Bilberry extract reduces UVA-induced oxidative stress in HaCaT keratinocytes: A pilot study

Alena Svobodová[*], Jana Rambousková, Daniela Walterová and Jitka Vostalová
Department of Medical Chemistry and Biochemistry, Faculty of Medicine and Dentistry, Palacký University, Olomouc, Czech Republic

Abstract. Exposure to UVA radiation is known to cause many adverse biological effects by inducing the stricken cells to produce reactive oxygen species (ROS). In recent years the use of botanicals has received considerable interest in the skin protection. Bilberry (*Vaccinium myrtillus* L.) fruit contains several polyphenols with strong antioxidant and anti-inflammatory properties. In this study we evaluated potential UVA preventive effect of *V. myrtillus* fruit extract (VME; anthocyanins, 25% w/w) in HaCaT keratinocytes. Pre-treatment (1 h) or post-treatment (4 h) of HaCaT with VME resulted in attenuation of UVA-caused damage. Application of the extract significantly reduced UVA-stimulated ROS formation in keratinocytes. VME also prevented/reduced UVA-caused peroxidation of membrane lipids and depletion of intracellular GSH. The observed cytoprotective effect may be linked to the antioxidant activity of the plant constituents, namely anthocyanins.

Keywords: *Vaccinium myrtillus*, UVA radiation, HaCaT, oxidative stress, free radicals

1. Introduction

The sun emits a wide spectrum of electromagnetic waves of which UV is the most aggressive towards cellular components [32]. The most damaging and cytotoxic part of solar UV light (100–295 nm) is eliminated in the stratospheric ozone layer. Nevertheless the rest of coming UV radiation, part of UVB (295–315 nm) and all UVA (315–400 nm), is responsible for manifold skin diseases [27]. The ratio of UVA/UVB depends on latitude, season and time of day. However, the sun is primarily a UVA source with a maximal terrestrial UVB content of about 5% [35]. Depending on wavelength, UV damage occurs via two different mechanisms. UVA primarily initializes massive production of reactive oxygen species (ROS) through interaction with endogenous photosensitizers. ROS are capable of oxidizing

---
*Address for correspondence: Alena Svobodová, Department of Medical Chemistry and Biochemistry, Palacký University, Hněvotínská 3, 775 15 Olomouc, Czech Republic. Tel.: +420585632324; Fax: +420585632302; E-mail: alf.svoboda@seznam.cz.

cellular macromolecules leading to the formation of oxidized products such as lipid hydroperoxides, protein carbonyls and 8-hydroxyguanosine which have been implicated in skin disorders. Both ROS and oxidized molecules can affect various cellular pathways and gene expression [27]. On contrary, DNA with its aromatic, heterocyclic bases is a strongly absorbing chromophore for UVB (absorbing maximum at 260–265 nm). Direct absorption of the UVB photons leads to disruption of DNA with cyclobutane pyrimidine dimers and pyrimidine-(6-4)-pyrimidone photoproducts formation as a result. Aromatic amino acids of proteins, mainly tryptophan and tyrosine, act as potent radiation absorbers within the UVB range and their UVB-caused modification may lead to affection of protein function and cellular pathways as well. Hence, both UVA and UVB play a role in the pathogenesis of photosensitive diseases such as sunburn, immunosuppression, premature skin aging so-called photoaging, and even photocarcinogenesis [33].

Even though UVA penetrates deep into the dermis of the skin, it also affects epidermal cells. The major cell type in the epidermis, the upper skin layer, is keratinocyte that comprises more than 90%. Keratinocytes are equipped with a complex of non-enzymatic and enzymatic antioxidant defence system. However, if excessive ROS generation induced by UVA radiation reaches a critical concentration, keratinocyte normal defence components are overwhelmed and it can lead to reversible or irreversible (photo)oxidative damage to all cellular components [27]. Throughout life oxidative protein and DNA modifications as well as lipid peroxidation and glycation products accumulate in cells, which lead to photoaging and can lead to skin carcinogenesis [32].

Fruits and vegetables contain many phytochemicals, which were recognized for their potential health benefits. Several epidemiological studies have revealed that high fruits and vegetables intake appears to be positively correlated with reduced mortality by some types of civilizational diseases including cancer. One possible mechanism responsible for the effect was attributed to the antioxidant activity of fruits and vegetables constituents [10]. For the same reason, the use of botanical supplements with antioxidant activity seems to be suitable way how attenuate the UV-caused oxidative stress and oxidative stress-mediated skin disorders [4]. In particular, polyphenols which are known antioxidants have been intensively studied for their UVA-protective activity.

Bilberry (*Vaccinium myrtillus* L.) also called blueberry, huckleberry or whortleberry is a well-known shrub belonging to the Ericaceae family. The berries represent a high nutritive food [18] and were reported to be one of the richest sources of antioxidant phytonutrients of fresh fruits and vegetables [15]. Furthermore fruit and leaves have traditional use in folk medicine of several European countries. Leave infusion was used among others as local application in cutaneous infection and burns. The berries were especially used in the treatment of gastrointestinal inflammations, mouth infections or urinary complaints. The berries contain different polyphenols like anthocyanins, cinnamic and benzoic acid derivatives, flavan-3-ols and flavonol glycosides [4]. Multiple pharmacological activities that have been reported seem to be related to the anthocyanidin fraction e.g. anti-atherosclerosis, anti-inflammatory, or wound-healing. Some of the activities may be associated with their antioxidant properties. Bilberry anthocyanins were reported to scavenge superoxide, reduce hydrogen peroxide-induced radicals, and inhibit lipid peroxidation *in vitro* [14,31] and *in vivo* [5].

The aim of this study was to investigate UVA (photo)protective activity of *Vaccinium myrtillus* extract (VME) *in vitro*. We specifically dealt with VME preventive or therapeutic effects on ROS generation, lipid peroxidation and GSH depletion.

2. Materials and methods

2.1. Materials

Human keratinocytes (spontaneously immortalized cell line HaCaT) were obtained from the Institute of Biophysics, Academy of Science of the Czech Republic, (Brno, Czech Republic). 2',7'- Dichlorodihy-drofluorescein diacetate (H_2DCFDA) was from Fluka Chemie (Germany). Dulbecco's modified Eagle's medium (DMEM), heat-inactivated fetal calf serum (FCS), stabilised penicillin-streptomycin solution, sterile dimethylsulfoxide (DMSO), neutral red (NR), 2,2'-dinitro-5,5'-dithiobenzoic acid (DTNB) and all other chemicals were purchased from Sigma-Aldrich (USA). Bilberry extract (Myrtilli fructus siccum, batch 171004MF015) was obtained from IVAX CR a.s., Opava, Czech Republic.

2.2. Bilberry extract analysis

The bilberry extract used contained anthocyanins (25.0%, w/w; analysed by HPLC using del-phinidin as external standard). Delphinidin-3-galactoside (2.9%), delphinidin-3-glucoside (3.3%), cyanidin-3-galactoside (4.7%) and cyanidin-3-glucoside (2.5%) were the most represented constituents, followed by petunidin-3-glucoside (2.2%), malvidin-3-glucoside (2.2%) and cyanidin-3-arabinoside (1.8%). Petunidin-3-galactoside (1.0%), paeonidin-3-glucoside (1.0%), malvidin-3-galactoside (0.8%), petunidin-3-arabinoside (0.7%) were also identified. Minor amounts of malvidin-3-arabinoside (0.5%), delphinidin (0.3%), cyanidin (0.3%), paeonidin-3-galactoside (0.2%), paeonidin-3-arabinoside (0.13%), petunidin (0.13%), malvidin (0.12%) and paeonidin (0.05%) were also found, as published previous-ly [10].

2.3. (Photo)stability of bilberry extract

To determine time and UVA-stability VME stock solution (10 and 5 mg/ml; DMSO) was diluted 200-times in phosphate saline buffer (PBS, pH 7.5). The blank samples were prepared with DMSO (0.5%, v/v, in PBS). **A**) Time-stability of VME (50 mg/l; 0.5% DMSO (v/v)) was determined after 1, 4 and 24 h incubation (37°C; dark). **B**) Photostability was monitored in the UVA region. VME solution (25 mg/l; 0.5% DMSO (v/v) was applied into a 6-well plate (2 ml per well) and irradiated (20 J/cm$^2 \sim$ 60 min). The sham samples were kept in the incubator for the time of irradiation.

The absorption spectrum was measured immediately between 200–600 nm in UV-VIS spectropho-tometer *UV-2401PC* (Shimadzu, Japan). Non-irradiated and irradiated samples (VME) were scanned in a quartz cuvette against non-irradiated and irradiated blanks (DMSO), respectively.

2.4. Cell culture

Keratinocytes were grown in DMEM supplemented with heat-inactivated FCS (7%; v/v), streptomycin (100 U/ml), penicillin (0.1 mg/ml) and glutamine (4 mmol/l). The cells were maintained in a humidified atmosphere with 5% (v/v) CO_2 at 37°C. Culture medium was changed twice a week. The cells were subcultured following trypsinization. For all experiments HaCaT were seeded in 96-well or 6-well plates at a density 1×10^5 cells per cm^2.

2.5. Treatment with UVA light and bilberry extract

Two protocols of irradiation and application VME were performed:

A) Keratinocytes were pre-treated with VME (5–100 mg/l) in serum free medium at 37°C for 1 h, irradiated and incubated in serum-free medium at 37°C for another 4 h.

B) Cells were irradiated and after UVA exposure VME (5–100 mg/l) in the serum-free medium was added to the keratinocytes for 4 h.

For all experiments stock solutions of VME (0.2–50 mg/ml) were prepared in DMSO. Before application, the solutions were diluted in serum-free medium so that the final concentration of DMSO in medium was 0.5% (v/v). Irradiated and non-irradiated control cells for each protocol were treated with serum-free medium containing the respective aliquot of DMSO (0.5%; v/v) instead of test compound stock solution.

2.6. UVA irradiation

Prior to UV irradiation, cells were washed with PBS and covered with a thin layer of PBS. Keratinocytes were irradiated on ice-cold plates to eliminate UVA thermal stimulation. In parallel, non-irradiated cells were treated similarly and were kept in the dark in an incubator for the time of UVA treatment. For irradiation, a solar simulator *SOL-500* (Dr. Hönle UV Technology, Germany), with spectral range (295–3000 nm) corresponding to natural sunlight, was used. For all experiments the simulator was equipped with a UVB-absorbing $H1$ filter (Dr. Hönle UV Technology, Germany) transmitting wavelengths of 315–380 nm. The UVA output measured by an UVA-meter (Dr. Hönle UV Technology, Germany) in direct contact with the cell culture dish was 6.0 mW/cm^2. Cells were irradiated with doses of 10–40 J/cm^2.

2.7. Cell viability

The effect of VME in the concentration range of 1–250 mg/l, various UVA doses (10–40 J/cm^2) or combinations of VME and UVA on keratinocytes cell viability was assessed after 4/24 h. Viability was determined photometrically by means of two methods, neutral red (NR) retention and lactate dehydrogenase (LDH) leakage assays, as described previously [28]. The NR uptake reveals the ability of living cells to incorporate water-soluble dye into lysosomes. The LDH leakage assay evaluates activity of LDH, a cytosolic enzyme, released into the medium which reflects cell membrane integrity.

2.8. ROS generation

Production of ROS was measured using a pre-fluorescent probe dichlorodihydrofluorescein diacetate (H$_2$DCFDA) as described previously [2]. Briefly, after treatment with VME, pre-treatment (VME+UVA) or post-treatment (UVA+VME), the probe H$_2$DCFDA (5 nmol/l; PBS) was added to the cells and keratinocytes were consecutively incubated for 15 min in an incubator. Then cells were washed with PBS and scraped into 2 ml of PBS and sonicated. The fluorescence was monitored at specific excitation/emission wavelengths 488/525 nm using a spectrophotometer (*LS 50 B*, Perkin-Elmer, USA). The protein concentration was determined by Bradford assay [6].

2.9. H_2O_2 induced damage

To assess the preventive effect of VME on hydrogen peroxide-caused damage, one of the most abundant ROS, keratinocytes cultured on two separate 96-well plates were pre-treated with VME (5–50 mg/l) for 1 h. After the pre-treatment period (1 h), cells on one plate were exposed to H_2O_2 (0.3 mM) in the presence of VME for 4 h (**A**). The medium with VME on the second plate was removed, serum-free medium was applied and H_2O_2 (0.3 mM) for 4 h (**B**). Control cells for both protocols were treated with appropriate volume of sterile water instead of H_2O_2. Cell viability was evaluated by NR retention assay as described previously [28].

2.10. Superoxide scavenging

Superoxide was generated from molecular oxygen in the presence of EDTA, $MnCl_2$, and mercaptoethanol and evaluated spectrophotometrically as the decrease in absorbance of NADH which is oxidized during the reaction [19]. The reaction mixture containing 190 μl of triethanolamine-diethanolamine buffer (100 mM, pH 7.4), 10 μl of NADH (7.5 mM), 10 μl of EDTA/$MnCl_2$ (100 mM/50 mM), 10 μl of VME or DMSO was incubated for 10 min at 25°C. The reaction was started with the addition of 25 μl of mercaptoethanol (10 mM), and the absorbance decrease was monitored at 340 nm for 15 min. The final concentrations of VME were 1–50 μg/ml.

2.11. Lipid peroxidation

Lipid peroxidation was determined in the cell lysates of various treatment groups using the thiobarbituric acid reaction method [7]. The cells were washed with cooled PBS, scraped into trichloroacetic acid (2.8%; w/v), sonicated and aliquots were used for protein determination by Bradford assay [6]. The suspension was mixed with thiobarbituric acid (1%; w/v) in a ratio 2:1, heated (30 min; 95°C) and centrifuged (10 min; 13 000 rpm; 4°C). The amount of thiobarbituric acid reactive substances (TBARS) was determined spectrophotometrically on a microplate reader (*Sunrise*, Schoeller Instruments, Austria) at 535 nm.

2.12. Intracellular GSH level

Reduced glutathione in cells treated with VME alone, pre-treated with VME and irradiated or irradiated and post-treated with VME was measured using the reaction with 2,2'-dinitro-5,5'dithiobenzoic acid (DTNB) [24]. Briefly, the cells rinsed with cooled PBS were scraped into cooled perchloric acid (1%; v/v), and sonicated. The aliquots were used for protein determination by Bradford assay [6]. The suspension was centrifuged (10 min; 13 000 rpm; 4°C) and 50 μl of supernatant was mixed with 200 μl of 0.8 M Tris-HCl buffer (pH 8.9) and 10 μl of DTNB (4 mg.ml^{-1}). After shaking the reaction mixture, the absorbance was measured at 412 nm within 5 min.

2.13. Statistical analysis

The series of experiments were performed as three or four independent examinations with at least three replicates for each sample. Cytotoxicity of VME and UVA radiation to HaCaT was expressed as % of control. Data were expressed as means ± S.D. Statistical analyses were performed using Student's t-test. Statistical significance was determined at $p = 0.05$.
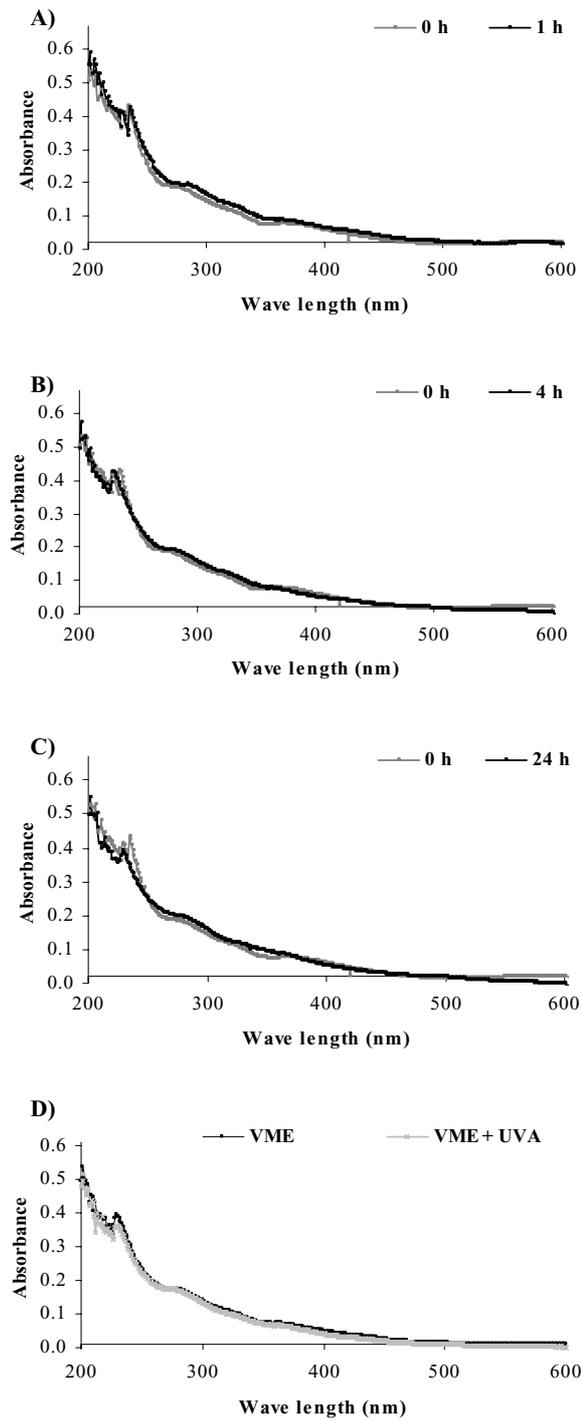
Fig. 1. (Photo)stability of VME. (A,B,C) Time-stability was performed in VME (50 mg/l; PBS; pH 7.5). Spectra were monitored after 0, 1, 4 and 24 h (37°C; dark). (D) Photostability was monitored in UVA region. The VME solution (25 mg/l; PBS; pH 7.5) was irradiated (20 J/cm^2 $\sim$ 60 min).
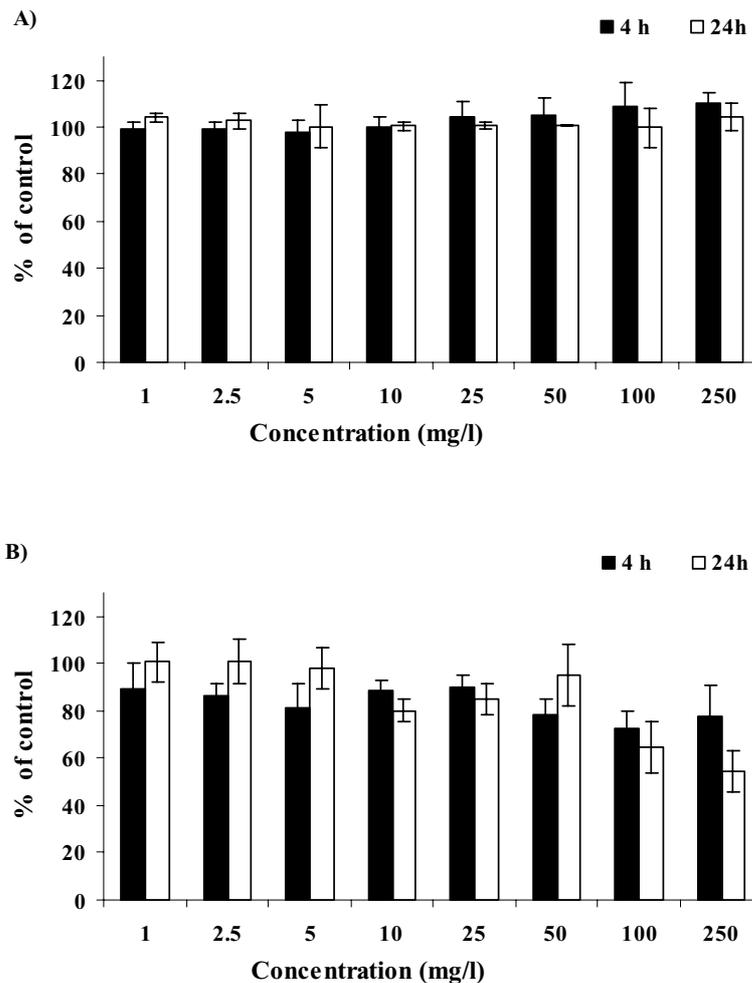
Fig. 2. Effect of VME on HaCaT viability. Keratinocytes were treated with VME (1–250 mg/l) for 4/24 h; control cells were incubated with DMSO (0.5%; v/v) under the same conditions. Cell damage was evaluated as NR retention (A) and LDH leakage into medium (B). Data are expressed as % of control and plotted as mean ± S.D. pre-treatment.

3. Results

3.1. (*Photo*)*stability of VME*

Time-stability of VME (50 mg/l; PBS, pH 7.5) was studied in the UV-VIS area of the electromagnetic spectrum. Time-stability spectra (Fig. 1A, B, C) showed no changes in absorbance in either the visible (400–600 nm) or the UV (200–400 nm) part after 1, 4 and 24 h (37°C, dark).

To assess the effect of UVA on VME, control experiments were performed on a non-cell model. *In vitro* irradiation (20 J/cm^2) of VME (25 mg/l; PBS, pH 7.5) did not change the absorption spectrum of VME indicating the photostability of its components (Fig. 1D). The measurements also showed that VME absorbs in both the UVA and UVB region spectrum. However its absorption ability is stronger in the UVB area.
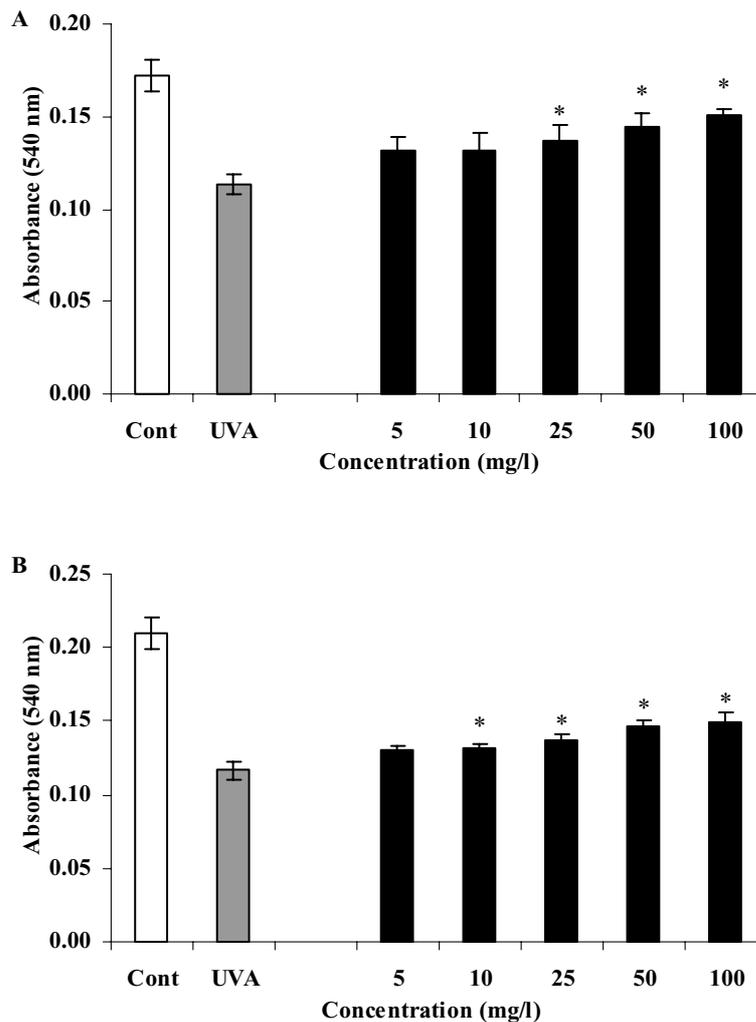
Fig. 3. Effect of VME on neutral red retention in UVA irradiated HaCaT. Cells were pre-treated (1 h) with VME (5–100 mg/l) (A) or post-treated (4 h) with VME (5–100 mg/l) (B). For irradiation the dose of 20 J/cm^2 was used. Irradiated and non-irradiated control cells were incubated with DMSO (0.5%; v/v) under the same conditions. Data are expressed as mean ± S.D. (*) $p < 0.05$ statistically different from irradiated cells.

3.2. Effect of VME and UVA irradiation on cell viability

First we evaluated the VME effect on HaCaT after 4 and 24 h treatment. VME at the concentrations tested (1–250 mg/l) did not affect cell viability, particularly NR incorporation and LDH leakage, after 4 h application (Fig. 2). NR uptake was not changed after 24 h either (Fig. 2A). However, VME treatment decreased LDH activity in medium samples containing 100 and 250 mg/l of VME after 24 h (Fig. 2B). The decrease should be caused due to inactivation or inhibition of LDH activity in consequence of high VME concentrations in the medium; however Valentová et al. assessed cytotoxicity of the VME on rat hepatocytes and find no adverse effect on LDH activity up to the concentration of 500 mg/l after 24 h [31]. We ascribe the effect to diminution of released LDH amount into the medium due to cell stabilization in the presence of VME. Moreover, we found no toxic effect on other parametres tested. For evaluation of
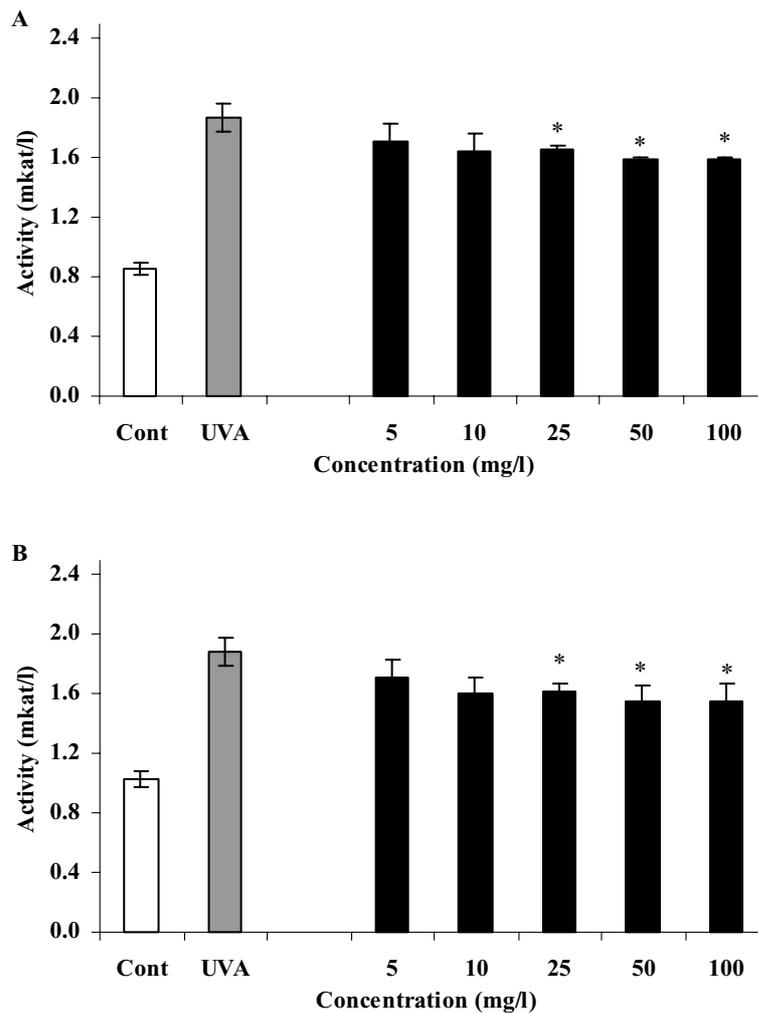
Fig. 4. Effect of VME on UVA-induced LDH leakage from HaCaT. Cells were pre-treated (1 h) with VME (5–100 mg/l) (A) or post-treated (4 h) with VME (5–100 mg/l) (B). For irradiation the dose of 20 J/cm^2 was used. Irradiated and non-irradiated control cells were incubated with DMSO (0.5%; v/v) under the same conditions. Data are expressed as mean $\pm$ S.D. (*) $p <$ 0.05 statistically different from irradiated cells.

protection on other parameters, the concentration range of 5–100 mg/l was chosen.

UVA (10, 20, 30 and 40 J/cm^2) cytotoxicity was also evaluated after 4 and 24 h. The results demonstrated that UVA light relevantly affected both cellular membrane integrity and keratinocyte viability at doses of 20–40 J/cm^2. Similar results were obtained for ROS generation. Depletion of intracellular GSH was significant already at the dose of 10 J/cm^2 and higher ones. In contrast, obvious increase in TBARS level was observed at the doses of 30 and 40 J/cm^2. Thus we used different UVA doses for individual markers (data not shown).

Pre-treatment (1 h) with of VME increased viability of UVA irradiated (20 J/cm^2) keratinocytes in a concentration dependent manner. In particular, the dose of 100 mg/l of VME provided nearly 50% protection (Fig. 3A). LDH release in pre-treated cells was attenuated as well (Fig. 4A). Application of VME after irradiation (4 h) also displayed significant diminution of HaCaT damage with a maximal
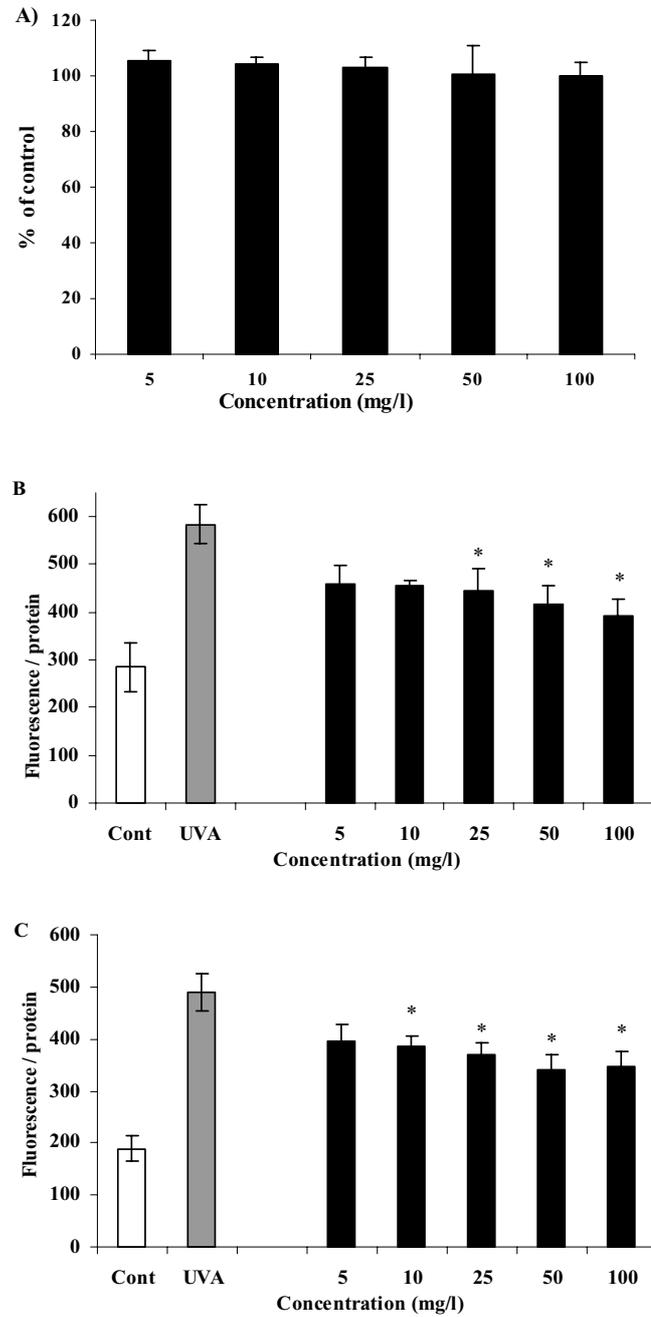
Fig. 5. Attenuation of UVA-induced ROS generation by VME. (A) Keratinocytes were treated with VME (5–100 mg/l) and ROS generation was assessed after 4 h. Data are expressed as % of control cells (non-irradiated, treated with DMSO). (B) Cells were pre-treated (1 h) with VME (5–100 mg/l) and irradiated or (C) exposed to UVA and post-treated (4 h) with VME (5–100 mg/l). For irradiation the dose of 20 J/cm^2 was used. Irradiated and non-irradiated control cells were incubated with DMSO (0.5%; v/v) under the same conditions. Data are expressed as mean $\pm$ S.D. (*) $p < 0.05$ statistically different from irradiated cells.
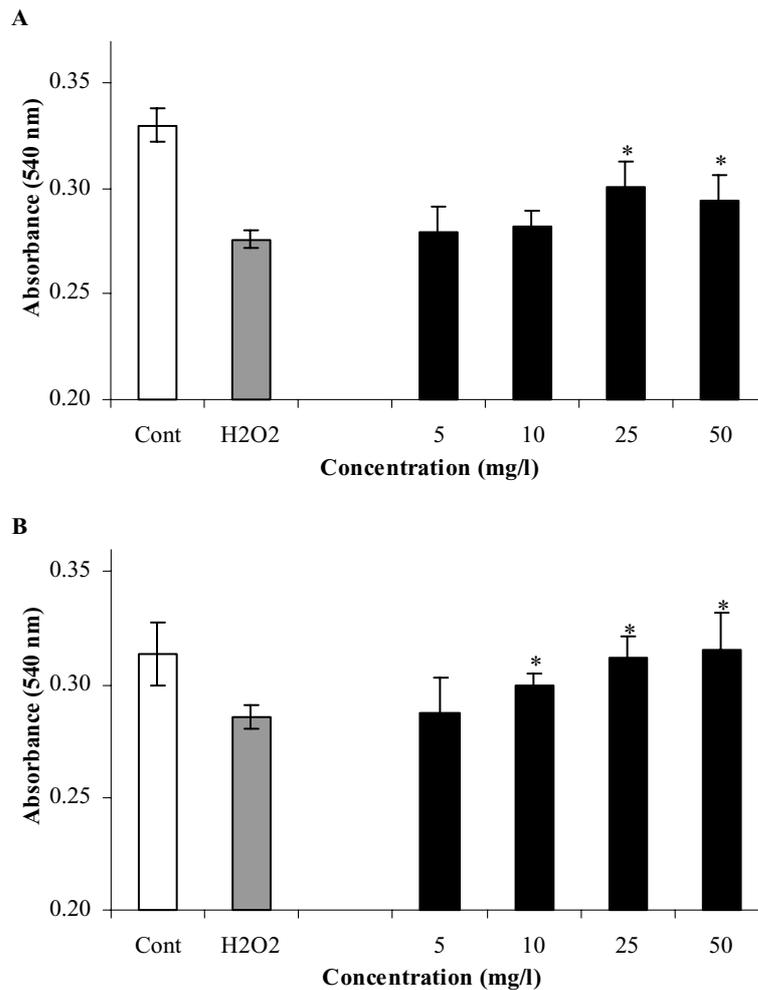
Fig. 6. Protection of H_2O_2-intoxicated HaCaT by VME application. After pre-treatment with VME (5–50 mg/l; 1h), cells were intoxicated (0.3 mM H_2O_2) and incubated for 4 h (A) or serum free medium was applied, cells were intoxicated (0.3 mM H_2O_2) and incubated for 4 h (B). Control cells were incubated with DMSO (0.5%; v/v) under the same conditions. VME effect on NR retention was evaluated. Data are expressed as mean $\pm$ S.D. (*) $p < 0.05$ statistically different from irradiated cells.

protection of 60% (NR retention; Fig. 3B) and 50% (LDH leakage; Fig. 4B) at the highest concentration tested.

3.3. VME treatment reduces UVA-induced ROS production

As shown in Fig. 5A, VME itself did not increase the level of intracellular ROS in non-irradiated cells. Also VME in PBS did not produce any significant fluorescent signal. UVA light (20 J/cm^2) dramatically increased ROS generation in keratinocytes. In HaCaT pre-treated with VME (5–100 mg/l) a decrease in fluorescence was evident. The maximal decline in ROS generation was at concentrations of 50 and 100 mg/l ($\sim$ 55%; Fig. 5B). VME application to UVA-treated cells led to a decline in ROS production. Concentrations of 50 and 100 mg/l VME were the most powerful (50%; Fig. 5C) as well. Both pre- and post-treatment were effective in a concentration dependent way.
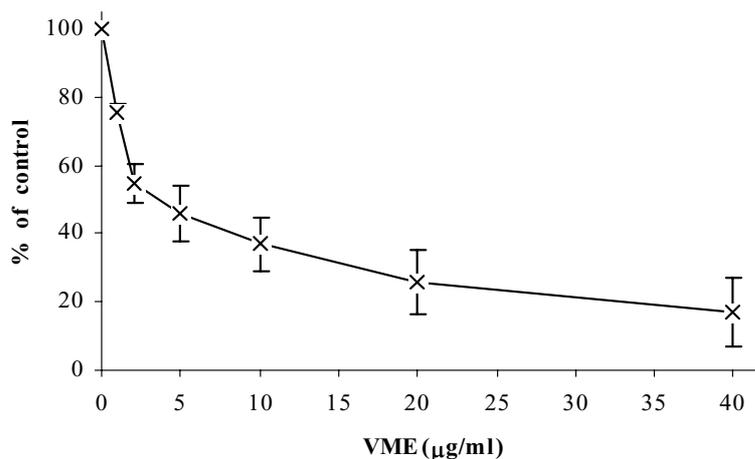
Fig. 7. Superoxide scavenging activity of VME. Superoxide anion was generated in a chemical system. Inhibition of NADH depletion, which is oxidised during the reaction, was calculated as follows: $(1-(\Delta A_S/\Delta dA_C))\cdot 100$ (%); $\Delta A_S-\Delta$ absorbance of sample (VME)/min, $\Delta Ac-\Delta$ absorbance of control (DMSO)/min.

3.4. VME application attenuates H_2O_2-induced damage

Hydrogen peroxide was used as a model reactive oxygen compound which is extensively generated during UVA exposure. Application of hydrogen peroxide in a concentration of 0.3 mM to HaCaT caused approximately 50% decrease in cell viability. Pre-treatment of keratinocytes with VME increased NR retention in a concentration dependent manner and the maximal effect was found at the concentration of 25 mg/l (50%; Fig. 6A). Treatment of cells with VME before (1 h) and during H_2O_2-exposure (4 h) led to more effective keratinocyte protection against damage (Fig. 6B) in comparison to 1 h-pre-treated with VME (Fig. 6A) and the protection reached 100% (25 and 50 mg/l).

3.5. Superoxide scavenging

To determine whether VME has the ability to scavenge superoxide, a non-cell system was utilized. As shown in Fig. 7 VME ability to eliminate superoxide was concentration dependent. The IC_{50} value was 2.90 ± 0.70 μg/ml.

3.6. VME prevents UVA-induced lipid peroxidation of cell membranes

The hallmark of UVA-induced damage is oxidation of various macromolecules including membrane lipids. Exposure of HaCaT to UVA light (30 J/cm^2) increased LPx by twofold compared to non-irradiated cells. VME (5–100 mg/l) alone did not induce LPx in non-irradiated cells (Fig. 8A). Pre-treatment of keratinocytes with VME significantly reduced UVA-caused LPx. The maximal protection was observed at a concentration of 50 mg/l (over 90%; Fig. 8B). Post-treatment with VME also markedly inhibited membrane lipid damage with maximum at concentrations of 25 and 50 mg/l (75–80%; Fig. 8C).

3.7. VME decreases UVA-induced GSH depletion

It is well-known that exposure to UV light leads to depletion of the antioxidant defence capability *in vitro* and *in vivo*. Using HaCaT cells as a model we also confirmed that UVA (10 J/cm^2) significantly
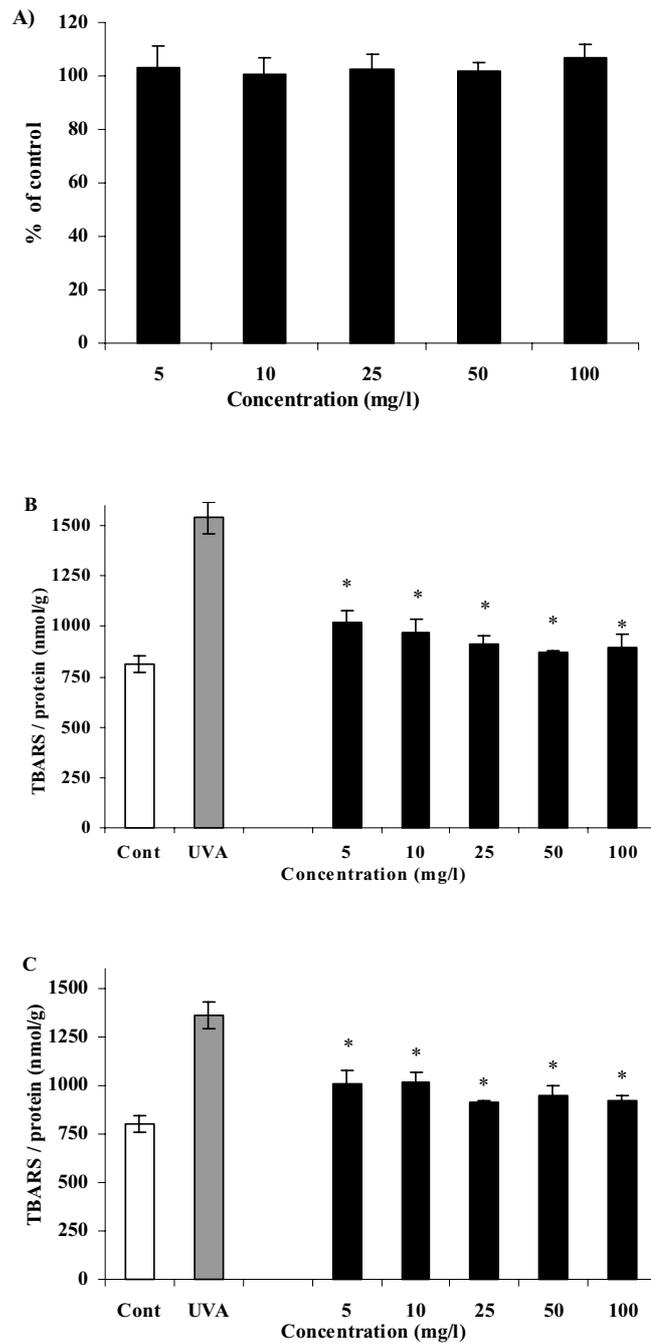
Fig. 8. Protection of UVA-induced lipid peroxidation in cell membranes by VME. (A) Keratinocytes were treated with VME (5–100 mg/l) and ROS generation was assessed after 4 h. Data are expressed as % of control cells (non-irradiated, treated with DMSO). (B) Cells were pre-treated (1 h) with VME (5–100 mg/l) and irradiated or (C) exposed to UVA and post-treated (4 h) with VME (5–100 mg/l). For irradiation the dose of 30 J/cm^2 was used. Irradiated and non-irradiated control cells were incubated with DMSO (0.5%; v/v) under the same conditions. Data are expressed as mean $\pm$ S.D. (*) $p < 0.05$ statistically different from irradiated cells.

reduced levels of intracellular GSH, an important non-enzymatic antioxidant. As shown in Fig. 9A, application of VME (5–100 mg/l) did not deplete intracellular GSH levels in HaCaT after 4 h. Conversely VME slightly increased GSH level in cells. Pre-treatment with VME significantly protects against UVA-caused GSH depletion, especially at a concentrations of 25 and 50 mg/l (55%; Fig. 9B). Post-treatment was the most effective in the concentration range of 50–100 mg/l (50%; Fig. 9C).

4. Discussion

A large number of independent researches have shown a correlation between consumption of fruits and vegetables with the prevention, delay or onset of chronic diseases including various kinds of cancer. Fruits and vegetables are rich in diverse nutrients, which can act as chemoprotectants. These structurally different components probably possess complementary and overlapping potential preventive actions such as antioxidant, anti-inflammatory, enhanced activity and/or expression of detoxification enzymes and strengthened immune system health [36]. It is no surprise that various component of human diets have been investigated for their potential UV protective ability. In this study we focused on the bilberry (*V. myrtillus*) fruit extract. Even though bilberry extract, as a whole, has not been studied to date, several components in the extract used, have UV protective potential such as delphinidin [1] or cyanidin-3-O-glucoside [8,9,29]. In order to examine the potential UVA preventive effect or therapeutic (regenerative) potential, keratinocytes were pre-treated (1 h) or post-treated (4 h) with VME (5–100 mg/l), respectively.

ROS overproduction is a pivotal characteristic phenomenon for UVA exposure. Increased ROS concentration, such as singlet oxygen, superoxide anion, hydrogen peroxide and hydroxyl radical [23], overwhelms the antioxidant mechanisms of targeted cells and contributes to further tissue damage. ROS are also known to impair the healing process and on the other hand antioxidants prevent tissue damage and stimulate wound healing [21]. Our experimental data, using H_2DCFDA, demonstrated that in both VME pre- and post-treated cells UVA-initiated, ROS generation was significantly prevented/reduced, respectively (Fig. 5). The VME effect of the both treatments was concentration dependent. In a non-cell model VME also showed the ability to eliminate chemically generated superoxide (Fig. 7), one ROS induced by UVA light. Superoxide is dismutated to hydrogen peroxide [20], which itself is not capable of causing damage. However, in the presence of metal cations (Fe, Cu) hydroxyl radicals are generated by the Fenton reaction [30]. Pre-treatment of HaCaT with VME also efficiently suppressed H_2O_2-induced damage, especially when VME was present after addition of hydrogen peroxide (Fig. 6).

Increased ROS formation after UVA radiation consecutively leads to oxidation of cell components such as proteins, lipids and DNA. Oxidative damage of lipids in biological membranes affects mainly polyunsaturated fatty acids and induces further pro-oxidants generation. Membrane destruction is linked to loss of fluidity, inactivation of membrane enzymes, to increase in cell membrane permeability to ions and eventually to rupture of membrane and release of organelles [11]. As we showed VME significantly inhibited membrane lipid oxidation, measured as TBARS level, when it was applied before or after irradiation (Fig. 8). In contrast to other parameters, VME effect was very potent even at low concentrations (5 and 10 mg/ml). We observed that after treatment with VME, coloured compounds were bound to keratinocyte membrane (even though we washed cells properly). It sems that compounds with higher molecular weight perhaps do not cross the phospholipid barrier and protect outer side of the cell membrane; this could explain powerful effectiveness of VME against UVA-induced membrane lipid peroxidation as well as H_2O_2-caused cell damage.

The preventive action of VME under conditions of oxidative stress may also be linked to its ability to modulate levels cell antioxidants. Recently, billberry anthocyanins were demonstrated to up-regulate the
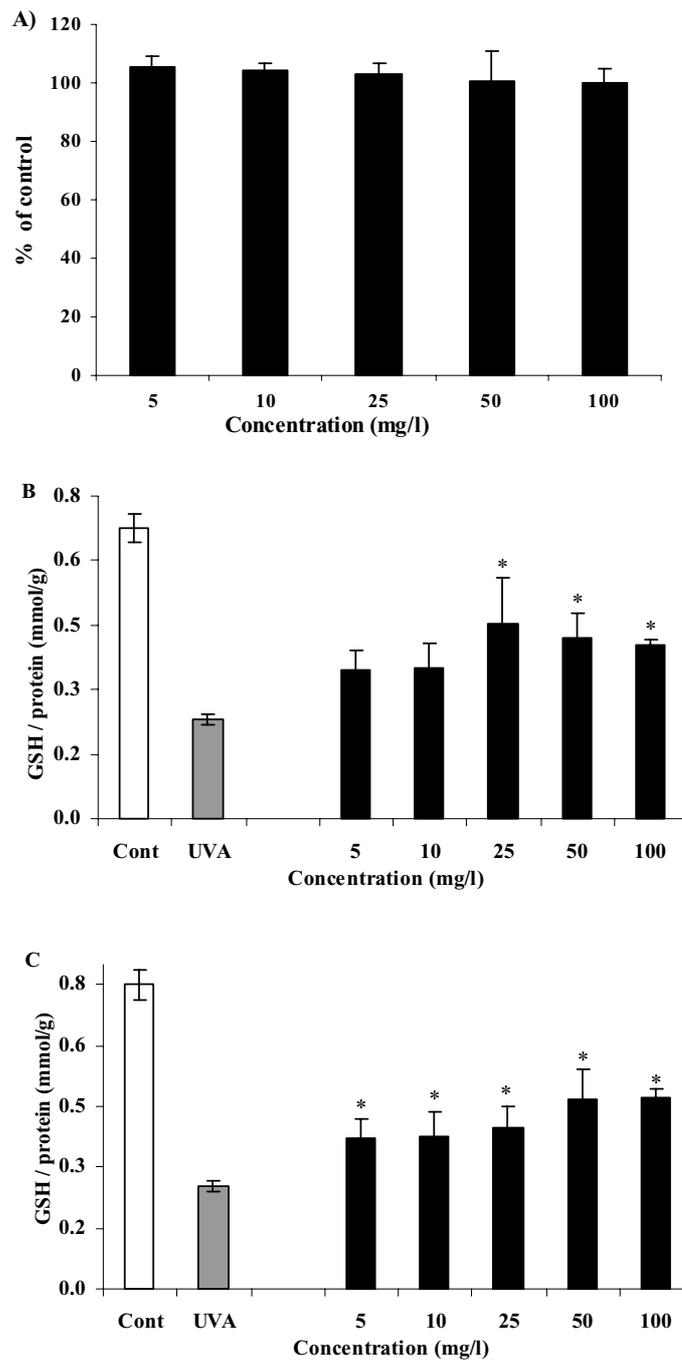
Fig. 9. Protection of UVA-induced GSH depletion by VME. (A) Keratinocytes were treated with VME (5–100 mg/l) and ROS generation was assessed after 4 h. Data are expressed as % of control cells (non-irradiated, treated with DMSO). (B) Cells were pre-treated (1 h) with VME (5–100 mg/l) and irradiated or (C) exposed to UVA and post-treated (4 h) with VME (5–100 mg/l). For irradiation the dose of 10 J/cm^2 was used. Irradiated and non-irradiated control cells were incubated with DMSO (0.5%; v/v) under the same conditions. Data are expressed as mean $\pm$ S.D. (*) $p < 0.05$ statistically different from irradiated cells.

oxidative stress defence enzymes heme oxygenase-1 and pi-class glutathione S-transferase in the human retinal pigment epithelial cell line (ARPE-19) [16]. The potential of *V. myrtillus* to protect against oxidative stress, especially lipid peroxidation and NO production, was also demonstrated in mice treated with $KBrO_3$, which induced ROS generation in kidney tissue [11]. Here we demonstrated that VME application markedly decreased GSH depletion in irradiated human keratinocytes cell line. The non-enzymatic defence system of skin cells consists of several scavenging molecules that are endogenously produced. Among these, GSH is the most important. GSH is necessary for maintaining of the intracellular redox state [22] and intracellular levels of GSH have been identified as a critical regulator of the induction of stress-activated signal transduction pathways [3,34]. Surprisingly pre-treatment with VME was the most efficient at a concentration of 25 mg/l (Fig. 9B). At the higher concentrations, protective ability of VME was decreased. However, post-treatment did not reveal this phenomenon and according to the absorption data VME appeared to be photostable. We assume that GSH depletion is probably connected with VME exposure to UVA light within keratinocytes. This phenomenon will be further studied.

As we demonstrated, VME components absorb photons in both UVA and UVB regions. Moreover the components displayed stability after UVA exposure (the dose of 20 J/cm^2; Fig. 1). For this reason, they presumably do not undergo photochemically induced reaction. These data indicate that compounds present in the used extract, in addition to antioxidant activity, have the potential to absorb UVA light and thus may act as UVA filters without production of toxic products. The UV screening properties of *V. myrtilus* polyphenols were studied by Jaakola et al. They demonstrated that direct sun exposure of *V. myrtilus* leaves stimulates the expression of flavonoid synthesis pathway genes. Concentration of cyanidin derivatives, catechin, epicatechin, quercetin, kaempferol, ferulic and caffeic acid conjugates were increased in exposed leaves. The authors suggested that these polyphenols play a predominant role in the defence against solar radiation in the leaves [12]. The photoprotective activity of VME that we observed may be attributed to anthocyanin fraction of the extract, mainly cyanidin, delphinidin and their derivatives which represent nearly 64% of all anthocyanins presented in the extract. Both cyanidin-3-glucoside and delphinidin have been shown to protect HaCaT against UVA- or UVB-caused damage [1,8,29,36]. Cyanidin-3-glucoside was also the predominat anthocyanidine (60%) in *Lonicera caerulea* fruit phenolic fraction (anthocyanins, 18.5% w/w), in which UVA-photoprotective effect was recently demonstrated [26]. However, penetration of anthocyanin glucosides through the membrane into keratinocytes may be quite difficult due to their molecular weigh. As mentioned above, during VME treatment some components coloured the membrane. So our ongoing experiments try to reveal which VME constituents and what amount get into the cells. Finally VME contains 75% of other phenolic constituents, except anthocyanins, thus it is predictable that other components contribute to the overall efficacy of the extract and contribution of individual compounds is speculative.

During outdoor activities, people are exposed to considerable UV doses. Extreme UV exposure in sports such as skiing, cycling, mountaineering or triathlon has been documented in a series of dosimetric studies. Moreover, sweating induced by physical exercise or heat may significantly contribute to UV-relate skin injury as it enhances skin photosensitivity, facilitating the risk of sunburn [17]. Although public health authorities recommend the consistent use of sunscreen preparations apart from avoidance of sunlight and the wearing of textiles and hats, people generally know little about the risk of sunlight [27]. Identification of novel agents with efficient full-spectrum protection (both UVA and UVB) is required. Perhaps testing of extracts or fractions isolated from plants, fruits or vegetables which are composed of structurally different components, is one way of discovering the most suitable prevention. Several studies have demonstrated excellent prevention by extracts *in vivo* [13,25]. Our results have shown that UVA-induced ROS-mediated cell responses in HaCaT keratinocytes can be modified by pre- and

post-treatment with VME. These effects of VME are mainly due to its antioxidant properties. Our results provide a confirmation for the photochemopreventive activity of VME and suggest that it may be a useful agent in photoprotective and/or regenerative cosmetics. However our *in vitro* model allows only investigation of response to VME application in monolayer. Thus further investigation is necessary on other skin cell types and skin/animal models.

Acknowledgements

References

[1] F. Afaq, D.N. Syed, A. Malik, N. Hadi, S. Sarfaraz, M.H. Kweon, N. Khan, M.A. Zaid and H. Mukhtar, Delphinidin, an anthocyanidin in pigmented fruits and vegetables, protects human HaCaT keratinocytes and mouse skin against UVB-mediated oxidative stress and apoptosis, *J Invest Dermatol* **127** (2006), 222–232.

[2] S.D. Angelo, D. Ingrosso, V. Migliardi, A. Sorrentino, G. Donnarumma, A. Baroni, L. Masella, M.A. Tufano, M. Zappia and P. Galletti, Hydroxytyrosol, a natural antioxidant from olive oil, prevents protein damage induced by long-wave ultraviolet radiation in melanoma cells, *Free Radic Biol Med* **38** (2005), 908–919.

[3] D. Bagchi, S. Roy, V. Patel, G. He, S. Khanna, N. Ojha, C. Philips, S. Ghosh, M. Bagchi and C.K. Sen, Safety and whole-body antioxidant potential of a novel anthocyanin-rich formulation of edible berries, *Mol Cell Biochem* **281** (2006), 197–209.

[4] M.S. Baliga and S.K. Katiyar, Chemoprevention of photocarcinogenesis by selected dietary botanicals, *Photochem Photobiol Sci* **5** (2006), 243–253.

[5] L. Bao, X.S. Yao, D. Tsi, C.C. Yau, C.S. Chia, H. Nagai and H. Kurihara, Protective effects of bilberry (*Vaccinium myrtillus* L.) extract on KBrO$_3$-induced kidney damage in mice, *J Agric Food Chem* **56** (2008), 420–425.

[6] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal Biochem* **72** (1976), 248–254.

[7] J.A. Buege and S.D. Aust, Microsomal lipid peroxidation, *Methods Enzymol* **52** (1978), 302–310.

[8] F. Cimino, R. Ambra, R. Canali, A. Saija and F. Virgili, Effect of cyanidin-3-O- glucoside on UVB-induced response in human keratinocytes, *J Agric Food Chem* **54** (2006), 4041–4047.

[9] M. Ding, R. Feng, S.Y. Wang, L. Bowman, Y. Lu, Y. Oian, V. Castranova, B.H. Juany and X. Shi, Cyanidin-3-glucoside, a natural product derived from blackberry, exhibits chemopreventive and chemotherapeutic activity, *J Biol Chem* **281** (2006), 17359–17368.

[10] A.J. Faria, J. Oliveira, P. Neves, P. Gamerio, C. Santos-Buelga, V. de Freitas and N. Mateus, Antioxidant properties of prepared blueberry (*Vaccinium myrtillus*) extracts, *J Agric Food Chem* **53** (2005), 6896–6902.

[11] A.W. Girotti and T. Kriska, Role of lipid hydroperoxides in photo-oxidative stress signaling, *Antioxid Redox Signal* **6** (2004), 301–310.

[12] L. Jaakola, K. Määttä-Riihinen, S. Kärenlampi and A. Hohtola, Activation of flavonoid biosynthesis by solar radiation in bilberry (*Vaccinium myrtillus* L.) leaves, *Planta* **218** (2004), 721–728.

[13] S. Katiyar, C.A. Elmets and S.K. Katiyar, Green tea and skin cancer: photoimmunology, angiogenesis and DNA repair, *J Nutr Biochem* **18** (2007), 287–296.

[14] S.B. Martín-Aragón, B. Basabe, J.M. Benedí and A.M. Viklat, Antioxidant action of *Vaccinium myrtillus* L., *Phototerapy Res* **12** (1998), S104–S106.

[15] P.E. Milbury, G. Gao, R.L. Prior and J. Blumberg, Bioavailablility of elderberry anthocyanins, *Mech Ageing Dev* **123** (2002), 997–1006.

[16] P.E. Milbury, B. Graf, J.M. Curran-Celentano and J.B. Blumberg, Bilberry (*Vaccinium myrtillus*) anthocyanins modulate heme oxygenase-1 and glutathione S-transferase-pi expression in ARPE-19 cells, *Invest Ophthalmol Vis Sci* **48** (2007), 2343–2349.

[17] M. Moehrl, Outdoor sports and skin cancer, *Clin Dermatol* **26** (2008), 12–15.

[18] P. Morazzoni and E. Bombardelli, *Vaccinium myrtillus* L., *Fitoterapia* **67** (1996), 3–29.

[19] F. Paoletti and A. Mocali, Determination of superoxide dismutase activity by purely chemical system based on NAD(P)H oxidation, *Methods Enzymol* **186** (1990), 209–220.

[20] D.I. Pattison and M.J. Davies, Actions of ultraviolet light on cellular structures, *EXS* **96** (2006), 131–157.

[21] T.T. Phan, L. Wang, P. See, R.J. Grayer, S.Y. Chan and S.T. Lee, Phenolic compounds of *Chromolaena odorata* protect cultured skin cells from oxidative damage: implication for cutaneous wound healing, *Biol Pharm Bull* **24** (2001), 1373–1379.

[22] T. Rousar, Z. Červinková, V. Mužáková, O. Kužera, H. Lotková and P. Křiváková, Glutathione and glutathione assays, *Acta Medica* (*Hradec Kralove*) *Suppl* **48** (2005), 15–20.

[23] C.S. Sander, H. Chang, F. Hamm, P. Elsner and J.J. Thiele, Role of oxidative stress and the antioxidant network in cutaneous carcinogenesis, *Int J Dermatol* **43** (2004), 326–335.

[24] J. Sedlak and R.H. Lindsay, Estimation of total, protein bound, and non-protein sulfhydryl groups in tissue with Ellma's reagent, *Anal Biochem* **25** (1968), 192–205.

[25] S.D. Sharma, S.M. Meeran and S.K. Katiyar, Dietary grape seed proanthocyanidins inhibit UVB-induced oxidative stress and activation of mitogen-activated protein kinases and nuclear factor-kappaB signaling in *in vivo* SKH-1 hairless mice, *Mol Cancer Ther* **6** (2007), 995–1005.

[26] A. Svobodová, J. Rambousková, D. Walterová and J. Vostálová, Protective effects of phenolic fraction of blue honeysuckle fruits against UVA-induced damage to human keratinocytes, *Arch Dermatol Res* **300** (2008), 225–233.

[27] A. Svobodová, D. Walterová and J. Vostálová, Ultraviolet light induced alteration to the skin, *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub* **150** (2006), 25–38.

[28] A. Svobodová, A. Zdařilová, J. Mališková, H. Mikulková, D. Walterová and J. Psotová, Attenuation of UVA-induced damage human keratinocytes by silymarin, *J Derm Sci* **46** (2007), 21–30.

[29] A. Tarozzi, A. Marchesi, S. Hrelia, C. Angeloni, V. Anrdisano, J. Fiori, G. Cantelli-Forti and P. Hrelia, Protective effects of cyanidin-3-O-beta-glucopyranoside against UVA-induced oxidative stress in human keratinocytes, *Photochem Photobiol* **81** (2005), 623–629.

[30] J. Thiele and P. Elsner, *Oxidants and Antioxidants in Cutaneous Biology*, Karger, Switzerland 2001.

[31] K. Valentová, J. Ulrichová, L. Cvak and V. Šimánek, Cytoprotective effect of a bilberry extract against oxidative damage of rat hepatocytes, *Food Chemistry* **101** (2007), 912–917.

[32] P.K. Vayalil, A. Mittal, Y. Hara, C.A. Elmets and S.K. Katiyar, Green tea polyphenols prevent ultraviolet light-induced oxidative damage and matrix metalloproteinases expression in mouse skin, *J Invest Dermatol* **122** (2004), 1480–1487.

[33] L.S. Verschooten, S. Claerhout, A. Van Laethem, P. Agostinis and M. Garmyn, New strategies of photoprotection, *Photochem Photobiol* **82** (2006), 1016–1023.

[34] D. Wilhelm, K. Bender, A. Knebel and P. Angel, The level of intracellular glutathione is a key regulator for the induction of stress-activated signal transduction pathways including Jun N-terminal protein kinases and p38 kinase by alkylating agents, *Mol Cell Biol* **17** (1997), 4792–4800.

[35] A.R. Young, Acute effects of UVR on human eyes and skin, *Prog Biophys Mol Biol* **92** (2006), 80–85.

[36] S. Zafra-Stone, T. Zasmán, M. Bagchi, A. Chatterjee, J.A. Vinson and D. Bagchi, Berry anthocyanins as novel antioxidants in human health and disease prevention, *Mol Nutr Food Res* **51** (2007), 675–683.