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A Concentrated Mix of Verbascoside and Echinacoside Induces Whole Biological Photoprotection of Skin Tissue

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INTRODUCTION

As human skin is repeatedly exposed to excessive levels of UV radiation (UVR); protection of its cells depends on an elaborate antioxidant defense system of enzymes and antioxidants for the neutralization of induced reactive oxygen species (ROS), biotransformation and elimination of electrophilic species, and the maintenance of redox homeostasis [1]. However, protection against UVR alone does nott prevent deleterious dermal consequences. In fact, over 50% of these free radicals are generated in the visible and infrared spectral regions [2], with the exposure to infrared (IR) radiation inducing biological effects, including the activation of matrix metalloproteinase-1 (MMP-1), a decrease in collagen synthesis and premature skin aging [3]. Artificial sources of blue light or high-energy visible light (HEV) such as light-emitting diodes (LEDs) are also becoming an important issue. Blue light seems to induce oxidative stress in keratinocytes and in live skin [4] and is associated with the upregulation of metalloproteinase expression, DNA damage and keratinocyte proliferation. Production of proinflammatory cytokines (IL-1, IL-6, IL-8...) and increased expression of MMP-1 and MMP-9 in human skin subsequent to HEV exposure are also reported [5]. Eventually, all these changes lead to a gradual loss of skin elasticity resulting in the phenomenon of aging, wrinkles and premature aging. Thus, the goal is to delay the onset of aging and/or slow down the structural and visual appearance of skin photoaging with time through neutralization of ROS overproduction induced by light exposure, focusing on UV, IR and HEV radiation and their detrimental effects on the skin.

Abstract

Skin exposure to ultraviolet radiation may have many detrimental cutaneous effects, including erythema (or sunburn reaction), ocular damage, photoaging and even skin cancers. Ultraviolet radiation penetrates deeply through the epidermis into the dermis and is involved in the generation of radical oxygen species, including singlet oxygen, hydrogen peroxide and hydroxyl radicals, and is therefore considered to cause premature aging and wrinkles. Protection against ultraviolet radiation alone is not sufficient to prevent skin disorders: Approximately 50% of free radicals in the skin originate from visible and infrared light. The harmful effects of blue light from artificial sources, such as smartphones and screens, also become an important issue for public health and skin aging. There has been considerable interest in using botanical agents for the prevention of skin damage caused by exposure to solar ultraviolet radiation. Many photochemoprotective agents have recently been identified to fight exposure to ultraviolet radiation. From Buddleja officinalis, adapted to high levels of exposure to sunlight in Chinese mountains through exceptional richness in phenylpropanoids, we developed an innovative global photoprotector – 'BO Flower Extract' – concentrated in verbascoside and echinacoside that protects against the damaging effects of ultraviolet, blue and infrared light.

Various compounds with different antioxidant properties found in plants may be applicable as therapeutics to decrease and prevent free radical damage. Among them, plant-derived phenylpropanoids comprise the largest group of secondary metabolites produced

by taller plants mainly for protection against biotic or abiotic stresses such as wounding, UV radiation, exposure to ozone and pollutants, and numerous studies have focused on the molecular mechanisms of the biological activity of natural phenylpropanoids [6]. These mechanisms include suppression of both the production of IL-1β and its effects on the activation of NF-kB, activation of caspase 3 and inhibition of the transcriptional activity of the COX-2 gene [7]. All these results explain the attraction of cosmetic product manufacturers to natural phenylpropanoids.

Verbascoside, a phenylpropanoid, has several biological properties including photoprotection and antioxidant activity [8]. The anti-inflammatory activity of verbascoside was observed in an *in vitro* test performed on cell cultures of primary human keratinocytes in which verbascoside was able to significantly reduce, in a dose-dependent manner, the release of pro-inflammatory chemokines. An *in vivo* study conducted on inflammation of the intestinal mucosa demonstrated that verbascoside is able to inhibit the activation of pro-inflammatory proteins and consequently the enzymatic activity of metalloproteinases, the latter also being involved in the skin aging phenomena [9].

Based on these data, the aim of this present study was to evaluate the effect of active phenylpropanoid extracts from Buddleja officinalis (BO), a shrub in the Buddlejaceae family known for its wound healing, antiinflammatory, diuretic, antiallergic, antiviral and antibacterial properties. Using our experience with vegetal extraction and purification, we obtained Buddleja officinalis flower extract (BOFE) from Buddleja officinalis through a high-tech process, leading to a powerful cellular protector with an ultra-high concentration of verbascoside and echinacoside ***(Figure 1)***.

Our main objective was to evaluate the global protection of BOFE on the skin using three approaches. Firstly, we examined the high-energy visible (HEV) light-protection capacity of BOFE in human keratinocytes *in vitro*. Secondly, we evaluated the effect of BOFE on infrared-induced MMP-1 release in normal human dermal fibroblasts. Finally, the photoprotective effect of BOFE was evaluated in a pigmented reconstructed skin model. For this, the pigmented full-thickness model was subjected to UVB stress after pretreatment with BOFE. Consequently, we determined whether a specific product (BOFE) has a global protective effect in cells affected by IR, HEV and UVB radiation.

EXPERIMENTAL

Effects of BOFE in counteracting oxidative stress induced by HEV

Human spontaneously immortalized keratinocytes (HaCaT) cell lines [10] were cultured overnight on a 96-well plate in a growth medium (DMEM medium) at a density of 10,000 cells/well. Twenty-four hours later, the culture medium was removed and replaced by a new culture medium supplied with BOFE at different concentrations (0.01 %, 0.03 % and 0.1 %) for 24 hours. After treatment, the cells were irradiated on the cell culture plate for 20 minutes. The light source used was 6 cm from the cell surface, resulting in an approximate dose of 7 $mW.cm^{-2}$ at a wavelength of 420 nm. We used the LZC-420 nm Irradiator (Luczchem Research, Canada).

The LZC-4V houses six top lamps and eight side lamps on four circuits, allowing top, side, or top and side irradiation. Immediately after irradiation, the ROS detection buffer (Fluorometric Intracellular ROS kit, Sigma, France) was added to the cell culture medium and incubated for approximately 1 hour. The accumulated intracellular ROS reacted with a fluorogenic probe (DCFH-DA) localized in the cytoplasm, resulting in a fluorometric product in amounts proportional to the amount of ROS present. The fluorescence was measured at λex = 490 nm / λem = 525 nm. The mean background of fluorescence was subtracted from each measurement in each replicate. Then the mean values for the four replicates in the control (untreated, non-irradiated) and the control + HEV (untreated, irradiated) were calculated and used to normalize each fluorescence measurement of the replicates in the corresponding samples and conditions. Finally, to determine the HEV-induced damage protection of the different treatments, basal ROS levels of the control were subtracted from the ROS levels of the control + HEV, and this value was used to normalize each luminescence measurement of the replicates in the corresponding samples and conditions.

Effect of BOFE on infrared-induced MMP-1 release in normal human dermal fibroblasts

Normal human dermal fibroblasts (NHDF) (Bioalternatives reference PF2) were cultivated on a 24-well plate in a growth medium (DMEM with 2 mM L-glutamine, 50 $U.mL^{-1}$ penicillin, 50 µg. mL^{-1} streptomycin, 10 % fetal bovine serum). After 24 hours the culture medium was removed and replaced by a new culture medium supplied with dexamethasone (10^{-7}M) or BOFE at 0.1 % for 24 hours. After this pretreatment the growth medium was replaced by the ir-

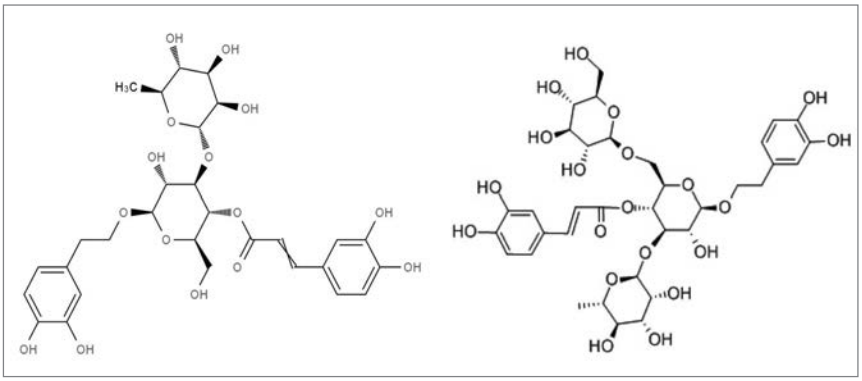

Figure 1 Structures of verbascoside and echinacoside.

radiation medium (EBSS with 0.264 g.L^{-1} $CaCl_2$, 0.2g/L^{-1} $MgSO_4$) and the cells were irradiated with infrared light (0.64 kJ.cm-^{2}) for 1 hour with a special lamp, a Hydrosun irradiator PhotoDyntype 505 (Medizintechnik, Müllheim, Germany) with an infrared barrier filter. After irradiation, the irradiation medium was removed and substituted with a new growth medium containing dexamethasone (10^{-7}M) or BOFE at 0.1 % for 48 hours.

Two experimental replicates (treated and controls) were evaluated for MMP-1 release in the culture medium supernatants using an ELISA kit according to the manufacturer's instructions (Duo set MMP-1, R&D Systems DY901, France). The percentage of protection from infrared-induced MMP-1 release was calculated according to the following equation:

Protection (%) = ($Mean_{irradiated\ control}$ – $Mean_{irradiated\ sample}$/$Mean_{irradiated\ control}$ – $Mean_{non\text{-}irradiated\ control}$Mean) * 100

Effect of BOFE on inflammation and MMP-1 induced by UVB in reconstructed skin

The 3D model was reconstructed with human primary cells (fibroblasts, keratinocytes, and melanocytes). Cell culture lasted 46 days. Treatments with BOFE were performed from Day 35 to Day 45 at 0.03 % and 0.1 %. The study was conducted in comparison with reconstructed skin grown in a normal medium without BOFE. For the irradiated condition, reconstructed skin was exposed to UVB 200 mJ.cm^{-2} on Day 45 (Stratalinker 1800 Bulbs 312 nm, 5 lamps, Agilent, United Kingdom). The culture media were then collected on Day 46. Tissues were collected on Day 46 and samples were fixed in buffered formalin 4% and embedded in paraffin or frozen and fixed in an OCT compound. Image processing and analysis were performed using the software ImageJ. Quantitative Immunoassays of TNF-α and prostaglandin E2 (PGE2) were performed to evaluate the inflammatory response of pigmented equivalent skins (ELISA human TNF-α, PGE2: R&D Systems, France). MMP-1 was assayed by ELISA in the supernatant of the reconstructed skin 24 hours after irradiation.

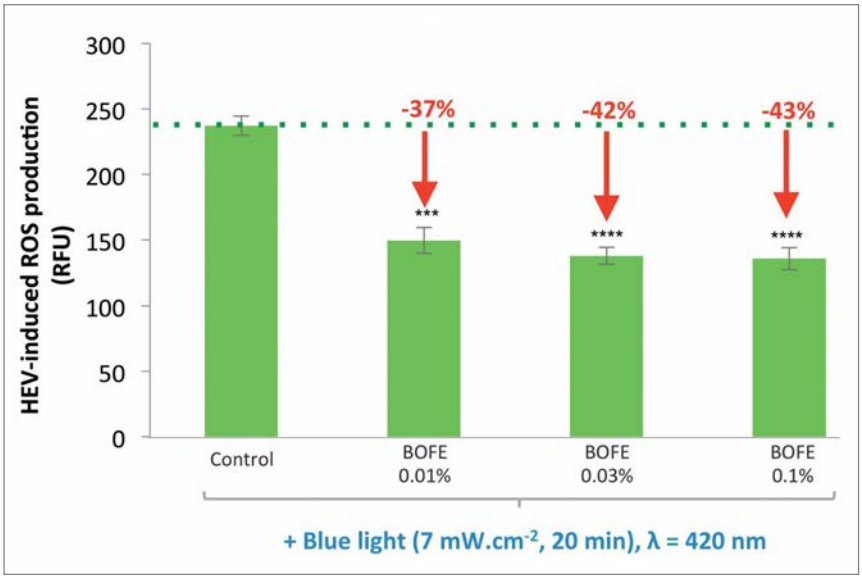

Figure 2 Bar graphs of the HEV-induced ROS accumulation in samples treated with BOFE (0.01 %, 0.03 % and 0.1 %). Control *vs.* treated: *** p < 0.001; **** p < 0.0001.

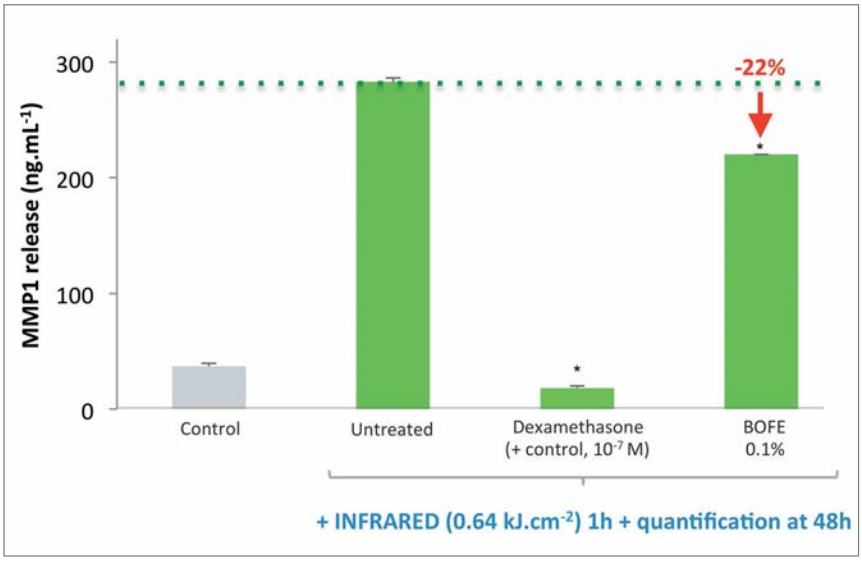

Figure 3 Bar graphs of the IR-induced MMP-1 release in NHDF treated with dexamethasone (10^{-7}M) or BOFE (0.1 %). Control *vs.* treated: * p < 0.05.

Statistical analysis

All data are presented as the mean ± standard deviation. Student's t-test was used in the first two experiments. The statistical significance was set at p < 0.05, 95 % confidence level.

RESULTS

Efficacy of BOFE in counteracting oxidative stress induced by HEV

Figure 2 depicts the HEV-induced ROS accumulation in samples treated with BOFE. Over the course of 20 minute, HEV light increased ROS levels in human keratinocytes *in vitro* by 23.5 ± 0.8 % compared with the non-irradiated control group. When human keratinocytes were treated with the products at each concentration and irradiated, results showed that BOFE at 0.01 %, 0.03 % and 0.1 %, significantly decreased ROS levels compared with the HEV control group, as shown in *Figure 2*. The results showed that the samples treated with BOFE at 0.01 %, 0.03 % and 0.1 %

protected significantly against HEV-induced oxidative stress by 37.0 ± 5.2 % ($p < 0.001$), 41.9 ± 4.1 % ($p < 0.0001$) and 42.9 ± 4.7 % ($p < 0.0001$), respectively.

Effect of BOFE on infrared-induced MMP-1 release in normal human dermal fibroblasts

Under non-irradiated conditions basal MMP-1 production by NHDF was 37 ng.ml-1. IR radiation induced an increase in MMP-1 release by 765 % compared with the control group (283 ng.ml^{-1}). Dexamethasone at 10^{-7} M drastically inhibits this increase (18 ng.ml^{-1}), leading to complete protection from IR radiation. BOFE at 0.1 % significantly inhibits the IR-induced MMP-1 release by 22 % (220 ng.ml^{-1}), as shown in ***Figure 3***.

Effect of BOFE on inflammation and MMP-1 induced by UVB in reconstructed skin

Figure 4 shows the secretion of TNF-α in reconstructed skin supernatant, irradiated or not irradiated and treated or not treated with BOFE. When reconstructed skin is treated with BOFE at 0.03 %, a significant decrease (36 % inhibition, $p < 0.05$) in TNF-α secretion was observed compared with the untreated control skin. A decrease in PGE2 secretion (at 0.03 % and 0.1 % BOFE: $p < 0.001$) was also noted.

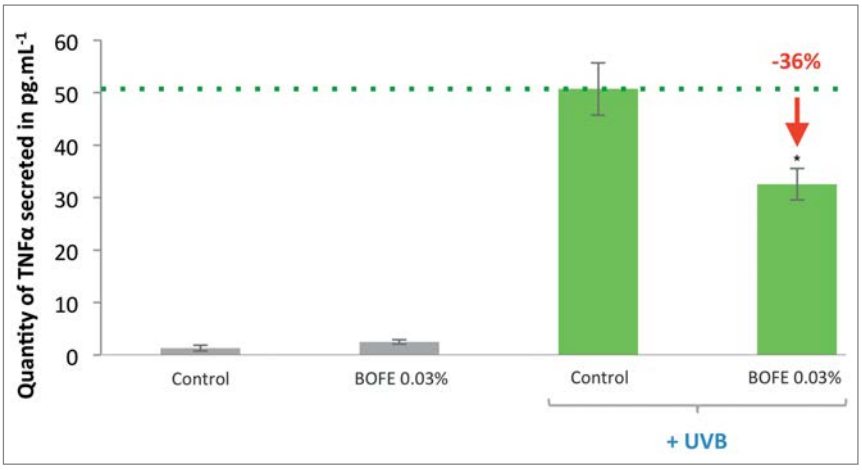

Figure 4 Secretion of TNF-α in reconstructed skin supernatant, irradiated or not irradiated and treated or not treated with BOFE 24 h after irradiation. Mean ± SEM, statistical significance *versus* UVB condition, t-test: * $p < 0.05$.

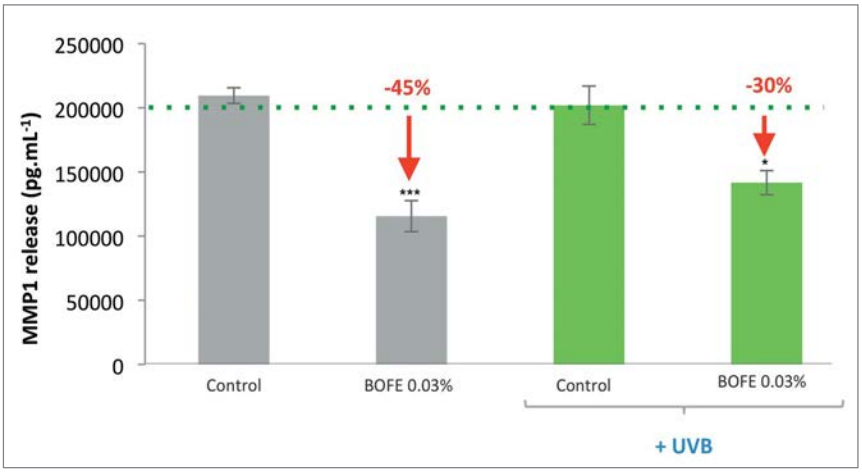

Figure 5 Secretion of MMP-1 in reconstructed skin supernatant, irradiated or not irradiated and treated or not treated with BOFE 24 h after irradiation. Mean ± SEM, statistical significance *versus* control or UVB condition: * $p < 0.05$, *** $p < 0.001$.

In the absence of UVB, secretion of MMP-1 is reduced by 45 % in reconstructed skin treated with BOFE at 0.03 % compared with the untreated control skin ($p < 0.001$). In the presence of UVB, secretion of MMP-1 is also reduced by 30 % in reconstructed skin treated with BOFE at 0.03 % compared with the untreated control skin ***(Figure 5)***.

DISCUSSION AND CONCLUSION

The skin is a major target of oxidative stress, which can develop into oxidative damage if not blocked. Despite an increase in UVA/B protection utilization, an increase in skin disease due to excessive solar exposure was reported in the last decade. The side effects of UV radiation are well known. On the other hand, it was recently demonstrated that visible light induced more hyperpigmentation than UV radiation [11]. The development of new ingredients able to protect skin against the global light is evident. In the dermocosmetic field, topical application of antioxidants is often suggested as a possible strategy to prevent and modulate oxidative skin damage. In recent years, there has been considerable interest in using botanical agents to prevent skin damage resulting from solar UV radiation. It appears that prolonged exposure of plants to solar exposure induces the production of specific secondary defense metabolites such as phenylpropanoids [12]. In this context, we investigated the photoprotective effect of active phenylpropanoid extracts from Buddleja officinalis using *in vitro* tests. Three major findings emerged from our study:

1) Results of the analysis of the HEV-induced damage showed that the treatments with BOFE at 0.01 %, 0.03 % and 0.1 % provide significant protection against HEV-induced oxidative stress.

2) BOFE at 0.1 % affords significant protection against IR-induced MMP-1 release by 22 % compared with the irradiated condition. Thus, it appears that BOFE preserves the extracellular matrix and prevents its premature degradation induced by infrared.

3) BOFE, by inhibiting TNF-α release and decreasing PGE_2, protects from inflammation induced by UVB exposure.

Moreover, we also confirmed, on reconstructed skin exposed to UVB radiation, the potential of BOFE in counteracting MMP-1 release.

Until recently, visible light (400 - 700 nm) was considered to have no significant cutaneous photobiological effect other than a thermal effect. During the last two decades developments in the field of photodynamic therapy and various dermatological treatments using lasers and light-emitting diodes in the visible gave rise to numerous studies and caused reconsideration of the cutaneous effects of visible light. It has been shown that irradiation with blue light leads to intracellular oxidative stress and toxic effects in a dose- and wavelength-dependent manner [13]. Furthermore, blue light at low doses reduces the antioxidative capacity of fibroblasts. Finally, some studies showed that irradiation of the human skin with blue-violet light results in a depletion of the epidermal antioxidants associated with a degradation of cutaneous carotenoid and long-lasting hyperpigmentation. Our results revealed that BOFE significantly reduces ROS production induced by blue light and thus protects from HEV-induced oxidative stress and damage ***(Figure 2)***.

IR radiation penetrates deeply into the skin and approximately half of the IR radiation is absorbed in the dermis. Even though the negative effect of solar radiation on human skin is usually associated with exposure to UVB and UVA radiation, many studies show that near-infrared (NIR) and especially IR radiation in high doses can negatively affect human skin [14]. Therefore, exposure to IR radiation induces biological effects which are similar to the action of UV radiation, including activation of MMP-1 and a decrease and disruption of collagen synthesis. Our results showed that phenylpropanoids extracted from Buddleja officinalis, in particular verbascoside and echinacoside, have the ability to reduce ECM degradation and photoaging. This was shown in our study after exposure to both UVB and IR radiation ***(Figures 3 and 5)***. These results are in line with those of *Hwang et al.* [15], who found that verbascoside reduced the level of metalloproteinase expression by suppressing NF-κB activation.

Inflammation produced by light exposure has been well documented clinically and histologically and leads to erythema and hyperpigmentation [16]. Inflammation is mediated by numerous mediators, such as cytokines like growth factors and interleukins, and eicosanoids, the latter being lipid mediators including prostaglandins and leukotrienes produced by cyclooxygenase and lipoxygenase enzymes, respectively. Our results show that BOFE limits inflammation.

Generally speaking, excessive exposure to solar radiation induces the free radical formation, inflammation, apoptosis and ECM degradation, which are the main causes of premature skin aging. This process is complex and can be triggered by various biological pathways, including receptor-initiated signaling, mitochondrial damage, protein carbonylation, lipid peroxidation, telomere-based DNA damage and apoptosis. Thus, the goal is to delay the onset of aging and/or slow down photoaging alterations, which are particularly mediated by reactive oxygen species. It appears that BOFE is an active ingredient that protects the skin from damage induced by light radiation.

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