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Lemon balm extract (*Melissa officinalis*, L.) promotes melanogenesis and prevents UVB-induced oxidative stress and DNA damage in a skin cell model

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ABSTRACT

Background: Solar ultraviolet (UV) radiation is one of the main causes of a variety of cutaneous disorders, including photoaging and skin cancer. Its UVB component (280–315 nm) leads to oxidative stress and causes inflammation, DNA damage, p53 induction and lipid and protein oxidation. Recently, an increase in the use of plant polyphenols with antioxidant and anti-inflammatory properties has emerged to protect human skin against the deleterious effects of sunlight.
Objective: This study evaluates the protective effects of lemon balm extract (LBE) (*Melissa Officinalis*, L) and its main phenolic compound rosmarinic acid (RA) against UVB-induced damage in human keratinocytes.
Methods: The LBE composition was determined by HPLC analysis coupled to photodiode array detector and ion trap mass spectrometry with electrospray ionization (HPLC-DAD-ESI-IT-MS/MS). Cell survival, ROS generation and DNA damage were determined upon UVB irradiation in the presence of LBE. The melanogenic capacity of LBE was also determined.
Results: RA and salvianolic acid derivatives were the major compounds, but caffeic acid and luteolin glucuronide were also found in LBE. LBE and RA significantly increased the survival of human keratinocytes upon UVB radiation, but LBE showed a stronger effect. LBE significantly decreased UVB-induced intracellular ROS production. Moreover, LBE reduced UV-induced DNA damage and the DNA damage response (DDR), which were measured as DNA strand breaks in the comet assay and histone H2AX activation, respectively. Finally, LBE promoted melanogenesis in the cell model.
Conclusions: These results suggest that LBE may be considered as a candidate for the development of oral/topical photoprotective ingredients against UVB-induced skin damage.
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1. Introduction

The skin is the largest organ of the human body, participates in sensitivity and temperature maintenance and offers protection from chemicals, microorganisms and UV radiation [1]. An excessive UV exposition can lead to several skin pathological disorders, including erythema, immunosuppression, edema, sunburn, hyperplasia, hyperpigmentation, premature aging and skin cancer [2]. UV radiation is divided into long wave UVA (315–400 nm, 90% of UV radiation), medium wave UVB (280–315 nm, 5% of UV radiation), and short wave UVC (200–280 nm). UVB is one thousand times more capable of

Abbreviations: 6-4PPs, pyrimidine (6-4) pyrimidone; CPDs, cyclobutane pyrimidine dimers; DDR, DNA damage response; DMSO, dimethyl sulfoxide; DSBs, double-strand brakes; H_2DCF-DA, 2′,7′-dichlorofluorescein diacetate; HNE, 4-hydroxy-2-nonenal; IBMX, 3-isobutyl-1-methylxanthine; LBE, lemon balm extract; MDA, malondialdehyde; ORAC, oxygen radical absorbance capacity; PBS, phosphate-buffered saline; RA, rosmarinic acid; ROS, reactive oxygen species; SSBs, single-strand brakes; TEAC, trolox equivalent antioxidant capacity; UV, ultraviolet.

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causing sunburn than UVA and is considered the most damaging and genotoxic [3].

Melanin is the main skin protective barrier that acts by absorbing and scattering UV radiation. Nevertheless, other intracellular molecules are targeted (DNA, RNA, lipids and proteins). The direct effect of UVB on DNA leads to the formation of cyclobutane pyrimidine dimers (CPDs) and to a lesser extent pyrimidine (6–4) pyrimidone (6-4PPs) photoproducts. When these alterations are not well repaired, the resulting substitution/transition mutations (cytosine-thymine) in the epidermal cells can lead to the development of skin cancer [4,5].

UVB, together with UVA, generates superoxide ($O_2^{\bullet}$), either directly or through enzyme activation [6,7]. This is the most promptly generated oxygen radical species (ROS) and is rapidly derived into H_2O_2, which forms OH upon the Fenton reaction [4]. UVB induced $^{\bullet}OH$ is postulated to be responsible for the formation of DNA single-strand breaks (SSBs) and also for lipid peroxidation through the generation of lipoperoxy radicals (ROO), malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE). Additionally, UVB-induced ROS interact with numerous cellular targets and receptors that regulate crucial pathways related to inflammation, cell survival, cell growth and differentiation in human keratinocytes: the NF-κB, the AP-1 transcription factor, the mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK1/2) pathways [8–10]. Most of these effects lead to extracellular matrix degeneration by proteases activation and skin photoaging [1].

Plant polyphenols possess strong free radical scavenging properties and have exhibited the capacity to modulate multiple cellular pathways [11]. Recently, their potential skin photoprotective effects have gained considerable attention [12]. The UVB protective effects of botanical extracts, such as *Punica granatum* [13], citrus and rosemary [14], green tea polyphenols [15] and pure compounds, such as resveratrol [16], genistein [17,18] and hydroxytyrosol [19] has been reported. Recently our group has demonstrated the synergistic protective effect of rosemary and citrus polyphenolic extracts both *in vitro* and *in vivo* [14].

Lemon balm (*Melissa officinalis,* L.) is a representative of the *Lamiaceae* family, native to Europe but with a worldwide distribution. This herb is used not only for ornamental purposes but also for medicine and cosmetics. It is commonly used for insomnia and anxiety [20], herpes [21], and indigestion [22], as an antioxidant [23] and as an antimicrobial agent [24]. Lemon balm extraction may lead to the essential oil and the lemon balm polyphenolic extract (LBE), which is enriched in phenylpropanoid derivatives and flavonoids. The major phenolic compound found in the polyphenolic extract is rosmarinic acid (RA), which is an ester of caffeic acid, and 3,4-dihydroxyphenyllactic acid [25] (Fig. 1, see insert). The antioxidant activity of LBE has been previously characterized in both *in vitro* and *in vivo* models [26–29].

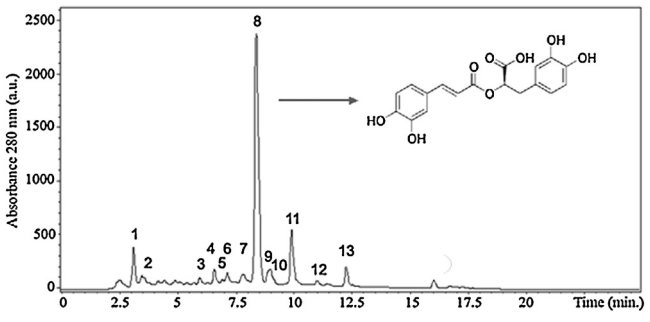

Fig 1. HPLC-DAD chromatogram for LBE at 280 nm. Insert shows RA structure.

In the present study, the UVB protective effects of LBE and its major polyphenol RA were explored and compared in human keratinocytes. The potential of LBE to protect human keratinocytes from UVB-induced oxidative stress and to alleviate DNA damage was studied. The protective effect through melanogenesis activation was also studied in a cellular model.

2. Materials and methods

2.1. Materials and LBE

Human keratinocytes (the spontaneously immortalized cell line HaCaT) were obtained from Cell lines Service GmbH, CLS (Eppelheim, Germany). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin were obtained from Gibco/Thermo Fisher Scientific (Waltham, MA, USA). RA (96%) and the rest of the reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). LBE standardized containing $18.0 \pm 0.3\%$ RA (w/w) was kindly provided by NUTRAFUR, SA – Frutarom Group (Alcantarilla, Murcia, Spain).

2.2. Cell culture

HaCaT cells were grown in DMEM with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin (0.1 mg/mL penicillin and 100 U/mL) in a humidified atmosphere with CO_2 (5% v/v) at 37 °C. The HaCaT cells were trypsinized every third day following the purchaser instructions and seeded in 96- or 6-well plates depending on the assay. B16-F10 mouse melanoma cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and grown using the same conditions described for the HaCaT cells.

2.3. HPLC-DAD-ESI-IT-MS/MS analysis of LBE

LBE was dissolved in DMSO:PBS (1:10) and filtered through a 0.45 μm nylon membrane. The extract was analyzed using a LC–MS system consisting of an Agilent LC 1100 series (Agilent Technologies, Inc., Palo Alto, CA, USA) controlled by the Chemstation software. The HPLC instrument was coupled to an Esquire 3000 (Bruker Daltonics, GmbH, Germany) mass spectrometer equipped with an ESI source and ion trap mass analyzer. The electrospray ionization source was operated in negative mode to generate $[M-H]^-$ ions using the following conditions: desolvation temperature was set at 300 °C, and the dry gas (nitrogen) and nebulizer were set at 10 L/min and 60 psi, respectively. The MS and MS/MS spectra were acquired over 100–1000 m/z at 10 ms, and the capillary voltage was 4.0 kV. A Teknokroma C-18 Tracer Excel 120 ODSB 5 μm 25 × 0.4 cm column was used for analytical purposes. The mobile phase was (A) formic acid (1%) and (B) acetonitrile, and the following linear gradient was used: 20% B at 0 min, 70% at 25 min, 70% at 30 min, 20% at 31 min and 5 more minutes for re-equilibration. The flow rate was 1 mL/min. Diode-array detection was set at 280, 320 and 340 nm. The MS data were acquired as full scan mass spectra at 50–1100 m/z using 200 ms for collection of the ions in the trap. Identification of the main compounds was performed by HPLC-DAD analysis. The retention times, UV spectra and MS/MS data of the peaks in the samples were compared with those of authentic standards or data reported in the literature. Quantitation of the RA content was performed using a commercial standard (Sigma–Aldrich, Europe) at 280 nm. The linearity range of the responses was determined on eight concentration levels of RA (ranging from 0.18 mg/mL to 5.4 mg/mL) with three injections for each level. Calibration graphs for the quantitative evaluation of the compounds were performed using a seven-point regression curve

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