immunosuppression, melanin is also affected and may have different effects on skin such as contributing to “dark CPD” generation [16].

4.5) Dark CPD

Both UVA and UVB produce reactive oxygen and nitrogen species (ROS and RNS), which together create a higher-energy form of melanin (chemoexcitation process). This new form of melanin contains the energy of a UV photon that is later transfered to DNA (chemiluminescence process) and may cause a delayed mutation, a “dark CPD” [16].

Since EPSE have antioxidant and chromophore abilities, we assessed its ability to protect skin cells from the “dark sun” that may cause melanin-induced delayed DNA alterations, the “dark CPD”. Melanocytes were thus exposed to solar irradiation (1.8 J/cm^2) and EPSE was later applied for 2 h. The relative number of “dark CPD” was then quantified.

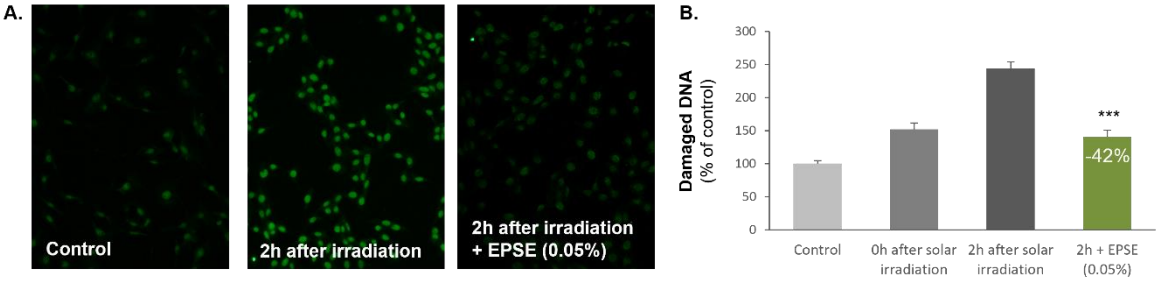

Figure 5 - EPSE prevents the apparition of Dark CPD.
Microphotographs of melanocytes exposed to sun light and then treated with ESPE for 2 h. Dark CPD were observed (A, in green) and quantified (B).

2 h after sun light exposure, a dramatic increase in the number of CPD was observed. The treatment with EPSE (0.05%) led to a 42% decrease in the number of CPD after 2 h of treatment (Figure 5A, 5B).

EPSE protects skin cells from the Dark sun as it reduces melanin-induced delayed DNA alterations.

4.6) Melanogenesis

While UCA and melanin are the main defensive mechanisms of the skin against UV, a sustained exposure to sunrays, chronic inflammation (inflamm'aging), oxidative stress, and/or hormonal changes may still trigger local melanin overproduction, resulting in non esthetic hyperpigmentary disorders, such as melasma, lentigines, dark circles or post-inflammatory hyperpigmentation [24].

The most widely used melanogenesis inhibitors (kojic acid, niacinamides, ascorbic acid and arbutin…) are inhibitors of tyrosinase, an enzyme that catalyzes the conversion of tyrosine to L-DOPA and further oxidizes it to dopaquinone, which is used for the ultimate formation of melanin. It was recently reported that entadamides, and especially entadamide A, show inhibitory activity for melanin production at the same level as arbutin [25].

So, in order to assess EPSE ability to inhibit melanogenesis, melanocytes received a 48 h treatment with EPSE (0.25%). Melanin production (Figure 6A) and tyrosinase activity (Figure 6B) were measured by spectrophotometry at 405 nm and 475 nm respectively.

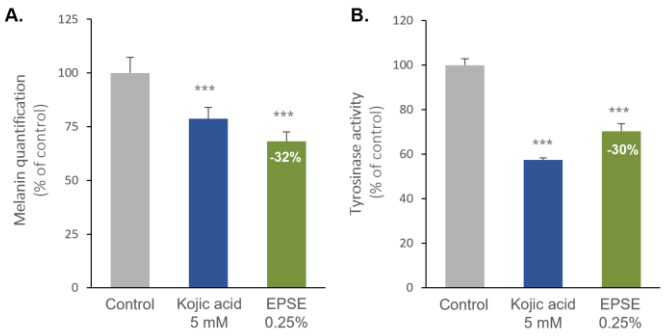

Figure 6 – EPSE reduces melanin production by inhibiting tyrosinase activity.
Quantification of melanin production (A) and tyrosinase activity (B) in melanocytes treated with EPSE.

The treatment with EPSE at 0.25% led to a drop in both the amount of melanin synthesized by melanocytes and tyrosinase activity with a 32% and 30% reduction respectively.

Taken together, these results suggest that EPSE is capable of reducing melanogenesis by inhibiting tyrosinase activity. Both observations support the use of EPSE to reduce skin colour (claims, skin brightener, skin lightener, skin tone unifier or skin whitening) or pigmentary disorders related to photo-ageing such as lentigines or pigmentary dark circles.

4.7) Blue light

It was shown that EPSE was capable of opposing the noxious effects of sun light (UVB and A). It is however known that the light affects skin from UVB to IR. As such, the visible spectrum of the light (44% of the solar spectrum) is also involved in the photoaging process. This is especially true for the blue light (that has the shortest wavelength of the visible spectrum and is therefore the most energetic) and which was described to promote matrix degradation by stimulating MMP-1 production [3]. Furthermore, the question of domestic artificial light participation to the aging process was raised.

In order to assess the ability of EPSE to protect the skin against artificial light, NHDF were exposed to blue light (30 J/cm^2) and incubated for 24 h in the presence or in the absence of EPSE (0.05%). MMP-1 expression was then quantified.

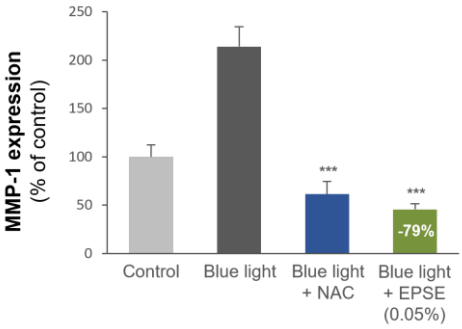

Figure 7 – EPSE protects skin from blue light.
Quantification of MMP-1 secreted by fibroblasts exposed to blue light in the presence or in the absence of EPSE (0.05%).

Blue light exposure led to a 2 folds increase in MMP-1 expression. The treatment with EPSE led to a 79% decrease in MMP-1 expression thus completely negating the effect of the blue light exposure (Figure 7).

This result suggests that EPSE is also capable of protecting the skin from an exposure to blue light, and more broadly from visible light.

5) Discussion & Conclusion

Our *in vitro* and *ex vivo* experiments have shown that EPSE, an extract obtained from *Entada phaseoloides* seeds, is effective against a large range of wavelengths (UVB, UVA, visible light). EPSE very efficiently limits UV-induced DNA damages, prevents the overexpression of key inflammatory cytokines (IL6, IL8…) and the setup of immunosuppressive mechanisms. Our mechanistic studies indicate that entadamide A, a specific UVB chromophore present in EPSE, is an important contributor to the photoprotective properties of the extract. Entadamide A is especially effective against photoimmunosuppression as it inhibits the isomerization of *trans*-UCA to the immunosuppressive *cis*-UCA. However, other constituents of EPSE significantly

participate to its photoprotective effect. Antioxidant polyphenols such as phaseoloidin efficiently scavenge ROS generated by UV and visible light, and anti-inflammatory constituents such as saponins contribute to reducing the formation of downstream inflammatory mediators. The combination of three mechanisms: UVB-absorption, antioxidation and anti-inflammation, enables a broad-spectrum photoprotection and supports the usefulness of EPSE for sun care or photoaging prevention. Furthermore, EPSE is also capable of inhibiting melanogenesis and may therefore prevent the apparition of melanin spots responsible for an uneven skin tone.

Taken together, the data presented here suggest that EPSE is an interesting active ingredient for any sun care products, by itself or in combination with sun filters, as it provides the skin with a broad-spectrum protection and prevent the apparition of melanin spots for a protected and even skin.

6) References

1. Passeron T and Ortonne JP (2003) le vieillissement cutané et sa prévention. Press Med 32 :1474-1482.

2. Krutmann J, Morita A, Chung JH (2012) Sun exposure: what molecular photodermatology tells us about its good and bad sides. J Invest Dermatol 132:976-984.

3. Liebel F, Kaur S, Ruvolo E, Kollias N, Southall MD (2012) Irradiation of skin with visible light induces Reactive Oxygen Species and matrix-degrading enzymes. J Invest Dermatol, 132:1901-1907.

4. Sinha RP, Häder DP (2002) UV-induced DNA damage and repair: a review. Photochem Photobiol Sci 1:225-36.

5. Halliday GM, Cadet J (2012) It's all about position: the basal layer of human epidermis is particularly susceptible to different types of sunlight-induced DNA damages. J Invest Dermatol 132:265-267.

6. Laethem AV *(*2005) The sunburn cell: regulation of death and survival of the keratinocyte. Int Biochem cell Biol 37:1547-1553.

7. Janda J, Burkett N, Blohm-Mangone K, Huang V, et al (2016) Resatorvid-based Pharmacological Antagonism of Cutaneous TLR4 Blocks UV-induced NF-κB and AP-1 Signaling in Keratinocytes and Mouse Skin. Photochem Photobiol 92: 816-825.

8. Schwarz T, Luger TA (1989) Effect of UV irradiation on epidermal cell cytokine production. J Photochem Photobiol B 4:1-13.

9. Yarosh DB, Boumakis S, Brown AB, Canning MT, Galvin JW et al (2002) Measurement of UVB-induced DNA damage and its consequences in models of immunosuppression. Methods 28: 55-62.

10. Amerio P, Carbone A, Auriemma M, Varatti S, Tulli A (2009) UV induced skin immunosuppression. Anti-inflamm Anti-Allergy Agents in Med Chem 8:3-13.

11. Gibbs NK, Norval M (2011) Urocanic in the skin: a mixed blessing? J Invest Dermatol 131: 14-17.

12. Yamaguchi T, Hiromasa K, Kabashima-Kub R, Yoshioka M, Nakamura M (2013) Galectin-7, induced by cis-urocanic and ultraviolet B irradiation, down-modulates cytokine production by T lymphocytes. Exp. Dermatol, 22:832-849.

13. Bernerd F, Sarasin A, Magnoldo T, (1999) Galectin-7 overexpression is associated with the apoptotic process in UVB-induced sunburn keratinocytes. Proc Natl Acad Sci USA 96:11329-11334.

14. Solano S, (2020) Photoprotection and Skin Pigmentation: Melanin-Related Molecules and Some Other New Agents Obtained from Natural Sources. Molecules 25 :1537; doi:10.3390.

15. Gillbro JM, Olsson MJ (2011) The melanogenesis and mechanisms of skin-lightening agents–existing and new approaches. Int J Cosmetic Sci 33: 210–221.

16. Premi S, Wallisch S, Mano CM, Weiner AB et al. (2015) Chemiexcitation of melanin derivatives induces DNA photoproduct long after UV exposure. Science 347: 842-847.

17. Nakashima Y, Ohta S Wolf AM (2017) Blue light-induced oxidative stress in lie skin. Free Radic Biol Med, 108: 300-310.

18. Deepa C, Shinde NW (2017) Proven activities of *Entada phaseoloides* (L.) Merr. Int J Curr Res Biosci Plant Biol 4:92-99.

19. Aktar F, Kuddus R, Faruque O, Rumi F, Quadir MA, Rashid MA (2011) Antioxidant, cytotoxic, membrane stabilizing and antimicrobial activities of bark and seed of *Entada phaseoloides* (L.) Merrill: A medicinal plant from Chittagong Hill tracts. J Pharm Nutr Sci 1:171-176.

20. Ikegami F, Sekine T, Aburada M, Fujii Y, Komatsu Y, Murakoshi I (1989) Synthesis of entadamide A and entadamide B isolated from *Entada phaseoloides* and their inhibitory effects on 5-lipoxygenase. Chem Pharm Bull 37:1932-1933.

21. Dawane JS (2011) Experimental evaluation of anti-inflammatory effect of topical application of *Entada phaseoloides* seeds as paste and ointment. North Amer J Med Sci 3:513-517.

22. Seguin MC (2021) Method for producing an *Entada* seed extract enriched with metabolites of interest, extract produced by such a method, and cosmetic and dermocosmetic applications of such an extract. United States Exsymol (Monaco MC) 20210106515; https://www.freepatentsonline.com/y2021/0106515.html

23. Clydesdale GJ, Dandie GW, Muller HK (2001) Ultraviolet light induced injury: Immunological and inflammatory effects. Immunology and Cell Biology 79: 547–568.

24. Ortonne J-P, Passeron T (2005) Melanin pigmentary disorders: treatment update. Dermatol Clin 23:209-226.

25. Sugimoto S, Matsunami K, Otsuda H (2018) Biological activity of *Entada phaseoloides* and *Entada Rheedei*. J Nat Med, 72:12-19.