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Flavonolignans from *Silybum marianum* moderate UVA-induced oxidative damage to HaCaT keratinocytes

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Apoptosis;
DNA damage

Summary

Background: UV radiation from sunlight is a very potent environmental risk factor in the pathogenesis of skin cancer. Exposure to UV light, especially the UVA part, provokes the generation of reactive oxygen species (ROS), which induce oxidative stress in exposed cells. Topical application of antioxidants is a successful strategy for protecting the skin against UV-caused oxidative damage.
Objective: In this study, silybin (SB) and 2,3-dehydrosilybin (DS) (1—50 μmol/l), flavonolignan components of *Silybum marianum*, were tested for their ability to moderate UVA-induced damage.
Methods: Human keratinocytes HaCaT were used as an appropriate experimental *in vitro* model, to monitor the effects of SB and DS on cell viability, proliferation, intracellular ATP and GSH level, ROS generation, membrane lipid peroxidation, caspase-3 activation and DNA damage.
Results: Application of the flavonolignans (1—50 μmol/l) led to an increase in cell viability of irradiated (20 J/cm^2) HaCaT keratinocytes. SB and DS also suppressed intracellular ATP and GSH depletion, ROS production and peroxidation of membrane lipids. UVA-induced caspases-3 activity/activation was suppressed by treatment with SB and DS. Lower concentrations of both compounds (10 μmol/l) significantly reduced cellular DNA single strand break formation.
Conclusion: Taken together, the results suggest that these flavonolignans suppress UVA-caused oxidative stress and may be useful in the treatment of UVA-induced skin damage.

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1. Introduction

Ultraviolet (UV) radiation (100—400 nm) has some beneficial health effects like vitamin D_3 formation or in combination with drugs it is used in the treatment of certain skin diseases such as psoriasis. However, it also causes a number of acute and chronic detrimental skin effects which can result in inflammation, immunosuppression, premature skin aging (photoaging) and the development of skin malignancies [1]. UVA radiation (315—400 nm), which is not absorbed in the ozone layer, comprises more than 95% of the UV light that reaches the earth. UVA penetrates the epidermis and affects both epidermal and dermal layers of the skin. At a cellular level, UVA-induced damage occurs mainly indirectly via oxidative processes initiated by endogenous photosensitization. UVA exposure causes significant oxidative stress via generation of reactive oxygen species (ROS) such as singlet oxygen, hydroxyl radical, superoxide anion and hydrogen peroxide as well as reactive nitrogen species (RNS), mostly nitric oxide and nitric dioxide [2]. ROS and RNS are rapidly removed by non-enzymatic (especially glutathione (GSH)) and enzymatic antioxidants (catalase, superoxide dismutase, thioredoxin reductase, glutathione peroxidase and glutathione reductase) that maintain the pro-oxidant/anti-oxidant balance resulting in cell and tissue stabilization. However, a surplus of ROS may overwhelm the skin anti-oxidant defence mechanisms causing pro-oxidant/anti-oxidant disequilibrium. Overproduction of ROS and RNS induces oxidation of nucleic acids, proteins and membrane lipids, which also lead to intracellular GSH and NADH/NADPH depletion and therefore energy loss from the cell. UV-generated ROS also affect the regulation of the gene expression of signalling molecules/cascades such as mitogen-activated protein kinases and interrelated inflammatory cytokines as well as NF-κB and activator protein-1 [3].

One approach for cutaneous photoprotection is exogenous application of substances to the skin that support the skin's own protective mechanisms or that attenuate the amount of UV penetrating the skin. In this regard, various natural substances and extracts have been investigated. Among these, plant phenolics have gained prominent importance. For a long time UVB light (280—315 nm) was considered to be responsible for the UV-induced deleterious effect and for this reason most studies were performed in the UVB region. Intense examination of UV influence on the skin, however, has revealed that the UVA part is also involved in induction and development of skin cancer [2,4,5]. To date only a number of substances/extracts have been demonstrated to be capable of protecting UVA-induced skin cells/skin injury, e.g. carnosic acid [6], quercetin [7], resveratrol [8], epikatechin [9,10], epigallokatechin-3-gallate [11,12] or *Polypodium leucotomos* extract [13], or suppressing UVA-caused damage, e.g. *Prunella vulgaris* extract [14] and silymarin [15].

Silybum marianum L. Gaertner (syn. *Carduus marianus* L., milk thistle; family of Asteraceae) is one of the oldest known herbal plants and has been widely used in traditional European medicine mainly for the treatment of liver disorders [16]. A standardized polyphenolic fraction of the extract from the seeds is called silymarin (SM) and contains approximately 70—80% flavonolignans and a 20—30% chemically undefined fraction, comprising mostly polymeric and oxidized polyphenolic compounds. Flavonolignans are mainly silybin (silibinin; SB; Fig. 1), isosilybin, silychristin and silydianin. Other substances present in lesser amount are flavonolignan 2,3-dehydrosilybin (DS; Fig. 1) and flavonoids taxifolin and quercetin [17]. However, SB is the major biologically active constituent of SM. Animal and human studies of both SB and SM have shown that they are well tolerated and do not cause any significant adverse health effects [18]. SB and SM, primarily known for their hepatoprotective and antioxidant activities, have been intensively studied for their UVB-photoprotective ability. A number of studies have shown that SM and SB have chemopreventive effects against UVB-induced photocarcinogenesis in animal models [19—22] and SB may suppress UVB damage to human keratinocytes as well [23]. Recently, we have demonstrated that SM and its flavonolignans SB and DS prevent hydrogen peroxide-induced oxidative stress in HaCaT

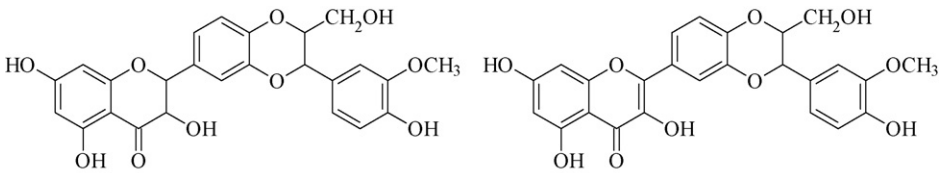

Fig. 1 Structure of silybin and 2,3-dehydrosilybin.

[24]. We also found that SM was able to attenuate UVA-caused damage to HaCaT [15].

In the current study, the ability of silymarin's constituents to ameliorate UVA-induced damage in HaCaT was investigated. SB, the major biologically active component, and DS, the minor flavonolignan with prominent antioxidant potency, were chosen. In particular, we focused on their effects upon ROS production and oxidative stress markers as well as DNA damage and apoptosis.

2. Material and methods

2.1. Material

Cell proliferation ELISA BrdU (colorimetric) kit and protease inhibitor cocktail tablete Complete^{TM} were purchased from Roche (Germany). 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA) was from Fluka Chemie (Germany). Caspase-3 fluorigenic substrate Ac-DEVD-AMC and inhibitor Ac-DEVD-CHO were obtained from Bachem AG (Switzerland). Western blotting luminol reagent for chemiluminiscent horseradish peroxidase detection, actin (I-19) goat polyclonal primary antibody, and goat anti-rabbit and rabbit anti-goat HRP conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (USA). Dulbecco's modified Eagle's medium (DMEM), heat-inactivated fetal calf serum (FCS), L-glutamine, stabilised penicillin—streptomycin solution, sterile dimethylsulfoxide (DMSO), neutral red (NR), agarose types I and VII, 2,2′-dinitro-5,5′dithiobenzoic acid (DTNB), ethidium bromide, rabbit anti-caspase-3 primary antibody and all other chemicals were purchased from Sigma—Aldrich (USA).

2.2. Test compounds

Silybin (batch 120692; $C_{25}H_{22}O_{10}$, Mr 482) was kindly provided by IVAX Pharmaceuticals s.r.o. (Opava, Czech Republic). 2,3-Dehydrosilybin (98%; $C_{25}H_{20}O_{10}$, Mr 480) was prepared in the Institute of Microbiology, Academy of Sciences of the Czech Republic (Prague, Czech Republic). Stock solutions of flavonolignans (0.20—10.05 mmol/l) were dissolved in DMSO. The final concentration of DMSO in the medium was 0.5% (v/v).

2.3. Cell culture

HaCaT keratinocytes, a spontaneously transformed human epithelial cell line, were grown in DMEM supplemented with FCS (7%; v/v), streptomycin (100 U/ml), penicillin (0.1 mg/ml) and glutamine (4 mmol/l) in a humidified atmosphere with CO_2 (5%; v/v) at 37 °C. The culture medium was changed twice a week. The cells were subcultured following trypsinization. For experiments, cells were seeded at a density of 1×10^5 cells/cm^2 and grown near to confluence.

2.4. Flavonolignans cytotoxicity

Culture medium was removed and keratinocytes were treated with serum-free medium containing flavonolignans (1—50 μmol/l) at 37 °C for 4 h. Control cells were treated with serum-free medium containing DMSO (0.5%; v/v) under the same conditions. Cell damage was evaluated using: neutral red (NR) retention, activity of lactate dehydrogenase (LDH) released into the medium, level of intracellular ATP and GSH, lipid peroxidation (LPx), ROS generation (dichlorodihydrofluorescein assay).

2.5. UVA irradiation and treatment with flavonolignans

Prior to UV irradiation, cells were washed with phosphate saline buffer (PBS) and covered with a thin layer of PBS. Dishes 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 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) transmitting the wavelengths of 315—380 nm. The UVA output measured by an UVA-meter (Dr. Hönle UV Technology) in direct contact with the cell culture dish was 6.2 W/cm^2. Cells were irradiated with doses of 20 or 30 J/cm^2.

After UVA irradiation PBS was removed, serum-free medium with flavonolignans (1—50 μmol/l) was applied and the cells were incubated thereafter at 37 °C for 4 h. Irradiated and non-irradiated control cells were treated with serum-free medium containing the same aliquot of DMSO (0.5%; v/v) instead of test compound stock solution. The following parameters were used for the monitoring flavonolignan effect on UVA treated keratinocytes: NR retention, LDH leakage, cell proliferation, intracellular ATP and GSH levels, lipid peroxidation (LPx), ROS generation (dichlorodihydrofluorescein assay), DNA breakage (Comet assay), caspase-3 activation (activity and protein level).

2.6. Neutral red assay

Neutral red (NR) is a water-soluble dye that incorporates into the lysosomes of living cells in a process

requiring cellular energy. NR uptake is measured spectrophotometrically at 540 nm after extraction of retained NR into acidic methanolic solution [25]. NR (0.03%, w/v) in PBS was added to the cells (2 h; 37 °C). Then the cells were washed with washing solution (formaldehyde (0.125%; v/v), $CaCl_2$ (0.25%, w/v)) and the retained NR was dissolved in extraction solution (methanol (50%; v/v), acetic acid (1%; v/v)). The absorbance was measured with a microplate reader (*Sunrise Remote*, Tecan, Austria).

2.7. LDH release assay

The activity of LDH (EC 1.1.1.27), released into the culture medium via damaged membrane, was measured spectrophotometrically. The method is based on LDH-catalysed reduction of pyruvate to lactate by an equimolar amount of NADH. Decrease in NADH is monitored at 340 nm [25]. After incubation, the sample (serum-free medium) was mixed with fresh LDH buffer (50 mmol/l Na_2HPO_4; 1.22 mmol/l sodium pyruvate; pH 7.5) containing NADH (0.33 g/l) on a microplate and the decrease in absorbance was monitored.

2.8. Cell proliferation

Cell proliferation was determined using a colorimetric immunoassay, based on the measurement of 5-bromo-2′-deoxyuridine (BrdU) incorporation in place of thymidine into the DNA during DNA synthesis. Proliferation was monitored using a Cell proliferation ELISA BrdU (colorimetric) kit according to the manufacturer's protocol.

2.9. Intracellular ATP level

Intracellular ATP content was determined by ion-pair chromatography analysis [26] of cell lysates. The cells were rinsed with cooled PBS and scraped into cooled perchloric acid (6%; v/v). The suspension was neutralised with cooled KOH (3 mol/l) and centrifuged (10 min; 13,000 rpm; 4 °C). The supernatant was used for quantification of ATP using HPLC *LC 10* (Shimadzu, Japan) equipped with HPLC *Supelcosil*™ *LC-18-T* column (4.6 mm × 250 mm, 5 μm) and guard column *Supesco C18* (20 mm) (Sigma–Aldrich, USA). HPLC equipment was maintained at 28 °C. The mobile phase (95% phosphate buffer (100 mmol/l; pH 7.0) with TBHS (2 mmol/l) and acetonitrile (5%; v/v)) was delivered at a rate of 1 ml/min and the diode array detector *SPD-M10AVP* (Shimadzu, Japan) operated at 259 nm. The ATP quantity in cell lysates was determined from the calibration curves.

2.10. Intracellular GSH level

Total GSH was estimated using a reaction with DTNB [27]. The cells were rinsed with cooled PBS, scraped into cooled perchloric acid (1%; v/v) and sonicated. The aliquots were used for protein determination by Lowry assay [28]. The suspension was centrifuged (10 min; 13,000 rpm; 4 °C) and supernatant was mixed with reaction mixture (800 mmol/l Tris/HCl, 20 mmol/l EDTA, pH 8.2; 20 mg/ml DTNB). A yellow chromophore was measured spectrophotometrically at 412 nm.

2.11. Lipid peroxidation

The extent of LPx was evaluated using TBARS assay [29]. Thiobarbituric acid (TBA) reacts with products of oxidative degradation of lipids named thiobarbituric acid reactive substances (TBARS), yielding red complexes, which absorb at 535 nm. The cells were washed with cooled PBS, scraped into trichloroacetic acid (2.8%; w/v), sonicated and aliquots were used for protein determination by Lowry assay [28]. The suspension was mixed with TBA (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 TBARS was determined spectrophotometrically.

2.12. Evaluation of ROS generation

The radical scavenging effect of flavonolignans in irradiated cells was monitored by dichlorodihydrofluorescein assay. The polar, pre-fluorescent dichlorodihydrofluorescein diacetate (H_2DCFDA) undergoes deacetylation by cytosolic esterases to form dichlorodihydrofluorescein, which reacts with ROS and gives rise to fluorescein. The fluorescence is monitored at specific excitation/emission wavelengths 488/525 nm.

After irradiation and treatment with flavonolignans, the cells were incubated with H_2DCFDA (5 nmol/l) 15 min in the incubator at 37 °C. Cells were then washed with PBS and scraped into 2 ml of PBS and sonicated [30]. The fluorescence was measured using a spectrophotometer (*LS 50 B*, Perkin-Elmer, USA). The protein concentration was determined by Bradford assay [31].

2.13. DNA single strand breaks (Comet assay)

Single strand breaks were monitored using the single cell gel electrophoresis (Comet assay). This method is based on monitoring DNA lesions in individual cells, after sandwiching a small number of cells between thin layers of agarose and subsequent lysis

and electrophoresis of cellular DNA. Damaged DNA is visualised by fluorescent stain as comet formation [32]. Standard melting-point agarose (1%; w/v) was applied on an agarose-coated slide, covered with a cover slip and placed on ice for 15 min. The cells were trypsinised, pelleted, resuspended in PBS and mixed with low-melting-point agarose (1%; w/v), then transferred to the layer of agarose, covered with a cover slip and the slides placed on ice. The cover slips were then removed and the samples were incubated in cold lysis buffer (2.5 mol/l NaCl, 100 mmol/l EDTA, 10 mmol/l Tris and 1% Triton X (v/v); 1 h; 4 °C). Electrophoresis was done (16 V; 20 min; 4 °C) in electrophoresis buffer (300 mmol/l NaOH and 1 mmol/l EDTA). The slides were washed three times with cold neutralising buffer (0.4 mol/l Tris), dried, stained with ethidium bromide and the nuclei were evaluated using an inversion fluorescent microscope Olympus IX 70 and analysed by an image analysis Olympus MicroImage (Olympus, Japan).

DNA damage was assessed in 100 nuclei per slide area by visual scoring from 0 (undamaged, no discernible comet tail) to 4 (almost all DNA in tail, insignificant head). Each comet was given a value according to its classification to produce an overall score for each slide ranging from 0 to 400 arbitrary units. Scores were calculated using the following formula [33]:

$$\text{total damage}\,(\%) = \frac{N_0 0 + N_1 1 + N_2 2 + N_3 3 + N_4 4}{N_0 + N_1 + N_2 + N_3 + N_4} \times 100$$

N_0, N_1, N_2, N_3 and N_4 are the number of cells in each group from 0 to 4.

2.14. Caspase-3 activity (Ac-DEVD-AMC cleavage assay)

Caspase-3 activity was measured as caspase-3-like protease activity using fluorogenic substrate Acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) [34]. The cells were washed with PBS and lysed in ice-cold lysis buffer (50 mmol/l HEPES, pH 7.4; Triton X-100 (0.5%; v/v), protease inhibitor cocktail tablet, 5 mmol/l dithiothreitol (DDT); 15 min; 4 °C). The lysate was cleared by centrifugation (14,000 rpm; 15 min; 4 °C). The protein concentration was determined by Bradford assay [31]. The lysate was mixed with assay buffer (20 mmol/l HEPES, pH 7.1, 2 mmol/l EDTA, protease inhibitor cocktail tablet, 5 mmol/l DDT) containing Ac-DEVD-AMC or inhibitor Acetyl-Tyr-Val-Ala-Asp aldehyde (Ac-DEVD-CHO) and incubated (37 °C; 60 min). Fluorescence was measured at 380/450 nm using a spectrophotometer (*LS 50 B*, Perkin-Elmer, USA).

2.15. Caspase-3 activation (Western immunoblot)

Activated caspase-3 protein amount was investigated using western immunoblot analysis. The cells were washed with PBS and ice-cold lysis buffer (20 mmol/l Tris, 5 mmol/l EGTA, 150 mmol/l NaCl, 20 mmol/l glycerol phosphate, 1 mmol/l NaF, Triton X-100 (1%; v/v), 1 mmol/l Na_3VO_4, Tween 20 (0.1%; v/v), protease inhibitor cocktail tablet) was added. After incubation (15 min; 4 °C), the cells were scraped and the lysate was cleared by centrifugation (14,000 rpm; 15 min; 4 °C). The supernatant protein concentration was determined by Lowry assay [28]. Samples were mixed with sample buffer (125 mmol/l Tris, pH 6.8; SDS (4%; m/v); glycerol (20%; v/v); 200 mmol/l DTT; bromophenol blue (0.02%; m/v); 4:1) and heated (95 °C; 5 min). Proteins were separated by 15% SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Residual binding sites on the membrane were blocked using blocking buffer (5% non-fat dried milk (w/v) in 100 mmol/l Tris-buffered saline (pH 7.5) with Tween 20 (0.05%; v/v)) for 1 h at room temperature. The membrane was then incubated with a primary antibody (rabbit anti-caspase-3 primary antibody or actin (I-19) goat polyclonal antibody) overnight at 4 °C and then with a secondary horseradish peroxidase conjugated antibody (goat anti-rabbit antibody or rabbit anti-goat antibody) for 2 h at room temperature. Caspase-3/actin expression was detected by chemiluminiscence using Western blotting Luminol Reagent and autoradiography with XAR-5 film [35].

2.16. Statistical analysis

The series of experiments were performed as three or more independent examinations with at least three replicates for each sample. Data were expressed as means ± S.D. Statistical comparison was performed using Student's *t*-test. Statistical significance was determined at $p = 0.05$.

3. Results

3.1. Cytotoxicity of flavonolignans

Possible harmful effects of flavonolignans were monitored in a concentration range of 1—50 μmol/l after 4 h treatment. SB itself caused no changes in NR retention or LDH leakage as well as no depletion of intracellular ATP or GSH at the concentrations tested (data published elsewhere; ref. [24]). SB treatment caused no increase in ROS generation and induced no

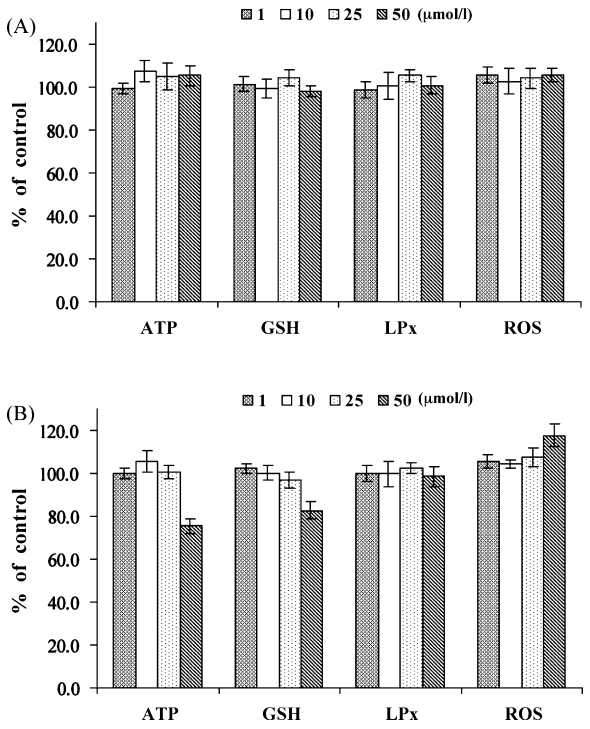

Fig. 2 Effect of SB and DS on HaCaT. Keratinocytes were treated with (A) SB or (B) DS (1—50 μmol/l) for 4 h; control cells were incubated with DMSO (0.5%; v/v) under the same conditions. Cell damage was evaluated using the following parameters: intracellular ATP level (ATP); intracellular GSH content (GSH); peroxidation of membrane lipids (LPx); ROS generation (ROS). Data are expressed as % of control and plotted as mean ± S.D. of three independent experiments performed in triplicate.

peroxidation of membrane lipids (Fig. 2A) either. DS showed slight toxic effects; it induced a disturbance in NR uptake, release of intracellular LDH and depletion of ATP and GSH at a concentration of 50 μmol/l (data published elsewhere; ref. [24]). DS application did not lead to LPx elevation, however, it slightly increased ROS creation in HaCaT at the highest concentration (Fig. 2B).

3.2. Effect of flavonolignans on cell viability in UVA-irradiated cells

The cell viability of keratinocytes was substantially reduced by UVA (20 J/cm^2) as assessed by NR (Fig. 3A) and LDH assays (Fig. 3B). Post-treatment with flavonolignans increased the cell viability in a dose-dependent manner. Compounds were the most effective at a concentration of 25 μmol/l. DS displayed significantly higher protection (48% for NR assay and 42% for LDH assay) at this concentration than SB (31% for NR assay and 25% for LDH assay). At a concentration of 50 μmol/l, both flavonolignans demonstrated a decrease in protection of NR uptake. In the case of DS, the decrease was more conspicuous in line with its cytotoxicity. The ability of DS to attenuate UVA-caused LDH leakage was again diminished at a concentration of 50 μmol/l.

3.3. Effect of flavonolignans on cell proliferation in UVA-irradiated keratinocytes

As shown in Fig. 4, cell exposure to UVA (20 J/cm^2) also significantly suppressed keratinocyte proliferation (approximately 70% reduction in comparison to non-irradiated cells). Application of SB and DS only non-significantly (5% increase) influenced BrdU incorporation into DNA in UVA-exposed cell.

3.4. Modulation of UVA-induced oxidative stress by flavonolignans

To investigate whether the addition of flavonolignans influences UVA-induced ROS generation (20 J/cm^2), a fluorescent assay using H_2DCFDA was

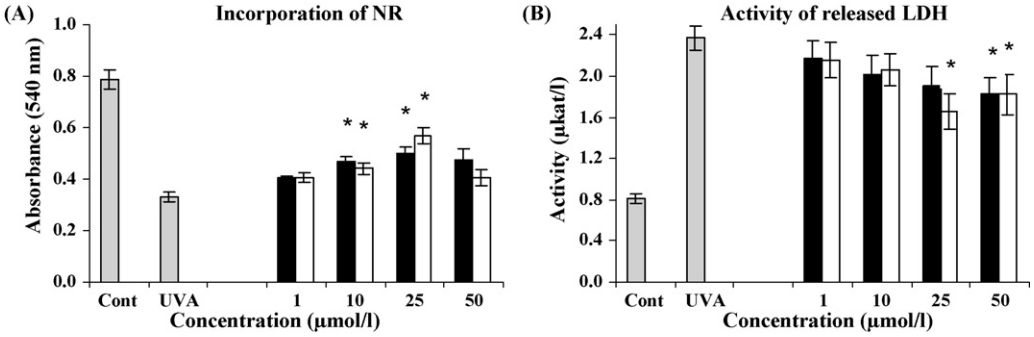

Fig. 3 Modulation of UVA-disturbed cell viability by SB and DS. Keratinocytes were irradiated (20J/cm^2) and treated with SB or DS (1—50 μmol/l) for 4 h. Irradiated and non-irradiated control cells were incubated with DMSO (0.5%; v/v) under the same conditions. Effect of SB (black bars) and DS (white bars) on UVA-induced alteration to (A) NR retention and (B) released LDH activity was evaluated. Data are expressed as mean ± S.D. of four independent experiments performed in triplicate. Statistical comparison analysis was performed using Student's *t*-test. (*) $p < 0.05$ statistically different from irradiated cells.

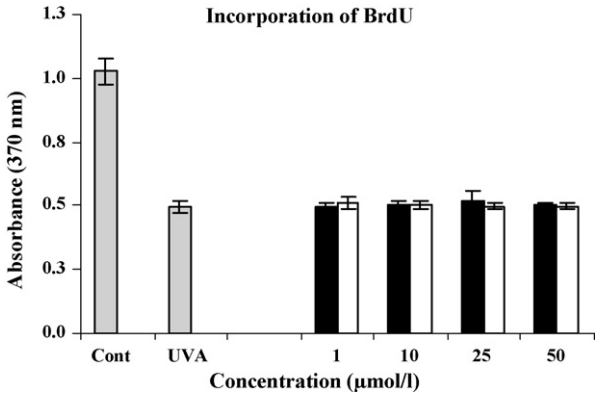

Fig. 4 Modulation of UVA-caused disruption of HaCaT proliferation by SB and DS. Keratinocytes were irradiated (20J/cm^2) and treated with SB or DS (1—50 μmol/l) for 4 h. Irradiated and non-irradiated control cells were incubated with DMSO (0.5%; v/v) under the same conditions. Effect of SB (black bars) and DS (white bars) on UVA-induced alteration to BrdU incorporation was evaluated. Data are expressed as mean ± S.D. of three independent experiments performed in triplicate. Statistical comparison analysis was performed using Student's *t*-test.

performed. Both substances exerted a substantial dose-dependent ability to eliminate ROS produced in UVA-irradiated keratinocytes (Fig. 5D). Flavonolignans efficacy was most powerful at a concentration of 25 μmol/l. DS was more potent than SB, in agreement with its more powerful antioxidant efficacy.

Development of LPx in irradiated HaCaT was measured as TBARS. Under our experimental conditions, obvious increase in LPx was caused by a UVA dose of 30 J/cm^2. As shown in Fig. 5C, TBARS amount was markedly reduced by the treatment with both test compounds. Surprisingly, DS was most powerful at a concentration of 50 μmol/l and showed protection of nearly 100%.

As demonstrated in Fig. 5B, in UVA-irradiated group of cells (20 J/cm^2) the GSH content was decreased to 30% of control cells. SB application to UVA-irradiated keratinocytes led to dose-dependent alleviation of GSH depletion. Maximal improvement (34%) was observed at a concentration of 25 μmol/l. The effect of DS was excellent at lower concentrations (1 and 10 μmol/l; 54% and 36%, respectively), however, at higher concentrations its cytotoxicity outweighed the benefits (Fig. 5B).

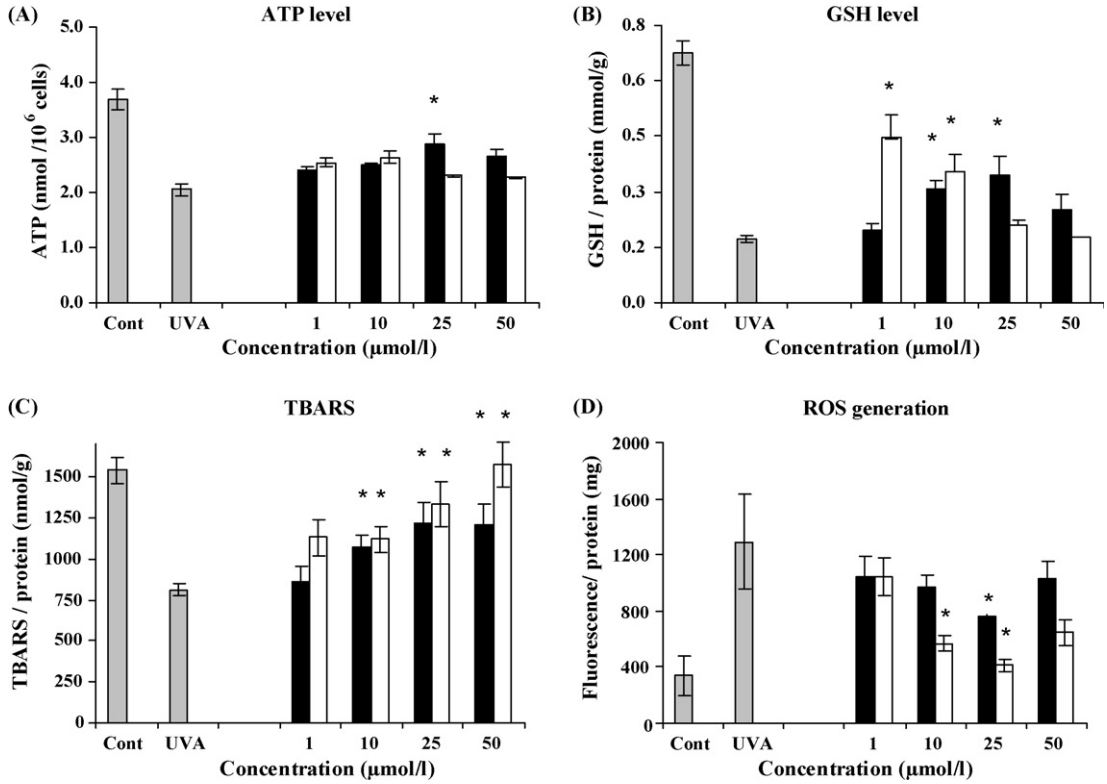

Fig. 5 Modulation of UVA-induced oxidative stress by SB and DS. Keratinocytes were irradiated (20 J/cm^2; in the case of lipid peroxidation 30 J/cm^2) and treated with SB or DS (1—50 μmol/l) for 4 h. Irradiated and non-irradiated control cells were incubated with DMSO (0.5%; v/v) under the same conditions. Effect of SB (black bars) and DS (white bars) on UVA-induced alteration to (A) intracellular ATP level, (B) intracellular GSH level, (C) lipid peroxidation and (D) ROS production was evaluated. Data are expressed as mean ± S.D. of three or more independent experiments performed in triplicate. Statistical comparison analysis was performed using Student's *t*-test. (*) $p < 0.05$ statistically different from irradiated cells.

Table 1 Modulation of UVA-induced DNA single strand breaks by SB and DS

Compound	Toxicity scale (%)					Total damage
	0	1	2	3	4	
Non-irradiated cells	98	2	0	0	0	2.0±0.05
Irradiated cells	65	7	8	9	11	92.9±8.5
SB (10 μmol l^{-1})	89	11	0	0	0	11.2±2.4[a]
SB (25 μmol l^{-1})	70	27	0	0	3	38.8±5.6[a]
DS (10 μmol l^{-1})	98	2	0	0	0	1.9±0.0[a]
DS (25 μmol l^{-1})	28	17	16	25	14	179.7±14.7[a]

Keratinocytes were irradiated (20 J/cm^2) and treated with SB or DS (1—50 μmol/l) for 4 h. Irradiated and non-irradiated control cells were incubated with DMSO (0.5%; v/v) under the same conditions. DNA damage was assessed by the Comet assay. The number of damaged nuclei in individual nuclei class was determined in 100 cells (nuclei) per slide. Total damage is expressed in arbitrary units, which range form 0 (undamaged cells) to 400 (totally damaged cells). The data are representatives of three independent experiments. Statistical comparison analysis was performed using Student's *t*-test.

[a] $p < 0.001$ statistically different from irradiated cells.

Similarly, the ATP level in irradiated cells was massively exhausted in comparison to control cells. Post-treatment with SB significantly prevented ATP depletion (Fig. 5A), most powerfully (43.5%) at concentrations of 25 μmol/l. The effect of DS was less remarkable (12.5% was the maximum).

3.5. Modulation of UVA-induced DNA damage and apoptosis by flavonolignans

As determined by the Comet assay, UVA exposure (20 J/cm^2) of keratinocytes led to extensive production of DNA single strand breaks in comparison to non-irradiated cells (Table 1). SB and DS themselves did not cause DNA damage at used concentrations (data not shown). In UVA-exposed cells treated with test compounds a powerful ability to attenuate comet appearance was evident (Table 1). SB as well as DS was more efficient at lower doses tested (10 μmol/l). DS at this concentration diminished "comet" incidence to control level. Protection of SB was about 95%. In contrast, DS protection at a concentration of 25 μmol/l was markedly reduced.

UVA-caused oxidative injury also leads to induction of apoptosis manifested by caspase-3 activation. Neither SB nor DS itself induced caspase-3 activation at used concentrations as demonstrated by Western blot analysis (Fig. 6A). In UVA-irradiated cells (20 J/cm^2) treated with DS an evident diminution of activated caspase-3 was observed (Fig. 6B) at all concentrations tested. In the case of SB, the reduction was more apparent at a concentration of 25 μmol/l. Application of test compounds to UVA-irradiated HaCaT dose-dependently reduced caspase-3 enzymatic activity, evaluated by Ac-DEVD-AMC cleavage assay (Fig. 6C). DS was more

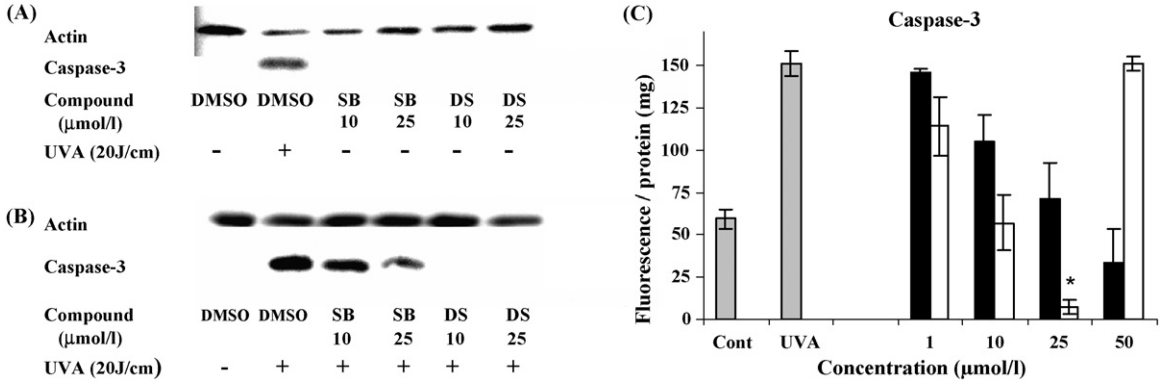

Fig. 6 Effect of SB and DS on basal and UVA-induced caspase-3 activation in HaCaT. (A) Keratinocytes were treated with SB or DS (10 and 25 μmol/l; 4 h) or (B) were irradiated (20J/cm^2) and treated with SB or DS (10 and 25 μmol/l; 4 h). Irradiated and non-irradiated control cells were incubated with DMSO (0.5%; v/v) under the same conditions. Basal and UVA-induced caspase-3 activation was evaluated by Western blot analysis. Data are representatives of three independent experiments. (C) Keratinocytes were irradiated (20 J/cm^2) and treated with SB or DS (1—50 μmol/l) for 4 h. Irradiated and non-irradiated control cells were incubated with DMSO (0.5 %; v/v) under the same conditions. Caspase-3 activity was evaluated by fluorimetric assay (the black bars represent SB; the black bars represent DS). Data are expressed as mean ± S.D. of three independent experiments performed in triplicate. Statistical comparison analysis was performed using Student's *t*-test. (*) $p < 0.05$ statistically different from irradiated cells.

potent in alleviation of caspase-3 activity at lower concentrations in comparison to SB. However, DS at a concentration of 50 μmol/l possessed no protection and controversially induced caspase-3 activity. SB protection was dose-dependent and was the most efficient at the highest concentration tested.

4. Discussion

Studies focused on the development of novel agents with protective activities against UV-induced damage, particularly from natural sources including various plants are intensively being carried out. In this study, we concentrated on the UVA part of UV light, whose relevance has been marginalized for a long period. A recent study indicates that UVA may play a pivotal role in human skin carcinogenesis [36]. The current explanation of UVA involvement in skin pathogenesis points to oxidative stress that itself manifests as wide-ranging biochemical changes in skin cells. Therefore, compounds exhibiting antioxidant activity may be beneficial for prevention/reparation of UVA-induced skin injury.

Our results provide the first evidence that flavonolignans SB and DS protect human keratinocytes from UVA-induced oxidative stress *in vitro*. Previous studies that involved only SB or its natural source silymarin have described their effectiveness against UVB light *in vitro* [23] and *in vivo* [19-22]. Recently, we showed protective activity of silymarin against UVA-caused damage to HaCaT cells [15]. However, the ability of SB against UVA-caused skin/skin cells damage and the ability of DS has not been studied yet. Here, we showed that post-treatment of irradiated HaCaT cells with SB and DS reduced intracellular ROS generation, DNA damage and apoptosis.

ROS-protective activity of SB and DS was previously demonstrated *in vitro* [37,38] and *in vivo* [39]. In the present study, we have revealed that the flavonolignans, especially DS, are likewise very effective in ROS elimination in UVA-irradiated skin cells. ROS overproduction is followed by rapid depletion of GSH, the most important non-enzymatic cellular antioxidant, whose level is directly associated with the degree of LPx in cell membrane [40]. We demonstrated that both compounds reduce GSH depletion in UVA-irradiated HaCaT. Similarly TBARS level, remarkably accumulated in UVA-exposed keratinocytes, was significantly reduced in cells post-treated with flavonolignans. These findings further support our assumption that SB and DS directly interact with ROS.

As mentioned, the plasma membrane is the main target of UVA-caused ROS production in keratinocytes; the mitochondrial membrane is affected as well. ROS-induced changes would promote the onset of mitochondrial permeability transition, a common event in both necrotic and apoptotic cell death [9,41]. Caspase-3 plays a primordial role in triggering the cascade of events leading to apoptosis. Since the caspase-3 activation is linked to ROS overproduction [42], radical scavengers could protect cells against oxidative damage and initiation of apoptosis. As we observed, SB and DS substantially decreased the UVA-elevated activation of caspases-3 in HaCaT. Their effects may be again explained by their ability to eliminate/alleviate UVA-induced ROS generation.

UVA-induced increase in ROS production among others can contribute to the formation of DNA oxidative lesions and strand breaks [3]. For DNA damage examination, we used the comet assay, a sensitive method for detecting DNA single strand breaks in individual cells [43]. Our observations also showed that post-treatment with SB and DS led to effective elimination of UVA-induced DNA single strand breaks close to the level of non-irradiated cells.

DS displayed a greater ability to diminish UVA-caused oxidative stress than SB. The higher efficacy of DS compared to SB is associated with its higher antioxidant potential, resulting from the presence of C_2—C_3 double bond in the heterocycle part of the molecule (B-ring; see Fig. 1). As has been demonstrated previously, dehydrogenation at C_2—C_3 in flavonolignans (SB versus DS) [44] or flavonoids (taxifolin versus quercetin) [45] strongly improves their antioxidant potency. Decrease in DS protective efficacy observed at higher concentrations is most probably a consequence of DS's low solubility in aqueous solutions at these concentrations or/and may be linked to its pro-oxidant effect. Quercetin, the structural analogue of DS, has pro-oxidant properties *in vitro* [46] and *in vivo* [47] resulting in cell damage. Furthermore, the intracellular metabolic activation of quercetin to *o*-quinone can be partially associated with radical formation and the observed concentration-dependent quercetin cytotoxic effect [48]. However, in the case of DS, which has no catechol moiety, this mechanism cannot exert. DS solubility can be improved by its oxidation to 2,3-dehydrosilybinic acid. However, this oxidation results in a slight reduction in antioxidant activity [44].

The data presented here suggest that studied the polyphenols may be useful as active components in dermatological creams and lotions to support repair and regeneration of UVA-irradiated skin. However, results that we obtained using the post-treatment of UVA-irradiated HaCaT keratinocytes cannot unequivocally prove whether the test compounds will be

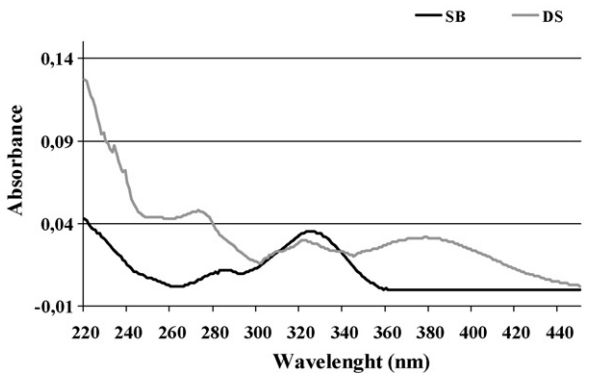

Fig. 7 Absorption spectra of studied polyphenols (10 μmol/l) at physiological conditions. Stock solutions of SB or DS (2.01 mmol/l; DMSO) were diluted in PBS (pH 7.5). The blank sample was prepared with DMSO (0.5%). The absorption spectrum was scanned in quartz cuvette against blank (DMSO) between 220 and 440 nm at medium speed scan in UV—VIS spectrophotometer UV-2401PC (Shimadzu, Japan).

able to prevent or reduce development of UVA-caused damage. Nevertheless, the preventive effect may be expected as the compounds absorb the UV light in UVA region. The absorbance data under physiological conditions showed that both SB and DS have an absorption peak in near UVA region (315—325 nm). DS absorbs also between 370 and 390 nm (Fig. 7). Recently, it was demonstrated that sunscreens absorbing in the long wave UVA part (maximum $\geq$360 nm) provide the optimal protection [49]. Thus, DS could be very effective not only as a radical scavenger but also as a UV filter.

At present, protection against solar UVA-induced oxidative damage is mainly based on the use of sunscreens. However, the current sunscreens formulations possess rather protection against UVB rather than UVA. One recent study demonstrated that sunscreen users are poorly protected against damaging effects of UVA present in sunlight [50]. Development of full-spectrum protective agents may help to prepare more effective sunscreen and thus to possess better protection. Our study reveals that SB and DS, the major and the minor flavonolignan components of silymarin have potency to reduce UVA-induced damage to keratinocytes. Both compounds seem to be the promising candidates for (photo)protection against skin UVB-induced injury, as was previously reported and may be efficient against UVA irradiation caused damage, as we proved here. However, skin is a complicated organ consisting of several layers and types of cells which influence each other during and after irradiation. Thus, further research is needed to specify the effect of flavonolignans on other skin cells such as fibroblasts or melanocytes, as well as to define their effect and safety *in vivo* in animal models and humans.

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