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Photoprotective properties of *Prunella vulgaris* and rosmarinic acid on human keratinocytes

Jitka Psotova [a,*], Alena Svobodova [a], Hana Kolarova [b], Daniela Walterova [a]

[a] *Institute of Medical Chemistry and Biochemistry, Faculty of Medicine, Palacky University, Hnevotínská 3, 775 15 Olomouc, Czech Republic*
[b] *Department of Medical Biophysics, Faculty of Medicine, Palacky University, Olomouc, Czech Republic*

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Abstract

UVA radiation provokes the generation of reactive oxygen species (ROS), which induce oxidative stress in the exposed cells leading to extensive cellular damage and cell death either by apoptosis or necrosis. One approach to protecting human skin against the harmful effects of UV radiation is by using herbal compounds as photoprotectants. This study evaluated the protective effects of *Prunella vulgaris* L. (Labiatae) and its main phenolic acid component, rosmarinic acid (RA), against UVA-induced changes in a human keratinocyte cell line (HaCaT). Human keratinocytes exposed to UVA (10–30 J/cm^2) were treated with an extract of *P. vulgaris* (1–75 mg/l) or RA (0.9–18 mg/l) for 4 h. *P. vulgaris* and RA exhibited ability to reduce the UVA-caused decrease in a cell viability monitored by neutral red retention and by LDH release into medium. The *P. vulgaris* extract and RA significantly suppressed UVA-induced ROS production, which manifests as a decrease in intracellular lipid peroxidation, elevation of ATP and reduced glutathione. Post-treatment with *P. vulgaris* extract and RA also significantly reduced DNA damage. In addition, UVA-induced activation of caspase-3 was inhibited by treatment with *P. vulgaris* and RA.

The *P. vulgaris* extract and RA demonstrated a concentration-dependent photoprotection (maximum at 25–50 mg/l and 9 mg/l, respectively). These results suggest that *P. vulgaris* and RA, used in skin care cosmetics, may offer protection against UVA-induced oxidative stress and may be beneficial as a supplement in photoprotective dermatological preparations.

Keywords: UVA radiation; Photoprotection; *Prunella vulgaris*; Rosmarinic acid; Keratinocytes

1. Introduction

The skin as an interface between the body and its environment, acts as a barrier to the harmful effects of various exogenous physical and chemical agents including ultraviolet radiation. Solar UV radiation is divided into three components, UVA, UVB and UVC. All UVC and partly UVB are blocked by the earth's ozone layer. UV reaching the earth's surface contains approximately 6% of UVB and 94% of UVA [1]. UV is responsible for numerous biological effects on the skin, including skin cancer, whose incidence is increasing dramatically. At a molecular level, UVA and UVB differ in how they generate pre-mutagenic skin lesions. UVB interacts directly with DNA bases and modifies them. Cyclobutane pyrimidine dimers are the main formed products, capable of interfering with DNA replication, which is important in photo-induced cancerogenesis. The deleterious UVA effects on cellular targets involve endogenous photosensitisers (chromophores), which generate reactive oxygen species (ROS) after UV excitation. Physiologically ROS are involved in the regulation of gene expression, and activation/deactivation of cellular pathways. Elevated levels on the other hand induce cellular oxidative stress and damage to macromolecules (lipids, proteins and DNA). Damaged DNA further activates

* Corresponding author. Tel.: +420585632316; fax: +420585632302.
E-mail addresses: psotova@tunw.upol.cz, j.psotova@email.cz (J. Psotova).

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tumor suppressor protein p53, leading to the initiation of apoptosis in keratinocytes, and this in turn leads to the disruption of the epithelial structure of the skin [2]. Although the skin possesses an elaborate antioxidant system to deal with UV-induced oxidative damage, the use of active photoprotectives is recommended.

In recent years, natural compounds, which possess antioxidant and anti-inflammatory properties, have created considerable interest as protective agents for reducing UV-induced skin damage [3–5]. *Prunella vulgaris* L. (Labiatae), known as the ''self-heal'', is rich in phenolic acids. It was popular in traditional European and Chinese medicine as a remedy for alleviating sore throat, reducing fever and accelerating wound healing [6–8]. More recently, the crude aqueous extract was used in the clinical treatment of herpetic keratitis [9]. The organic fraction of *P. vulgaris* (PV) exhibits antioxidative and antimicrobial activities [10]. Phytochemical studies indicate that PV contains oleanolic, betulinic, ursolic, 2α,3α-dihydroxyurs-12-en-28-oic and 2α,3α-ursolic acids, triterpenoids, anionic polysaccharide prunelline, flavonoids, tannins, rosmarinic and caffeic acids [11–13]. Rosmarinic acid (RA; Fig. 1), the major phenolic component of the plant, exhibits a wide spectrum of biological activities [14], mainly antioxidant [15] and anti-inflammatory [16]. Caffeic acid afforded a significant protection against UVB-induced erythema in vitro and in human volunteers [17]. Phenolic acids have also been shown to accelerate the wound healing [18]. Ursolic and oleanolic acids display anti-inflammatory activity [19]. PV extract and saponin are used as ingredients in cosmetic/dermatological preparations for their anti-ageing and anti-inflammatory activities [20].

In this study, we investigated the effect of PV extract and RA on the UVA modulated cellular pathways in human keratinocytes. We focused on their effects on ROS induced cellular oxidative stress, lipid peroxidation, DNA damage and caspase-3 activation.

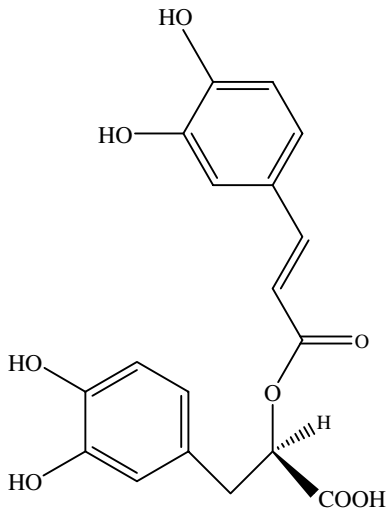

Fig. 1. Structure of rosmarinic acid.

2. Materials and methods

2.1. Materials

Human keratinocytes (spontaneously immortalised cell line HaCaT) were obtained from the Institute of Biophysics, Academy of Science of the Czech Republic, Brno. Protease inhibitor cocktail tablet (Complete™) and Cell Proliferation ELISA, BrdU (colorimetric) kit were purchased from Roche, Germany. Caspase-3 fluorescent substrate Ac-DEVD-AMC was purchased from Bachem AG, Switzerland. Western blotting luminol reagent, actin (I-19) goat polyclonal antibody, horseradish peroxidase conjugated goat anti-rabbit and rabbit anti-goat antibodies were received from Santa Cruz Biotechnology, USA. Phototope®-HRP Western blot detection system was from Cell Signalling, USA. Immun-Blot™ polyvinylidenefluoride (PVDF) membrane was from Bio-Rad Laboratories, Hercules, CA. XAR-5 film from Eastman Kodak, USA. 2,2′-Dinitro-5,5′-dithiobenzoic acid was purchased from Serva, Germany. Rabbit anti-caspase-3, Dulbecco's modified Eagle's medium (DMEM), heat-inactivated foetal calf serum (FCS) and all other chemicals were obtained from Sigma–Aldrich, USA.

2.2. P. vulgaris extract and rosmarinic acid

The aerial part of *P. vulgaris* was collected in bloom in July 2004. The cut dry plant (10.45 kg) was extracted with aqueous ethanol (30% v/v). The primary extract was concentrated on a vacuum rotary evaporator to dryness and further dried in a vacuum dryer (70 °C; 10 mbar) yielding 2.48 kg of the extract. The PV extract was characterised by an RA content (9.0% w/w; determined by HPLC). The extract (20 g) was fractioned on a silica (40–100 μm) column using a toluene–acetone gradient. RA was obtained from a toluene–acetone fraction (30:70) as a crystalline substance (1.6 g), m.p. 167–169 °C. The UV, IR, ^{1}H NMR, and ^{13}C NMR were used to confirm the identity of the acid.

2.3. Cell culture

HaCaT were grown in DMEM supplemented with FCS (7% v/v), streptomycin (100 U/ml), penicillin (0.1 mg/ml) and glutamine (4 mM) in a humidified atmosphere with CO_2 (5% v/v) at 37 °C. HaCaT were seeded in 96-, 12-, or 6-well plates at a density 1×10^5 cells/cm^2 and grown near to confluence for 2 days.

2.4. Cytotoxicity of test compounds

DMEM with FCS was removed and cells were treated with serum free medium containing PV extract (1–250 mg/l) and RA (0.9–18 mg/l) for 4 h. Stock solutions of PV (0.2–50 g/l) and RA (0.18–3.6 g/l) were prepared in DMSO before individual experiment. Final concentration