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Birch Sap (*Betula alba*) and Chaga Mushroom (*Inonotus obliquus*) Extracts Show Anti-Oxidant, Anti-Inflammatory and DNA Protection/Repair Activity *In Vitro*

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Abstract

OBJECTIVE: The skin interacts strictly with the surrounding environment. Despite an efficient system of protection, its integrity is continuously assaulted by a massive group of external stresses. UV irradiations represent one of the most harmful factors for the cutaneous tissue. Both UV-A and UV-B can induce deep modifications of the different layers of the skin, including a weakening of its barrier function properties, DNA damages and degradation of the extracellular matrix. The aim of this project was to assess the UV protection activity of two natural compounds, the birch sap from *Betula alba* and organic extract from *Inonotus obliquus* (chaga mushroom) used separately or in a complex. **METHODS**: The anti-oxidant (ROS and MDA quantification, catalase and SOD activity measurement), anti-inflammatory (IL-1β, IL-6, IL-8, IL-10, TNF-α and INF-γ dosages) and the DNA protection/repair activities (DNA lesion site analysis) of birch sap and chaga mushroom extracts tested separately or in a complex containing organic birch sap 5% and *Inonotus obliquus* extracts 2% were evaluated *in vitro* after exposure of cultured keratinocytes and fibroblasts or reconstructed epidermis to UV-A/UV-B irradiations. **RESULTS:** We observed that birch sap from *Betula alba* and extracts from *Inonotus obliquus* prevent the formation of ROS and decrease the oxidative stress induced under UV irradiations, suggesting a strong anti-oxidant activity. In addition, the tested products showed an immunomodulatory effect by reducing the quantity of pro-inflammatory cytokines upon UV irradiations. UV-induced DNA damages of keratinocytes were also reduced by birch sap and chaga mushroom extracts. **CONCLUSION:** Here, for the first time, we have shown the photo-protection activity of extracts ob-

tained from *Betula alba* and *Inonotus obliquus* mushroom on skin cells exposed to UV-A and UV-B irradiations. Due to their anti-oxidant, anti-inflammatory and DNA protection/repair activities, the tested products represent promising candidates in the development of cosmetic products with anti-photo-aging activity.

Keywords

Birch Sap, Chaga Mushroom, UV Irradiations, Oxidative Stress, Photo-Aging, Natural Compounds

1. Introduction

The skin is an indispensable biological barrier, providing an efficient line of defence against the continuous assaults of the external environment such as air pollutions, pathogenic microorganisms and ultraviolet (UV) irradiations.

UV irradiations represent one of the most hazardous environmental factors for human skin. Based on their wavelength, they can be classified as UV-A (315 - 400 nm), UV-B (280 - 315 nm) and UV-C (100 - 280 nm).

The ambient sunlight is composed mainly by UV-A (90% - 95%) and UV-B (5% - 10%), while UV-C cannot reach the Earth's surface due to the absorbing properties of ozone layer.

Since UV penetration in the skin depends strictly on their wavelength [1], UV-A penetrate profoundly into the dermis, reaching the upper reticular dermis. Nevertheless, they can react also with both *stratum corneum* and epidermis. Inversely UV-B are absorbed mainly by the different layers of the epidermis, where they trigger a cell damage response.

UV-A are commonly associated with the induction of oxidative stress in the different cutaneous compartments through the induction of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide and singlet oxygen.

ROS can react easily with nucleotides, causing DNA mutations. One of the most common ROS-induced DNA damages is the formation of 8-hydroxy-2'-deoxyguanine (8-OHdG), which consists in the oxidation of guanine at the 8th position [2] leading to the guanine to thymidine transversion.

On the contrary, UV-B show a direct genotoxic effect since they are strongly absorbed by the DNA. Consequently, several photo-lesions can be induced upon UV-B exposure including cyclobutane pyrimidine dimers or pyrimidone photoproducts [3].

The deleterious effects of both UV-A and UV-B on skin barrier properties have also been demonstrated.

As previously described, UV-A irradiations reduce the level of skin anti-oxidants, such as vitamin E in the *stratum corneum* [4], induce lipid peroxidation [5] and decrease the activity of enzymes with anti-oxidant functions such as catalase and superoxide dismutase (SOD), affecting the protective role and the

anti-oxidant properties of the whole skin tissue [6].

In parallel UV-B cause abnormalities in lamellar body secretion [7] and in lipid cohesion [8] resulting in altered skin barrier functions. In addition, like UV-A irradiation, UV-B are also able to decrease the activity of enzymes with anti-oxidant roles [9].

More recently, thanks to the development of methods allowing the simultaneous monitoring of a wide number of genes, the impact of UV-A and UV-B on the skin transcriptome has also been characterized.

As well described by Zheng and collaborators [10], repetitive exposure of dermal fibroblasts to UV-A induces the modulation of 607 genes, with 238 up-regulated and 369 down-regulated genes. In particular, the expression of genes encoding for structural proteins of the extracellular matrix, including elastin, was decreased. Interestingly, the expression of *SPRY*1 was significantly induced upon UV-A irradiation. *SPRY*1 encodes for a protein, named sprouty, involved in cell signalling and able to induce the expression of proteins playing a key role in degradation of extracellular matrix, including matrix metalloproteins (MMPs) [11].

As expected, these data converge with the largely described mechanism of skin photo-aging [12], giving new insights in the cellular process leading to structural and physiological changes of the whole cutaneous tissue.

In a previous work, the team of Li and collaborators [13] have analysed the impact of acute UV-B irradiations on the transcriptome of epidermal keratinocytes *in vitro*. Following UV-B exposure, 198 genes were differentially regulated in a time-dependent manner. More specifically, three waves of time points were observed: an early (0.5 to 2 hours), an intermediate (4 to 8 hours) and a late (16 to 24 hours) point of regulation. In addition, seven main categories of genes were modulated upon UV-B irradiation including genes encoding for proteins involved in DNA protection and repair such as gadd 45, cyclin G1, and BTG2, genes encoding for signal transducers and transcriptional factors like junB, junD, c-fos and ETR101 and genes encoding for chemokines, cytokines and growth factors. In particular, five members of CXCL8 chemokine family were induced, including interleukin-8 (IL-8).

Overall, UV-B are able to induce the over-expression of other class of cytokines with anti-inflammatory activity, including IL-1, IL-6 and *tumor necrosis factor*-(TNF)-α [14].

Due to the multiple deleterious effects of both UV-A and UV-B irradiations on skin biology and physiology, the development of active ingredients and end-products with an efficient activity against UV exposure remains a current concern of the cosmetic industry.

The mushroom *Inonotus obliquus* (IO), also commonly called Chaga, is a parasitic fungus growing on trees such as birches and is used in several folk medicines. The pharmacology of Chaga mushroom extract has been studied for its antioxidant activity as free radicals scavenger on human keratinocytes [15], antiviral activity against hepatitis C virus [16], and more recently for its cytotoxicity

activity on cancerous cells [17]. In the dermocosmetic field, *Inonotus obliquus* extract has shown anti-aging [18] and anti-melanogenic effects that are of interest for the treatment of hyperpigmentation [19].

Birch sap (BS) has a broad ethnobotanical use and is consumed as a simple beverage because of the richness of its composition (organic acids, vitamins, carbohydrates, and mineral substances) offering wide benefits in medicine and cosmetic [20] [21]. However, very little is known and published about the biological properties of *Betula alba* (white birch) sap on skin cells.

Recently, the use of molecules of plant and marine origin in cosmetic products targeted against the damages caused by UV irradiation increased considerably [22].

The aim of the present work was to assess the skin protective effects against UV irradiations of the two natural compounds, the aqueous organic extract from *Inonotus obliquus* and the birch sap collected from *Betula alba*. Given the relevant properties of the two compounds used separately it was of great interest to study the combined biological properties of these two compounds in a complex composed by 5% of organic birch sap and 2% of chaga mushroom extracts. Unexpectedly, the complex revealed to have increased protective and repairing activities on several skin cells models.

2. Experimental Procedure

2.1. Complex Manufacturing Process

Birch sap has been harvested in Cantal and Puy-de-Dôme, in the heart of the Volcansd' Auvergne Regional Nature Park. It was collected in the spring, every morning, by light excision of the tree.

Organic *Inonotus obliquus* has been also harvested in the region of Auvergne in the department of Cantal and Puy de Dome directly on the birch tree.

Aqueous concentrated cryoextract of organic *Inonotus obliquus* was obtained by cryo-grinding followed by aqueous extraction and clarification.

For both extracts, citric acid and preservatives were added before decontamination by filtration (0.2 µm) and the final complex described in this study was composed of organic birch sap 5% and *Inonotus obliquus* 2%.

2.2. Cell Isolation and Culture

Keratinocytes were isolated from foreskin of a two-year-old Caucasian boy obtained by surgery. Cell viability was estimated about 96% with a doubling time of 28 hours. The following study was performed on cells grown in a 6-well plates, with 2.10^6 cells per well. Cells were grown in a keratinocyte medium supplemented with 0.4% bovine pituitary extract, 0.125 ng/mL human recombinant epithelial growth factor, 5 µg/mL insulin, 0.33 µg/mL hydrocortisone, 10 µg/mL human transferrin, 0.39 µg/mL epinephrine and 0.15 mM calcium chloride.

Foreskin fibroblasts were obtained after routine circumcision and grown in

RPMI 1640 medium (Gibco®-Thermo Fisher Scientific, Les Ulis, France) supplemented with Foetal Bovine serum (FBS), L-Glutamine and Gentamicine. Fibroblasts were used exclusively between the 2nd and 4th passages.

For keratinocytes/fibroblasts co-culture, cells were grown in KGM2 medium (Clonetics™-Thermo Fisher Scientific, Les Ulis, France).

2.3. Reconstructed Human Epidermis

Human epidermis was reconstituted using the CellSystem® model (CellSystem®, Troisdorf, Germany). Briefly, human keratinocytes were grown on 0.5 cm^2 polycarbonate filters in a modified MCDB 153 medium (Sigma Aldrich, Saint-Quentin Fallavier, France). Cells were grown for 14 days at the air-liquid interface and growth medium was changed every other day. Epidermis were used after 17 days of culture.

2.4. Cell Viability Assay

The cytotoxicity of each complex was assessed using the colorimetric MTT [3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide] assay (Abcam, Cambridge, USA). Reconstructed epidermis treated for 24 hours with the different tested products were incubated for 3 hours at 37˚C with the MTT reagent (**Figure S1**). Dimethyl sulfoxide was then added to dissolve formazan crystals and the absorbance was measured at 570 nm.

2.5. Malondialdehyde (MDA) Extraction and Quantification

The lipid peroxidation was determined by measuring MDA content in reconstructed epidermis. After 24 hours of treatment with the tested products and UV-A (5 J/cm^2)-UV-B irradiations (150 mJ/cm^2) (**Figure S1**), epidermis homogenates were resuspended in a specific extraction buffer containing Tris-HCl 50 mM, NaCl 0.1 M, EDTA 20 mM, HCl 0.1 N and thiobarbituric acid (TBA) 0.67%. Cells were incubated for one hour at 50˚C in the dark, chilled in cold water and n-butanol was added. Samples were centrifuged at 10,000 g at 0˚C for 10 min and the upper phase was collected for MDA detection. The MDA-TBA complex was separated on a High Performance Liquid Chromatograph (HPLC) equipped with a Bischoff Model 2.200 pump (Bischoff Chromatography, Leonberg, Germany), on a Ultrasep C18 (30 cm × 0.18 cm, 6 mm porosity) column. The MDA-TBA complex was eluted with methanol: H_2O (40:60, v/v) and monitored by fluorescence detection through a 821-F detector (Jasco, Easton, USA) with excitation at 515 nm and emission at 553 nm. A standard curve was used for the quantification.

2.6. Reactive Oxygen Species (ROS) Quantification

ROS were quantified after keratinocyte UV-irradiation [(UV-A (5 J/cm^2) + UV-B (100 mJ/cm^2)] following a 0 min, 30 min and 3 hours kinetics using a solar simulator (VilberLourmat, Collégien, France) (**Figure S1**). For mitochondrial

ROS (mROS) quantification, human keratinocytes were incubated with MitoSOX™ Red reagent (Thermo Fisher Scientific) for 15 min at 37˚C, in the dark. Cells were then washed twice with Phosphate Buffer Saline (PBS) and analysed by flow cytometry. In parallel, cells were incubated with the CM-H_2DCFDA probe (Thermo Fisher Scientific) for 15 min at 37˚C, in the dark, for the cytoplasmic ROS detection. Cells were washed, as previously, twice in PBS 1X and 1 × 10^4 cells were collected for flow cytometry analysis.

2.7. Superoxyde Dismutase (SOD) and Catalase Activities Quantification

According to the same experimental procedure used for ROS quantification (**Figure S1**) Cells were sonicated twice in Tris-HCl (pH 7.5), for 30 sec, and centrifuge for 10 min at 12,000 g. Samples were kept at −80˚C and total protein concentration was measured by BCA kit (Thermo Fisher Scientific). SOD activity was measured using a SOD-WST kit (Dojindo Molecular Technologies, Munich, Germany) and catalase activity by the Amplex Red Assay Kit Catalase (Thermo Fisher Scientific). Calibration curves were prepared using purified enzyme preparation. One unit of catalase will decompose 1 μmole of H_2O_2 per minute in optimal enzymatic conditions (pH 7.0, 25 ˚C). One unit of SOD activity corresponds to the enzyme quantity required to inhibit 50% of WST-1 formazan per minute.

2.8. Cytokine Detection and Quantification

Fibroblasts/keratinocytes co-cultured cells were plated one day before the experiment in a serum-free growth medium. The different compounds were applied to the cells for 2 hours before cell activation by UV-A/UV-B exposure (**Figure S1**). Cell culture supernatant (25 μL) was collected after 48 hours and analysed with FlowCytomix for pro- and anti-inflammatory cytokines detection (Bender Med-Systems, Wien, Austria).

2.9. Oxidized Protein Quantification

Protein concentration was determined on reconstructed epidermis using Bradford protein quantification assay (Bio-Rad, Hercules, USA) and absorbance values were measured at 595 nm using bovin serum albumin (BSA, 10 μg/mL) as standard solution.

Protein oxidation in BSA standard and in samples treated for 24 hours and irradiated by UV-A (5 J/cm^2) and UV-B (100 mJ/cm^2) (**Figure S1**) with the complex were analysed using the OxiSelect™ Protein Carbonyl ELISA Kit (Clinisciences, Nanterre, France) following manufacturer's instructions.

2.10. Mitochondrial DNA (mDNA) Extraction, Amplification and DNA Damage Quantification

To assess the complex protective effect on keratinocytes, cells were treated with

the complex (5% BS + 2% IO for 150 min), UV-B irradiated (100 mJ/cm^2) and collected 24 hours later (Figure S1). In order to study the repairing effect, cells were first irradiated with UV-B, grown for 3 hours and the complex was then added to the culture medium for 24 hours (Figure S1).

DNA was extracted using Invisorb® Spin DNA Extraction kit (Eurobio, Les Ulis, France) following manufacturer's instructions. Due to a low proportion in the total DNA extract, mDNA was amplified using REPLI-g Mitochondrial DNA (Qiagen, Courtaboeuf, France) and DNA damage [apurinic/apyrimidinic (AP) sites] were quantified in the samples using DNA Damage quantification kit (Biovision, Mountain View, USA) following manufacturer's instructions. The optical density (OD) was analysed at 450 nm and sample DNA readings were applied to the calibration curve prepared with standard Aldehyde Reactive Probe-DNA (ARP/DNA) solutions (Dojindo Molecular Technologies).

2.11. Statistical Methods

The data were analyzed statistically using Microsoft Excel®. Error bars represent the standard deviation of three independent experiments. Differences were considered statistically significant when p-value < 0.05 using Student's t-test.

3. Results

3.1. Cell Viability Assay

Cells metabolic activity was measured by MTT assay after 24 hours of treatment on reconstituted epidermis. No significant effect was observed on cell proliferation for the organic BS at 5%, IO extracts at 2% or with the combination of the two compounds (BS 5% + IO 2%) compared to the control (Figure 1).

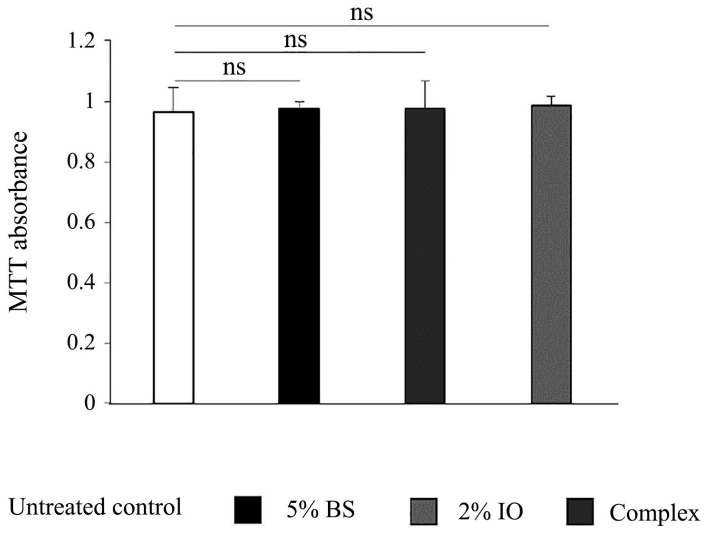

Figure 1. Effect of birch sap, *Inonotus obliquus* extracts or the complex on cell viability. The cytotoxicity of each compound was tested on reconstituted epidermis after 24 hours of treatment using MTT assay. Bars show the absorbance measured for each condition. ns: not significant. BS: Birch sap, IO: *Inonotus obliquus*. Complex: BS 5% + IO 2%.

3.2. Antioxidant Effect

The antioxidant effect was studied on UV-irradiated reconstituted epidermis after 24 h treatment with the different compounds. Lipid peroxidation was measured by MDA detection. Increased MDA detection was significantly reduced by epidermis treatment with the BS 5% (−22%, $p < 0.05$) or the IO 2% alone (−28%, $p < 0.01$) before oxidative stress induction by UV-irradiation. The complex of the two compounds showed higher reduction of lipid peroxidation revealing a cumulative effect (−41%, $p < 0.01$) (Figure 2(a)). Detection of protein oxidation was also measured in the same conditions and showed significant reduction of the oxidative stress in the BS 5%-, IO 2%- or complex-treated samples (−31%, −35%, −49%, respectively) with higher antioxidant effect of the complex.

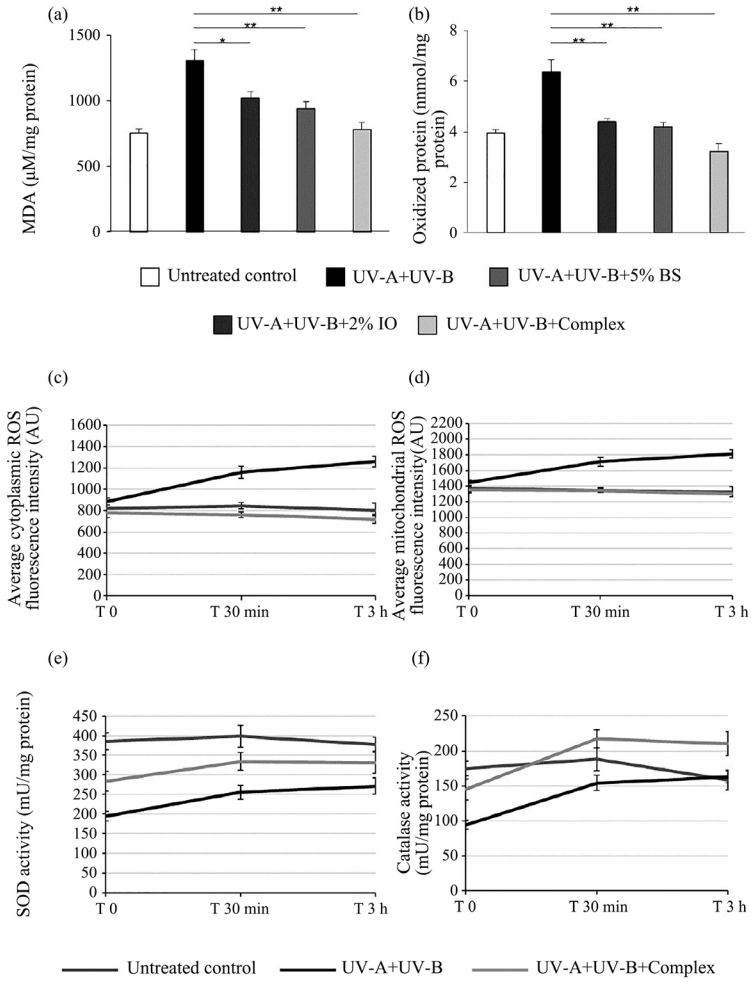

Figure 2. Anti-oxidant activity analysis on reconstituted epidermis or cultured keratinocytes. (a) (b) Bar graphs represent MDA (a) and oxidized protein (b) quantification after UV-A (5 J/cm^2) + UV-B (100 mJ/cm^2) irradiation or not (untreated control). Reconstituted epidermis were treated for 24 hours with the indicated compounds before irradiation. (c)-(f) Cytoplasmic (c) and mitochondrial ROS (d) production kinetics, superoxyde dismutase (e) and catalase (f) activity kinetics after keratinocytes UV irradiation for 0, 30 minutes or 3 hours. BS: Birch sap, IO: *Inonotus obliquus*. Complex: BS 5% + IO 2%.

Cytoplasmic and mitochondrial ROS kinetics were followed 0 min, 30 min and 3 hours after UV-A + UV-B irradiation. The keratinocytes treatment with the complex significantly reduced the production of both cytoplasmic (**Figure 2(c)**) and mitochondrial ROS (**Figure 2(d)**) (−43%, $p < 0.001$ and −28%, $p < 0.001$ respectively in comparison with the irradiated batch) measured after 3 hours incubation, and showed a kinetic similar to the unirradiated cells (untreated control). In parallel, the antioxidant enzyme activity of the SOD (**Figure 2(e)**) and the catalase (**Figure 2(f)**) were determined in the same conditions. Both enzymes activities were significantly increased by the BS 5% + IO 2% complex treatment prior keratinocytes irradiation (+22%, $p < 0.001$ and +29%, $p < 0.001$ respectively) revealing the protective property of the complex on SOD and catalase activity. Together these results showed the antioxidant properties of the BS 5% + IO 2% complex against UV-induced oxidative stress.

3.3. Immunomodulatory Effect on Human Keratinocytes/Fibroblasts Co-Culture

Skin UV irradiations induce keratinocytes activation with an inflammatory response and cytokines production that can further damage the cells. Immunomodulatory effect was investigated on human keratinocytes/fibroblasts co-culture after 2 hours of treatment with the different compounds followed by keratinocytes activation in response to UV irradiation (UV-A + UV-B). Flow cytometry quantitative detection in the growth medium of various interleukins [Il-1β, IL-6, IL-8, IL-10, TNF-α and interferon-γ (IFN-γ)] showed a significant reduction of secretion in the BS or IO extracts keratinocytes treated conditions, with an even stronger decrease with the BS + IO complex treatment (**Figures 3(a)-(f)** and **Table 1**). These cytokines are involved in acute and chronic inflammatory response after UV irradiation, revealing the anti-inflammatory activity and the modulation of immune response by the organic birch sap and chaga mushroom extract used separately or in a complex.

3.4. UV-B Protective and Repairing Effect

UV-B irradiation-induced DNA damage, measured by the number of apurinic/apyrimidinic sites, were significantly reduced (−41%, $p < 0.01$) in comparison with the control after 150 min treatment with the complex revealing the protective effect on human keratinocytes (**Figure 4(a)**). Additionally, the BS 5% + IO 2% complex showed a regenerative effect with a significant lower (−27%, $p < 0.01$) DNA damage quantity when the complex was applied for 24 hours on the cells after UV-B-induced DNA damage (**Figure 4(b)**).

4. Discussion

As described by Krutmann and collaborators in 2017 [23], the skin exposome regroup the totality of internal and external factors that affect the cutaneous compartment, causing the acceleration of skin ageing process.

Table 1. Cytokines quantification on keratinocytes/fibroblasts co-culture after UV-A + UV-B irradiation in the different conditions. Percent change from untreated UV-irradiated control. BS: Birch sap, IO: *Inonotus obliquus*.

Interleukins	Il-1β	Il-6	Il-8	Il-10	TNF-α	IFN-γ
BS 5%	−28%	−26%	−22%	−24%	−24%	−22%
IO 2%	−35%	−32%	−27%	−30%	−29%	−24%
BS 5% + IO 2%	−48%	−49%	−38%	−40%	−48%	−38%

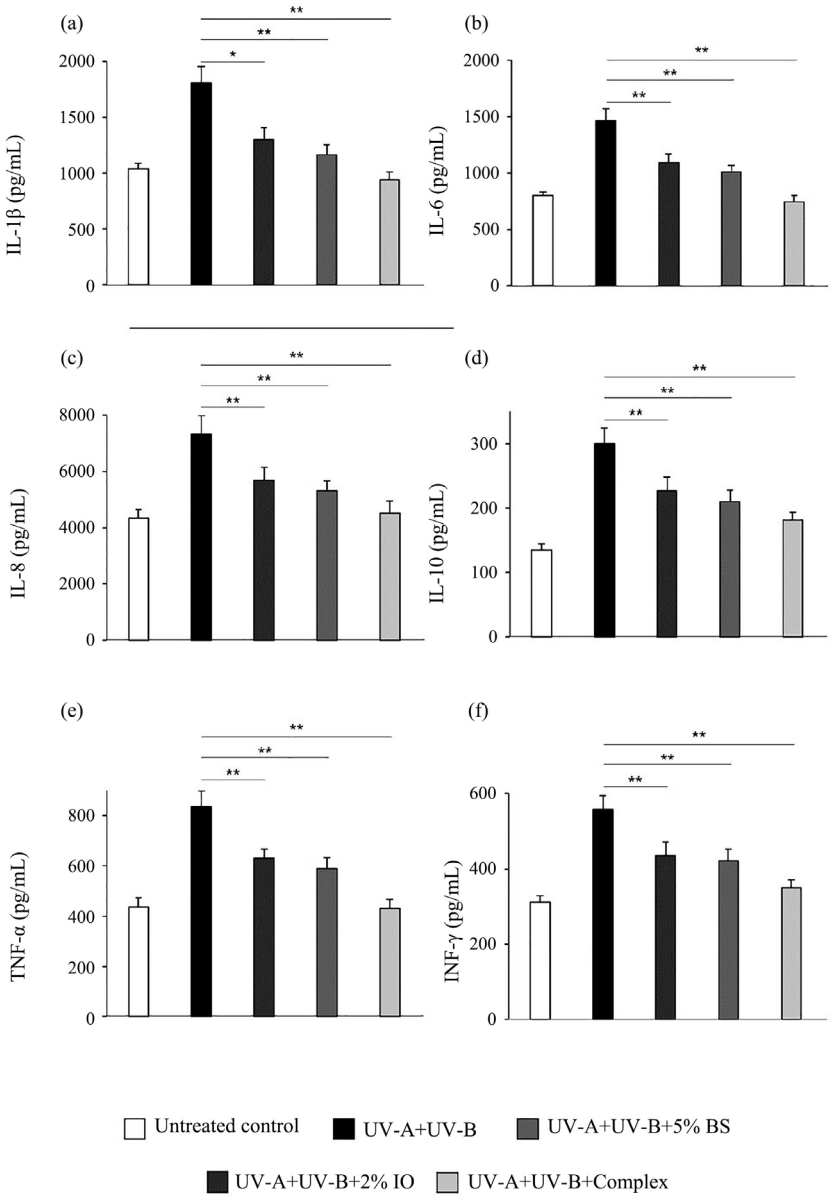

Figure 3. Complex immunomodulation effect on keratinocytes/fibroblasts co-culture after UV-A+UV-B irradiation. (a)-(f) Bar graphs represent interleukin IL-1β (a), interleukin IL-6 (b), interleukin IL-8 (c), interleukin IL-10 (d), TNF-α (e) and IFN-γ (f) quantification after UV-A (5 J/cm^2) + UV-B (100 mJ/cm^2) irradiation or not (untreated control). Cells were treated for 2 h with the indicated compounds before cells activation. BS: Birch sap, IO: *Inonotus obliquus*. Complex: BS 5% + IO 2%.

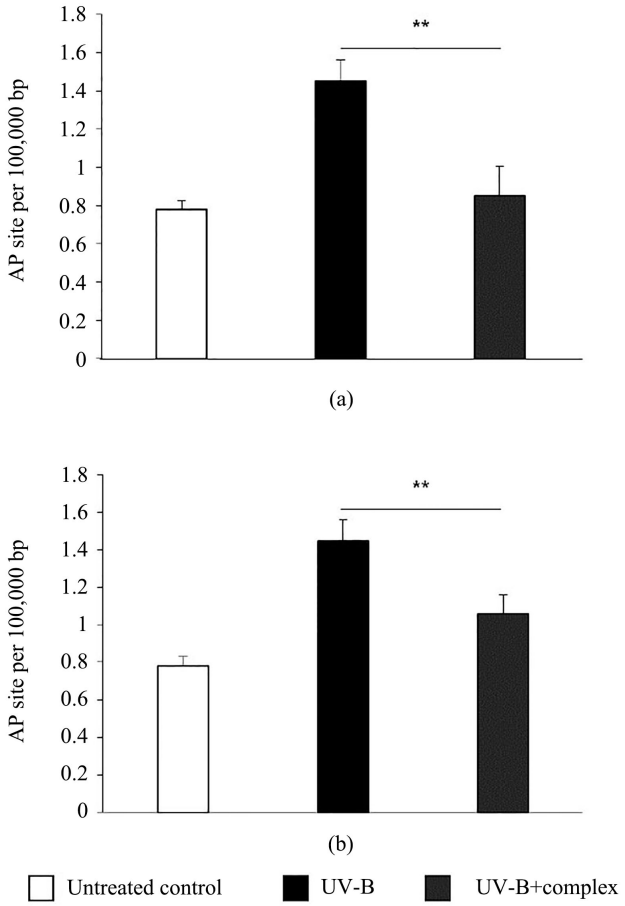

Figure 4. UV-B protective and regenerative effect on UV-B irradiated human keratinocytes. (a) (b) Complex (BS 5% + IO 2%) protective (a) and regenerative (b) effect against UV-B induced mDNA damages.

UV irradiations, including UV-A and UV-B, is a major component of skin exposome altering the physiology and integrity of epidermal cells at different levels.

One of the first effect of both UV-A and UV-B irradiations is the formation of free radicals, leading to the generation of a deleterious oxidative stress [24].

In particular, the abnormal production of ROS triggered by UV exposure may cause protein and lipid peroxidation inducing the formation of highly reactive carbonyl species including MDA and 4-hydroxy-2-nonenal [25].

In the present work we observed that birch sap, chaga mushroom extracts and the tested natural complex composed by 5% of organic birch sap and 2% of chaga mushroom extracts were able to decrease significantly the content of both cytoplasmic and mitochondrial UV-induced ROS in cultured keratinocytes.

As expected, the tested products decreased strongly also the content of MDA and oxidized proteins following UV irradiations.

Interestingly, we observed that BS and IO extracts protect keratinocytes *in vitro* against the SOD and catalase decrease activity provoked by UV irradiations.

SOD and catalase are important enzymes showing a redoubtable anti-oxidant

activity, which protect the skin against the oxidative stress induced by UV irradiations [26] by reducing free radical content.

In this context it should be noted that birch sap extract shows a relatively high content of different metallic elements, including copper and zinc [27], both metals presenting an important anti-oxidant activity. In particular, it has been previously shown that copper is able to induce SOD transcription and activity in cultured fibroblasts [28] [29]. The presence of copper and zinc in birch sap extracts could explain the strong anti-oxidant activity of the BS 5% - IO 2% complex against UV irradiations.

In parallel chaga mushroom extract are rich in polyphenols [27], previously described as potent anti-oxidant [30]. As a consequence, the association of BS and IO extracts in the natural complex tested in the present work presents an important activity against the oxidative stress induced by UV-A and UV-B irradiation *in vitro.*

As previously mentioned, the exposure of the skin to UV-A and UV-B can produce important damages to both genomic and mitochondrial DNA [2] [3] contributing profoundly to skin-age associated modifications [31]. Here we demonstrated that birch sap and chaga mushroom extracts exhibit a significant DNA protection and repair activity at both nuclear and mitochondrial levels.

Different mechanisms could be involved in the observed activities. Firstly, organic BS extracts are rich in zinc. As previously described, zinc is able to protect against apoptosis and DNA damages induced by UV-A and UV-B irradiation in HaCat keratinocytes and fibroblasts *in vitro* [32] [33]. As a result, the presence of zinc in the natural complex tested herein could explain in part the observed activity.

Secondly, polyphenols of chaga mushroom extract in addition to their anti-oxidant activity could act as a sunscreen due to their ability to absorb the whole UV-B spectrum and a part of UV-A spectrum [34]. In parallel, polyphenols are able to repair the UV-induced cyclobutane pyrimidine dimers *via* the stimulation of IL-12 expression [35] and by inducing the expression of genes encoding for proteins involved in nucleotide excision repair [36].

The role of UV exposure in the initiation of an important inflammatory process in the cutaneous tissue has also been documented [37]. The response of the skin to UV irradiation starts with the synthesis of IL-1, IFN-γ and TNF-α which in turn induce the synthesis of other pro-inflammatory molecules in keratinocytes such as IL-6 and IL-8. In addition, UV irradiation can induce the synthesis of IL-10, a cytokine acting to attenuate inflammatory damages in the skin [38].

Moreover, the cytokine cascade induced upon UV irradiation triggers the activation of the transcriptional factors AP-1, which in turn regulates positively the expression of several MMPs, including MMP-1, MMP-3 and MMP-9 resulting in extracellular matrix degradation [39].

Here we observed that birch sap and chaga mushroom extracts tested separately or in combination in a natural complex decrease significantly the content

of different pro-inflammatory cytokines, including IL-1β, IL-6, IL-8, TNF-α and IFN-γ, following the exposure of human keratinocytes/ fibroblasts co-culture to UV-A and UV-B. Consequently, by reducing the inflammatory response of keratinocytes to UV irradiations, lower levels of IL-10 were also detected.

On one hand, this activity could be explained by the presence in birch sap extracts of botulin and betulinic acid, two molecules with a described anti-inflammatory activity [40].

On the other hand, *Inonotusoubliquus* extracts are rich in compounds presenting an anti-inflammatory activity including ergosterols, lanosterol, inotodiol and trametenolic acid [41] [42] which could be able to reduce the inflammatory cascade induced in skin cells after UV irradiations.

5. Conclusions

In the work described here, we demonstrated for the first time that birch sap and chaga mushroom extracts protect the skin against the UV-induced damages. In particular we have shown that these natural compounds present a strong DNA protection and repair activity as well as anti-oxidant and anti-inflammatory properties on UV-exposed keratinocytes and fibroblasts *in vitro*. Nevertheless, this study has been conducted on cellular models lacking the biological complexity of the skin tissue. In this sense, the photo-protection activity of birch sap and chaga mushroom extracts should be confirmed on 3D skin models, such as reconstructed epidermis or human skin explants *ex vivo*. These biological models are composed of different types of skin cells and their use could help to better characterize the protection activity of birch sap and chaga mushroom extracts against UV.

Moreover, the use of human skin explant *ex vivo* as experimental model could elucidate the kinetics of penetration of both extracts across the *stratum corneum*.

The results obtained here, in particular the anti-oxidant and the anti-inflammatory properties, suggest that birch sap and chaga mushroom extracts complex might present additional activities of interest for the dermocosmetic field, including anti-pollution activity. To confirm this hypothesis, the protective role of birch sap and chaga mushroom complex against environmental pollutants should be demonstrated following the exposure of 3D skin model to external aggressions including heavy metals, volatile organic compounds and polycyclic aromatic hydrocarbons.

To conclude, taken together our findings suggest that *Betula alba* and *Inonotus obliquus* extracts represent valid natural ingredients for the development of cosmetic products with photo-protection activity.

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Conflicts of Interest

MS and BP are employees of Inderma laboratory.

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Supplementary

Experiment	Model	Conditions	Technique	Figure
Cell viability	Reconstituted epidermis from human keratinocytes	24 h treatment	MTT assay	Fig 1
Lipid oxydation		24 h treatment +UV-A&B irradiation	MDA assay	Fig 2A
Protein oxidation		24 h treatment +UV-A&B irradiation	OxiSelect™ Protein Carbonyl ELISA Kit	Fig 2B
Anti-oxidant kinetics	Human keratinocytes	Complex pre-incubation +UV-A&B irradiation	Cytoplasmic ROS	Fig 2C
			Mitochondrial ROS	Fig 2D
			SOD activity	Fig 2E
			Catalase activity	Fig 2F
Immunomodulation	Co-culture human fibroblasts/ keratinocytes	2 h treatment +UV-A&B irradiation + 48h culture	Flow cytometry cytokines detection	Fig 3
DNA protection	Human keratinocytes	2h30 treatment +UV-B irradiation +24 h culture	Mitochondrial DNA damage quantification	Fig 4A
DNA damage repair		UV-B irradiation +3 h culture +24 h treatment		Fig 4B

Figure S1. Schematic of the experimental procedures used in this study.