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ORIGINAL PAPER

A pilot study of the UVA-photoprotective potential of dehydrosilybin, isosilybin, silychristin, and silydianin on human dermal fibroblasts

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Abstract
The exposure of naked unprotected skin to solar radiation may result in numerous acute and chronic undesirable effects. Evidence suggests that silymarin, a standardized extract from *Silybum marianum* (L.) Gaertn. seeds, and its major component silybin suppress UVB-induced skin damage. Here, we aimed to investigate the UVA-protective effects of silymarin's less abundant flavonolignans, specifically isosilybin (ISB), silychristin (SC), silydianin (SD), and 2,3-dehydrosilybin (DHSB). Normal human dermal fibroblasts (NHDF) pre-treated for 1 h with flavonolignans were then exposed to UVA light using a solar simulator. Their effects on reactive oxygen species (ROS), carbonylated proteins and glutathione (GSH) level, caspase-3 activity, single-strand breaks' (SSBs) formation and protein level of matrix metalloproteinase-1 (MMP-1), heme oxygenase-1 (HO-1), and heat shock protein (HSP70) were evaluated. The most pronounced preventative potential was found for DHSB, a minor component of silymarin, and SC, the second most abundant flavonolignan in silymarin. They had significant effects on most of the studied parameters. Meanwhile, a photoprotective effect of SC was mostly found at double the concentration of DHSB. ISB and SD protected against GSH depletion, the generation of ROS, carbonylated proteins and SSBs, and caspase-3 activation, but had no significant effect on MMP-1, HO-1, or HSP70. In summary, DHSB and to a lesser extent other silymarin flavonolignans are potent UVA-protective compounds. However, due to the in vitro phototoxic potential of DHSB published elsewhere, further studies are needed to exclude phototoxicity for humans as well as to confirm our results on human skin ex vivo and in vivo.

Keywords Cell culture · Flavonolignan · UVA · Oxidative damage · Heat shock protein · Metalloproteinase-1

Introduction

Although solar radiation, particularly its ultraviolet (UV) part, possesses some health benefits, such as the production of cholecalciferol, and is often used in the treatment of some skin pathologies, such as vitiligo and psoriasis, it is mainly associated with the initiation and development of several skin disorders. Most incoming UV radiation (90–95%) belongs to the UVA (315–400 nm) waveband. Compared to UVB, which is absorbed mostly in the epidermis, UVA penetrates deep into the skin and, thus, affects the epidermis and dermis and their principal cells, keratinocytes, and fibroblasts. UVA photons provoke a large production of reactive oxygen and reactive nitrogen species (ROS and RNS) through interacting with endogenous chromophores. ROS/RNS oxidize cellular molecules and thus generate oxidized products of lipids [e.g., (hydro)peroxides], proteins (e.g. carbonyls), and DNA lesions [e.g., single-strand breaks

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(SSBs) or altered DNA bases]. ROS/RNS and oxidized products affect various cellular pathways and the expression of numerous signaling molecules that initiate the development of pathological changes in skin tissue, such as altered epidermal cell proliferation and differentiation, a decrease in collagen synthesis, the upregulation of extracellular matrix-degrading enzymes that are responsible for collagen and elastin degradation, and the accumulation of amorphous material. Repeated exposure to UV radiation causes the gradual accumulation of damaged molecules, resulting in skin hyperpigmentation, skin photoaging, and/or carcinogenesis [20].

The rise in solar UV radiation on the earth's surface has led to a depletion of stratospheric ozone over recent decades, thus accelerating the need to protect human skin against the harmful effects of UV radiation. The topical application of sunscreens represents the most popular way of the skin photoprotection. Recently, compounds derived from natural sources have attracted remarkable attention for their implementation in sunscreen products to substitute for or to reduce the amounts of synthetic molecules. Natural compounds can possess additional properties such as antioxidant, anti-inflammatory, and immunomodulatory, which provide further protection against the damaging effects of UV radiation [1, 14]. Silymarin (SM) is one of the most frequently studied polyphenolic mixtures for photoprotective activity. Several reports documented the ability of SM and its major component silybin (SB) (see Fig. 1) to reduce UVB-stimulated skin damage, including carcinogenesis [19, 25]. We recently reported that SM and SB reduced UVA-stimulated damage to normal human dermal fibroblasts (NHDF) [15]. However, this ability in less abundant components, the flavonolignans isosilybin (ISB), silychristin (SC), silydianin (SD), and 2,3-dehydrosilybin (DHSB) has not been studied yet. All the compounds show higher antioxidant potential compared to most copious SB [26] and might provide photoprotective activities. Therefore, we examined here whether ISB, SC, SD, and DHSB can prevent UVA-induced damage to NHDF, and compare their efficacy and participation on SM UVA-photoprotective effects.

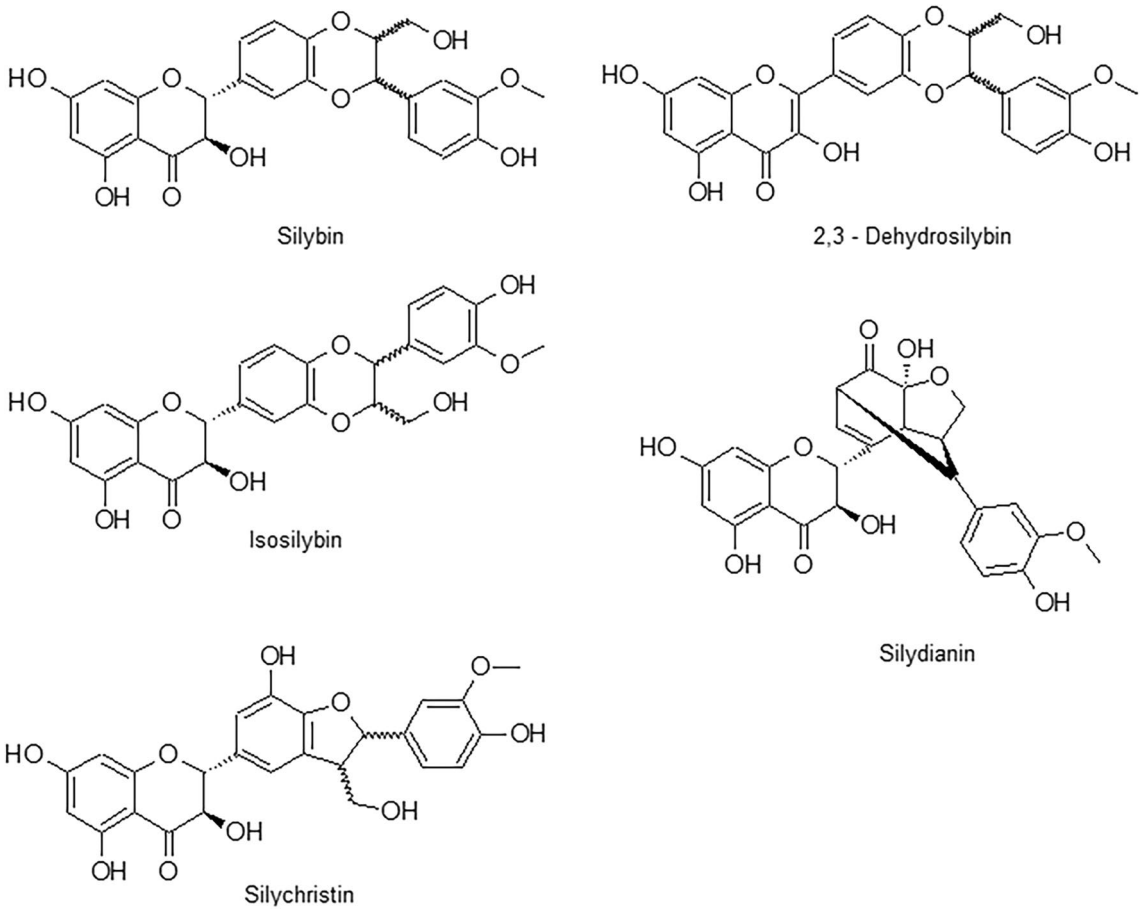

Fig. 1 Chemical structures of tested flavonolignans

Materials and methods

Chemicals

Protease inhibitor cocktail tablets Complete™ and Sybr Green were purchased from Roche Diagnostic (Germany). Caspase-3 fluorigenic substrate/inhibitor (Ac-DEVD-AMC/Ac-DEVD-CHO) was obtained from Bachem AG (Switzerland). Agarose for DNA electrophoresis was from Serva (Germany). Western blotting luminol reagent for chemiluminescent horseradish peroxidase (HRP) detection, rabbit anti-hem oxygenase-1 (HO-1; 32 kDa), rabbit anti-metalloproteinase-1 (MMP-1; 52 kDa), goat anti-heat shock protein (HSP; 70 kDa) and goat anti-actin (I-19; 42 kDa) primary antibodies, and goat anti-rabbit and rabbit anti-goat HRP conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (USA). The Pierce BCA Protein Assay Kit was from Thermo Fisher Scientific (Czech Republic). Dulbecco's modified Eagle's medium (DMEM), Ham-F12 nutrient mixture, heat-inactivated fetal calf serum (FCS), stabilized penicillin–streptomycin solution, amphotericin B, hydrocortisone, adenine, insulin, epidermal growth factor, 3,3′,5-triiod-L-thyronin, trypsin, ampicillin, trypsin–EDTA (0.25%), dimethyl sulfoxide (DMSO), neutral red (NR), dichlorodihydrofluorescein diacetate (H_2DCFDA), 2,2′-dinitro-5,5′dithiobenzoic acid (DTNB), fluorescein-5-thiosemicarbazide (FTC), and other chemicals were from Sigma-Aldrich (Czech Republic).

Test compounds

ISB (mixture of diastereomers ca. 95:5), SC (mixture of diastereomers ca. 9:1), and SD were isolated from SM by Sephadex LH-20 column chromatography as described previously [9]. DHSB was prepared as described by *Gažák* et al. [8]. Briefly, SB was refluxed in acetic acid with iodine and potassium acetate and precipitated with water. Acidic hydrolysis and crystallization with ethanol gave DHSB in a good yield and purity. The chemical structures of the studied flavonolignans are shown in Fig. 1.

Cell culture

NHDF were obtained from the skin fragments of medically healthy adult donors. The tissue specimens were obtained from patients undergoing plastic surgery at the Department of Plastic and Aesthetic Surgery (University Hospital, Olomouc). The use of skin tissue complied with the Ethics Committee of the University Hospital in Olomouc and Faculty of Medicine and Dentistry, Palacký University, Olomouc (date: 6.4.2009, ref. number: 41/09). All patients had given their written informed consent.

The skin fragments and NHDF were cultured as described earlier [15]. Fibroblasts were used between the second and fourth passages. For experiments, cells were seeded on 96-, 24-, or 6-well plates at a density of 0.5×10^5 cells per cm^2.

Cytotoxicity of test compounds

Fibroblasts seeded on 96-well plates were treated with DHSB, ISB, SC, or SD (1.6–100 µmol/l; the highest soluble concentration of flavonolignans) in serum-free DMEM for 24 h. Control cells were treated with serum-free medium-containing DMSO (0.5%, v/v) under the same conditions. Cell viability was then evaluated as the retention of neutral red (NR) that was measured spectrophotometrically as described recently [15].

Photoprotection potential of test compounds

Fibroblasts were pre-treated with DHSB, ISB, SC, or SD in serum-free DMEM for 1 h. For basic parameters (GSH and ROS level and caspase-3 activity), a wide range of concentrations (6.25, 12.5, 25, and 75 µmol/l) was used to check phototoxic potential of flavonolignans reported by *Narayanapillai* et al. [10]. More specific markers (comet assay and western blot analysis) were measured only at two selected concentrations with a significance to potential practical use. Compounds were dissolved in DMSO, so that the final concentration of DMSO in the medium was 0.5% (v/v). After incubation, fibroblasts were washed twice with PBS and then PBS supplemented with glucose (1 mg/ml) was applied. Cells (+UVA) were exposed to UVA radiation (7.5 or 10 J/cm^2) on ice-cold plates to eliminate UV-induced thermal stimulation. A solar simulator *SOL 500* (Dr. Hönle UV Technology, Germany) with a spectral range (295–3000 nm) corresponding to natural sunlight was used for irradiation. The simulator was equipped with an H1 filter transmitting wavelengths of 315–380 nm. The UVA output before each experiment was measured with an UVA meter (Dr. Hönle UV Technology, Germany). The UVA doses used were chosen according to pilot experiments (data not shown). In parallel, non-irradiated cells (-UVA) were treated similarly and kept in the dark. After irradiation, serum-free DMEM was applied and NHDF were then incubated for 1–24 h according to the measured parameter.

Cell viability

The UVA-photoprotective effect of DHSB, ISB, SC, or SD was evaluated 24 h after UVA irradiation (10 J/cm^2) by retention of NR. The lysosomal accumulation of NR was measured spectrophotometrically [15].

ROS level

The effect of compounds on the UVA-caused (10 J/cm^2) production of ROS in fibroblasts was measured by dichlorodihydrofluorescein diacetate assay as described recently [15].

GSH level

Effect of flavonolignans and UVA radiation (10 J/cm^2) on the GSH level in fibroblasts was measured spectrophotometrically using the reaction with 2,2′-dinitro-5,5′-dithiobenzoic acid (DTNB) 4 h after irradiation as described recently [15].

Caspase-3 activity

Caspase-3 activity stimulated by UVA radiation (10 J/cm^2) was evaluated by a fluorescent assay using the fluorogenic substrate acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) 4 h after irradiation as described recently [15].

Level of carbonylated proteins

The level of carbonylated proteins was measured using fluorescein 5-thiosemicarbazide (FTC), which specifically reacts with carbonyl groups in oxidized proteins and not in oxidized lipids [6]. The level of carbonylated proteins was measured 24 h after UVA exposure (10 J/cm^2). NHDF were washed once with PBS, scraped into PBS, and centrifuged (280×*g*; 10 min; 4 °C). The cell pellet was dissolved in ice-cold lysis buffer (50 mM HEPES, pH 7.4; Triton X-100 (0.5%, v/v), 5 mmol/l dithiothreitol, protease inhibitor cocktail tablet; 50 µl), and then, FTC solution (0.2 µM; 50 µl) was added. After overnight incubation in the dark, proteins were precipitated by the addition of four volumes of ice-cold TCA (20%, v/v; 400 µl). After 10 min of incubation on ice, the tubes were centrifuged (12,000×*g*; 10 min; 4 °C). The supernatants were carefully decanted; precipitates were washed three times by vortexing with 1 ml of acetone and centrifuged immediately (12,000×*g*; 10 min; 4 °C). Finally, the acetone supernatant was carefully decanted and protein precipitates were air-dried, solubilized with guanidyl hydrochloride (50 µl; 6 mol/l), and diluted tenfold by the addition of 0.1 M NaH_2PO_4, pH 7.0 (450 µl). The fluorescence was measured at 485 nm (excitation) and at 535 nm (emission) using a microplate spectrophotometer (INFINITE M200, Tecan Trading AG, Switzerland). Protein concentration was measured by bicinchoninic acid assay. The results were expressed as relative fluorescence per mg of protein. Each experiment was performed in duplicate and in four independent experiments using NHDF from different donors.

DNA single-strand breaks

The alkaline Comet assay was used for the evaluation of DNA SSBs. The fibroblasts were harvested 4 h after UVA exposure (10 J/cm^2) and processed as described previously [15]. An Axiovert 40 CFL inversion fluorescent microscope (ZEISS, Germany) was used for nuclei analysis and the fluorescent stain Sybr Green was used to visualize DNA. The 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:

$$\text{Total damage } (\%) = \frac{N_0 \times 0 + N_1 \times 1 + N_2 \times 2 + N_3 \times 3 + N_4 \times 4}{N_0 + N_1 + N_2 +_3 N_4} \times 100,$$

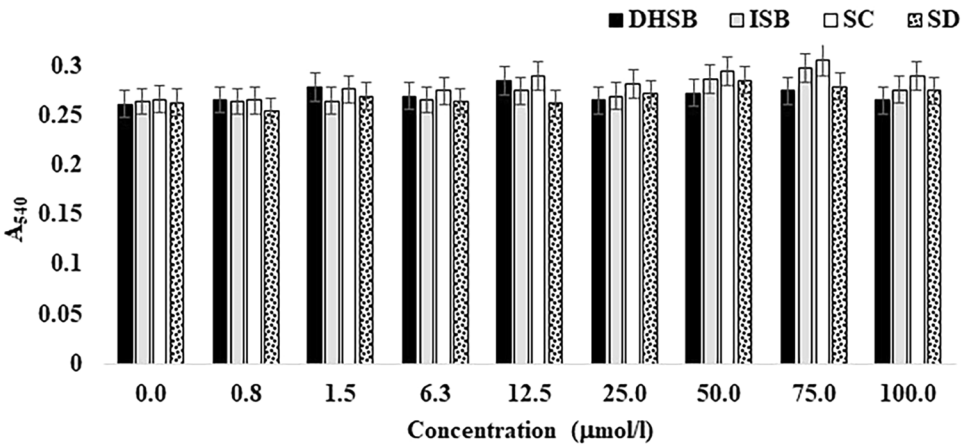

Fig. 2 Effect of flavonolignans on NHDF viability. Cells were treated for 24 h with DHSB, ISB, SC, or SD (six replicate wells per concentration) and then viability was evaluated by neutral red assay. Results are mean ± SD of four experiments employing cells from four donors

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

Western immunoblot analysis

The level of selected proteins in fibroblasts pre-treated with flavonolignans and UVA irradiated (7.5 J/cm^2) was evaluated at four and 24 h after exposure. Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane. After blocking (2 h, room temperature) using non-fat dry milk [5% (w/v) in 100 mM Tris-buffered saline, pH 7.5 with Tween 20 (0.05%, v/v) (TBS/T)], the membrane was then incubated with a specific primary antibody (rabbit anti-MMP-1, rabbit anti-HO-1 and goat anti-HSP-70, goat anti-actin (I-19) antibody; BSA (5%, w/v) in TBS/T) overnight at 4 °C, and then with a secondary horseradish peroxidase conjugated antibody (goat anti-rabbit antibody/rabbit anti-goat antibody; BSA (5%, w/v) in TBS/T) for 1 h at room temperature. The proteins

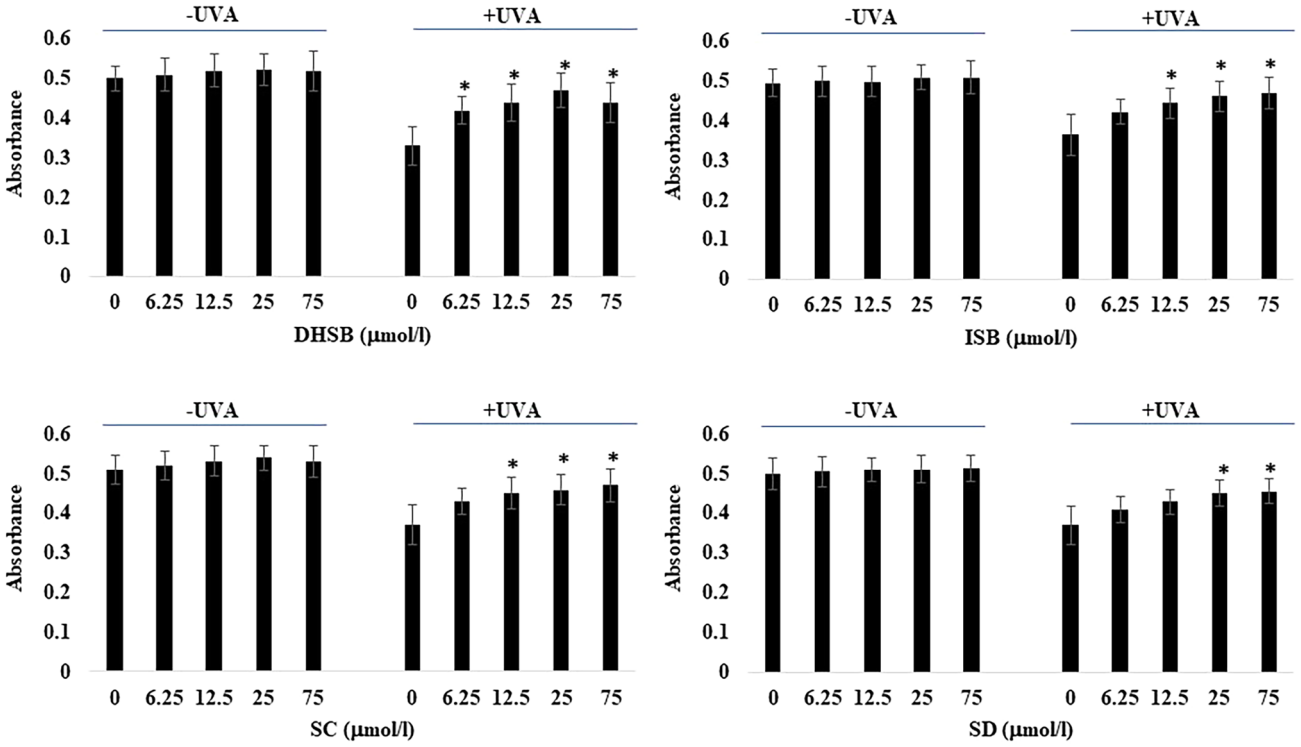

Fig. 3 Effect of flavonolignans on UVA-induced toxicity. Cells pre-treated with DHSB, ISB, SC, or SD were non-irradiated (-UVA) or exposed to 10 J/cm^2 (+UVA) and after 24 h of incubation, cell viability was evaluated as described in "Materials and Methods". Results are mean ± SD of four experiments employing cells from four donors. *Significantly different from irradiated cells at $p = 0.05$

expression was detected by chemiluminescence and autoradiography. The quantification of the proteins was performed by a densitometry analysis using the image processing program ImageJ (more details were described previously) [15].

Statistical analysis

Experiments were performed in at least four independent examinations (four different donors). Data were expressed as mean ± SD. Statistical comparison was performed using Student's *t* test. Statistical significance was determined at $p = 0.05$.

Results

Cytotoxic potential of test compounds

In non-irradiated fibroblasts, possible damage provoked by DHSB, ISB, SC, and SD was evaluated in the concentration range of 1.6–100 µmol/l. The highest concentration was chosen according to the solubility of flavonolignans in the serum-free DMEM used in experiments. All flavonolignans themselves caused no morphological changes in fibroblasts that were controlled microscopically (data not shown). No significant changes were found in NR retention after 24 h treatment with compounds, as shown in Fig. 2.

Cytoprotective effect

UVA radiation (10 J/cm^2) reduced cell viability of fibroblasts. All flavonolignans were able to diminish UVA toxicity and their protective effect was dose-dependent. However, only DHSB significantly prevented decline of cell viability at the lowest concentration of 6.25 µmol/l (see Fig. 3).

Elimination of ROS

UVA radiation (10 J/cm^2) stimulated ROS generation in NHDF after only 1 h of exposure, as shown by the dichlorodihydrofluorescein diacetate assay. Pre-treatment with DHSB resulted in a concentration-dependent reduction in ROS generation (Fig. 4). The protective effect was 51% at the lowest concentration (6.25 µmol/l) and nearly 100% at the highest concentration (75 µmol/l). Similarly, SC stimulated a concentration-dependent decrease in ROS. A significant effect was found at the concentration of 12.5 µmol/l and at higher ones. SC also non-significantly reduced basal ROS level in fibroblasts. SD reduced ROS level, as well; however,

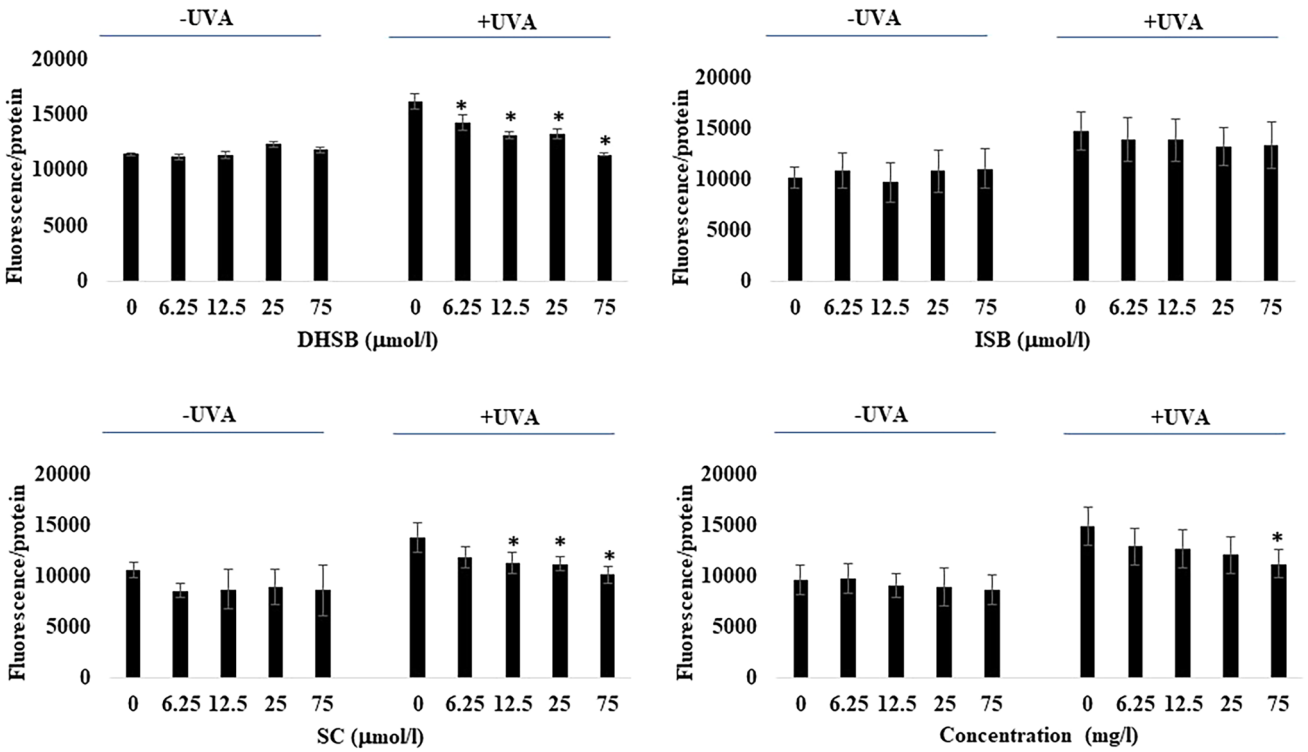

Fig. 4 Effect of flavonolignans on UVA-induced ROS generation. Cells pre-treated with DHSB, ISB, SC, or SD were non-irradiated (-UVA) or exposed to 10 J/cm^2 (+UVA), and after 1 h of incubation, ROS level was evaluated as described in "Materials and Methods". Results are mean ± SD of four experiments employing cells from four donors. *Significantly different from irradiated cells at $p = 0.05$

the effect was only significant at the highest concentration. The protective effect of ISB was minimal.

Prevention of GSH depletion

As shown in Fig. 5, UVA irradiation (10 J/cm^2) also caused a significant depletion in intracellular GSH level in dermal fibroblasts. Pre-treatment with DHSB prevented the GSH depletion. The protection reached its maximum at a concentration of 12.5 µmol/l (85%) and decreased at the higher concentrations. Moreover, at the highest concentration (75 µmol/l) DHSB amplified the damage caused by UVA radiation, although no GSH depletion (Fig. 5) and stimulation of ROS generation (Fig. 4) was evident in the non-irradiated DHSB treated cell. All other flavonolignans exhibited a concentration-dependent increase in GSH level in irradiated cells. Compared to DHSB, their effect was significant at the concentration of 12.5 µmol/l (ISB) or 25 µmol/l (SC and SD). With SC and SD, an increase in GSH was also found in non-irradiated cells, but the effect was significant only with SD.

Reduction of caspase-3 activity

Caspase-3 is a frequently activated death protease, catalyzing the specific cleavage of many key cellular proteins in the process of apoptosis [11] that may be triggered by oxidative damage. The application of DHSB significantly reduced caspase-3 activity stimulated by UVA radiation (10 J/cm^2). The trend corresponds to that in the GSH level. The protection was high (70%) at the lowest concentration of 6.25 µmol/l, reached its maximum (nearly 100% protection) at the concentrations of 12.5 µmol/l and then decreased. At the highest concentration (75 µmol/l), DHSB pre-treatment together with UVA exposure stimulated caspase-3 activity twice as much as UVA radiation alone despite minimal caspase-3 activity being found in non-irradiated fibroblasts treated by the highest concentration. SC and SD exhibited a concentration-dependent effect; however, it started to be significant at the higher concentration of 12.5 µmol/l (SC) and 25 µmol/l (SD). ISB reduced caspase-3 activity; however, it was not significant at any concentration (Fig. 6).

Modulation of DNA single-strand breaks

UVA radiation (10 J/cm^2) stimulated SSBs production (expressed as total DNA damage) in NHDF at 4 h after exposure (Fig. 7). Pre-treatment with flavonolignans reduced the amount of SSB. ISB, SC, and SD exhibited concentration-dependent effects. In cells pre-treated with DHSB, a reduction in protective effect was found at the higher concentration tested (12.5 µmol/l).

Prevention of carbonyl proteins production

The basal level of carbonylated proteins in NHDF varied depending on the donor of skin tissue, and the value range was 175.2–259.9 F.U./g of protein. Due to different donor sensitivities, UVA irradiation also induced different cell responses and the level of protein carbonyls was increased by about 35.5–116.7% of the basal level (305.7–490.9 F.U./g of protein) (see Fig. 8). All flavonolignans decreased the basal level of carbonylated proteins dose-dependently, as demonstrated in Fig. 8. In irradiated fibroblasts, all flavonolignans except SC reduced the UVA-induced level of carbonylated proteins. At the concentration of 12.5 µmol/l, DHSB and ISB were the most potent. At the higher concentration tested (25 µmol/l), ISB and SD were the most efficient. The effectiveness of DHSB disappeared at this concentration (Fig. 8).

Effect on protein level of MMP-1, HO-1, and HSP70

Flavonolignans were also studied for their potential effect on selected protein levels (MMP-1, HO-1, and HSP70). Their effect was evaluated 4 and 24 h after UVA exposure. The level of MMP-1 in UVA-irradiated cells was significantly increased at both time points, but the effect was more pronounced after 24 h. Pre-treatment with DHSB caused a significant reduction in MMP-1 level in both non-irradiated and irradiated cells 4 h after irradiation. With SC and ISB, there was only a non-significant reduction in irradiated cells (4 h). However, at the higher concentration, ISB and SC reduced the MMP-1 level in non-irradiated cells (after 4 h). SD had no effect on MMP-1 level (Fig. 9).

UVA radiation also increased the HO-1 protein level; the effect was more obvious 24 h after irradiation (around 180% of control). The pre-treatment of fibroblasts with DHSB resulted in a significant decrease in HO-1 level in both non-irradiated and irradiated cells after 4 h. ISB only significantly elevated the HO-1 level in non-irradiated fibroblasts; its effect was not significant in UVA-exposed cells. On the other hand, after 24 h, ISB increased HO-1 level, but there was only a significant effect in non-irradiated cells. The other two flavonolignans, SC and SD, had a minimal effect on HO-1 level (Fig. 10).

The effect of UVA exposure on HSP70 level in fibroblasts was clearly smaller than that on MMP-1 and HO-1. The increase in HSP70 level was more evident 24 h after irradiation (around 120% of control). All flavonolignans slightly reduced the amount of HSP70 to the control level in irradiated cells, but it was not significant (Fig. 11).

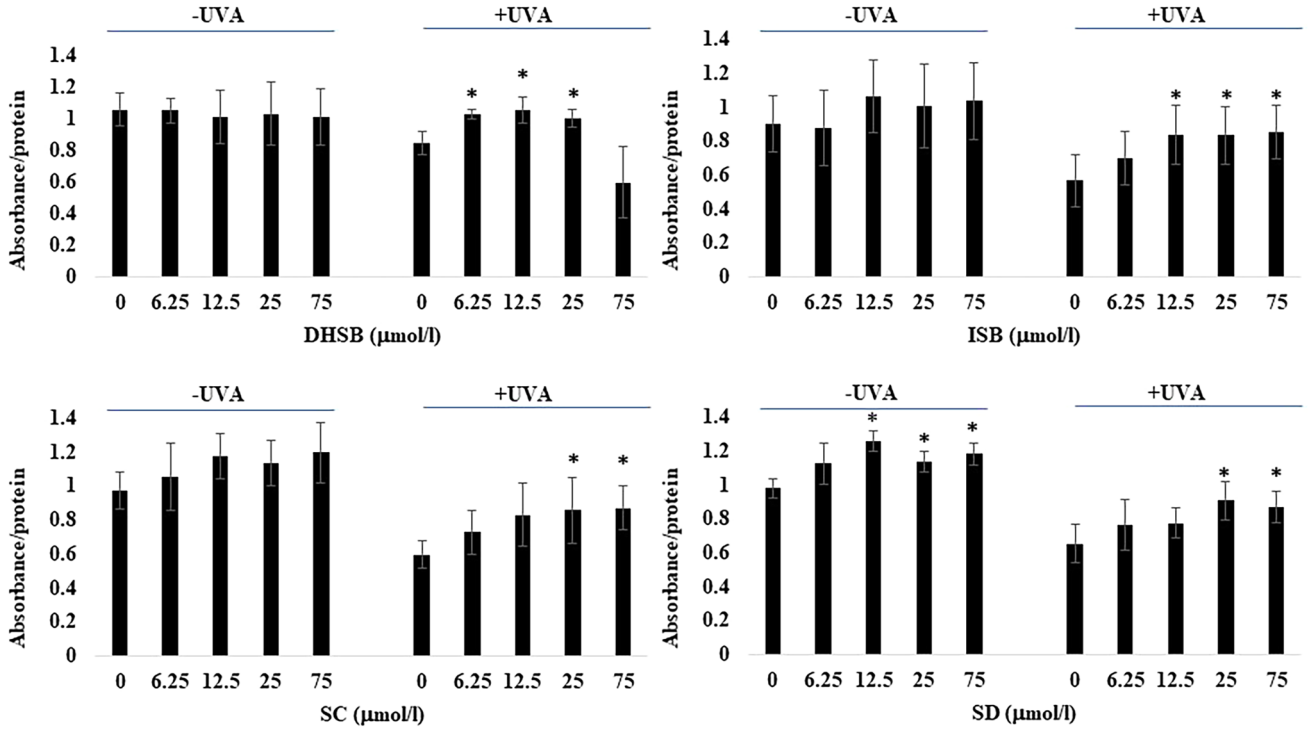

Fig. 5 Effect of flavonolignans on UVA-induced GSH depletion. Cells pre-treated with DHSB, ISB, SC, or SD were non-irradiated (-UVA) or exposed to 10 J/cm^2 (+UVA) and after 4 h of incubation, the level of intracellular GSH was evaluated as described in "Materials and Methods". Results are mean ± SD of four experiments employing cells from four donors. *Significantly different from irradiated cells at $p = 0.05$. #Significantly different from non-irradiated cells at $p = 0.05$

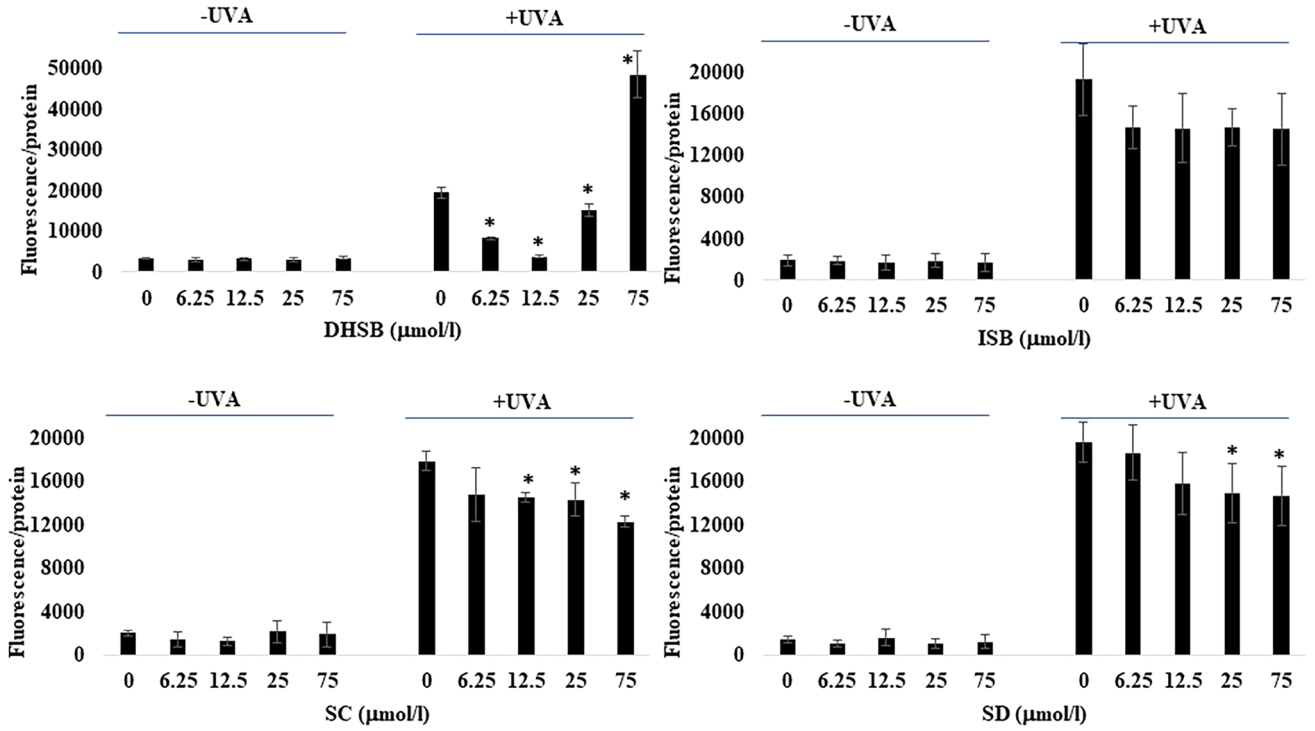

Fig. 6 Effect of flavonolignans on UVA-induced caspase-3 activity. Cells pre-treated with DHSB, ISB, SC, or SD, were non-irradiated (-UVA) or exposed to 10 J/cm^2 (+UVA), and after 4 h of incubation, caspase-3 activity was evaluated as described in "Materials and Methods". Results are mean ± SD of four experiments employing cells from four donors. *Significantly different from irradiated cells at $p = 0.05$

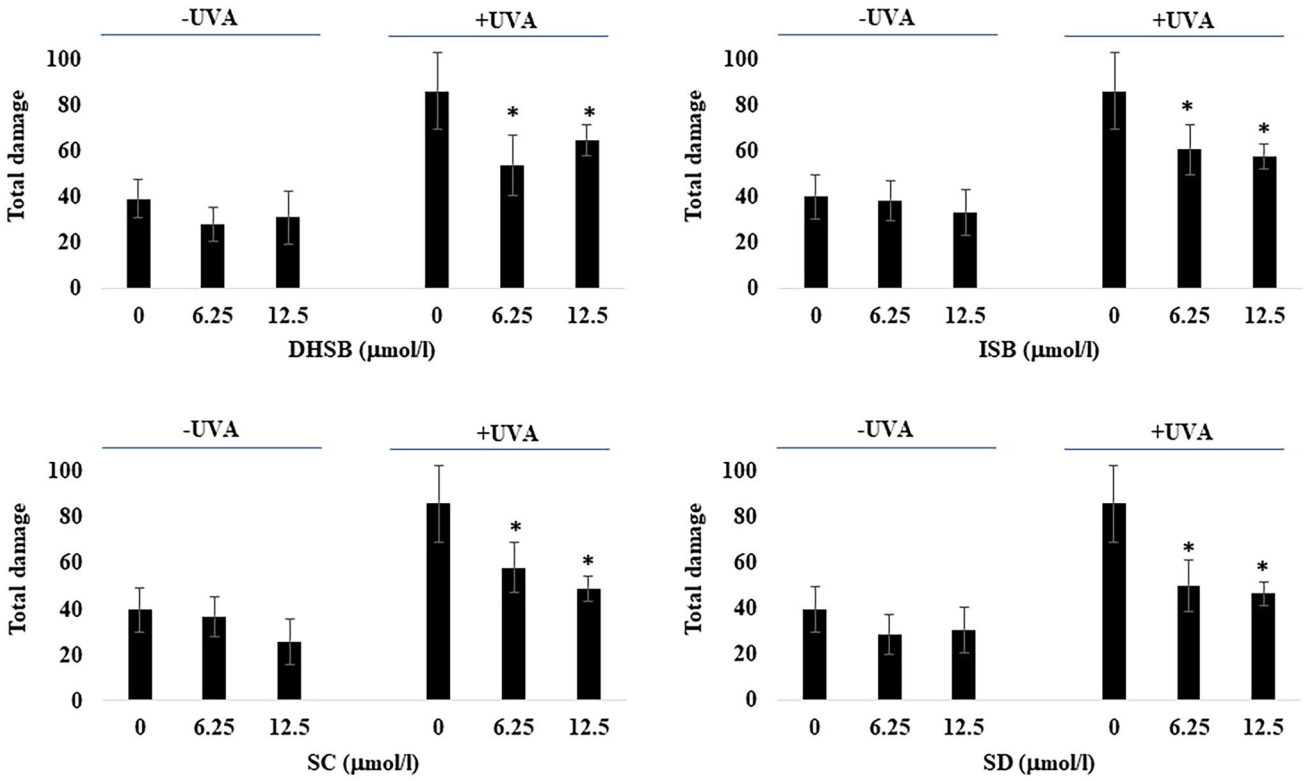

Fig. 7 Effect of flavonolignans on UVA-induced single-strand break level. Cells pre-treated with DHSB, ISB, SC, or SD were non-irradiated (-UVA) or exposed to 10 J/cm^2 (+UVA), and after 4 h of incubation, DNA damage was evaluated by Comet assay as described in "Materials and Methods". Results are mean ± SD of four experiments employing cells from four donors. *Significantly different from irradiated cells at $p = 0.05$

Discussion

Silymarin, a standardized extract of *Silybum marianum* seeds, has been an interesting source of biologically active compounds for several decades. Due to its antioxidant, anti-inflammatory, immunomodulatory, regenerative, and cytoprotective activities, SM has gained attention as an agent for various dermatological applications. SM and its major flavonolignan SB have been intensively studied for their beneficial effects on UVB-induced skin damage in vitro and in vivo [19, 25]. Recently, we have shown that SM and its most abundant flavonolignan SB reduced UVA-induced damage to dermal fibroblasts [15]. As the other flavonolignans present in SM, only DHSB has been previously tested on skin cells for potential dermatological benefits [21, 22]. However, several in vitro studies demonstrated a higher antioxidant potential of the less abundant flavonolignans present in SM compared to that of SB [2, 12, 13]. Therefore, we decided to evaluate the ability of DHSB, ISB, SC and SD to reduce UVA-stimulated cellular damage to primary human dermal fibroblasts.

The principal mechanism of UVA radiation stimulated damage to skin cells is associated with the excessive generation of ROS [20]. A previous study showed that the pre-treatment of human keratinocytes HaCaT with flavonolignan SB (75 µmol/l) subsequent irradiation with UVA (5 J/cm^2) increased the ROS level and caspase-3 activation [10]. For this reason, such a high concentration as well as the same parameters were also used in our experiments. In fibroblasts pre-treated with DHSB and SC, a concentration-dependent reduction in ROS level was found. The other two flavonolignans (SD, ISB) were less effective and except for SD at a concentration of 75 µmol/l, their effects were not significant. However, none of the tested flavonolignans amplified ROS level in irradiated cells, even at the highest concentration of 75 µmol/l, which contradicts the results of *Narayanapillai* et al. [10], who published significant increase in ROS formation in cells pre-treated with SB.

The caspase-3 that plays a central role in the activation of apoptotic cell death [11] was reduced by DHSB, SC and SD. The effect of SC and SD was concentration-dependent and more pronounced at higher concentrations, which corresponds to the effect reported for SB [15]. DHSB was most effective at the two lowest concentrations (6.25 and 12.5 µmol/l) and then its ability decreased. At the highest

concentration (75 µmol/l), DHSB conversely stimulated caspase-3 activity in UVA-treated cells more than UVA radiation alone (Fig. 6). Such a similar effect was reported for SB at the same concentration by *Narayanapillai* et al. [10], but we did not confirm it in a recent study [15]. The stimulation of caspase-3 found for DHSB herein is most likely associated with the phototoxic effect of DHSB that we recently demonstrated on various cell types, including NHDF and HaCaT in other papers [16]. It is known that DHSB is excellent antioxidant and, therefore, highly reactive compound with pro-oxidant potential. In cells stressed by UVA radiation, this may result in pro-oxidant phototoxic action of DHSB (mainly at higher concentrations). This phenomenon is probably linked to an obvious failure of GSH protection found in NHDF treated with the highest concentration of DHSB (Fig. 5). All other flavonolignans exhibited a concentration-dependent protection of intracellular GSH, the most important non-enzymatic antioxidant, in UVA-irradiated cells. SD was also able to stimulate a significant GSH level increase in non-irradiated fibroblasts (Fig. 5).

An example of UVA-provoked ROS-mediated oxidative damage in living cells is the formation of SSBs of DNA. An SSB is a discontinuity in the sugar-phosphate backbone of one strand of a DNA duplex. If SSBs are not repaired, they can be converted into potentially mutagenic DNA double-strand breaks. SSBs arise from DNA damage, primarily from attacks on DNA sugar residues and bases by ROS and other electrophilic molecules [3]. Here, we evaluated the number of SSBs by the classical alkaline comet assay that quantifies SSBs together with alkali-labile sites, related not only to ROS-mediated oxidation but also to the repair process of DNA [4]. As demonstrated in Fig. 7, all flavonolignans reduced the number of SSBs (expressed as total DNA damage) in irradiated fibroblasts. ISB, SC, and SD exhibited a concentration-dependent effect in contrast to DHSB. DHSB was more effective at the lower concentration tested (6.25 µmol/l). This agrees with our previous study where HaCaT cells exposed to UVA were post-treated with DHSB [22].

UVA radiation can provoke irreversible oxidative modification of some amino acids in proteins to form carbonyls groups (aldehydes and ketones) by generation of ROS and/or other reactive compounds such as lipid peroxidation breakdown products. Such oxidative damage results in loss of the protein activity and function [23]. All studied flavonolignans except SC reduced the level of protein carbonyls in UVA-treated fibroblasts pre-incubated with the compounds

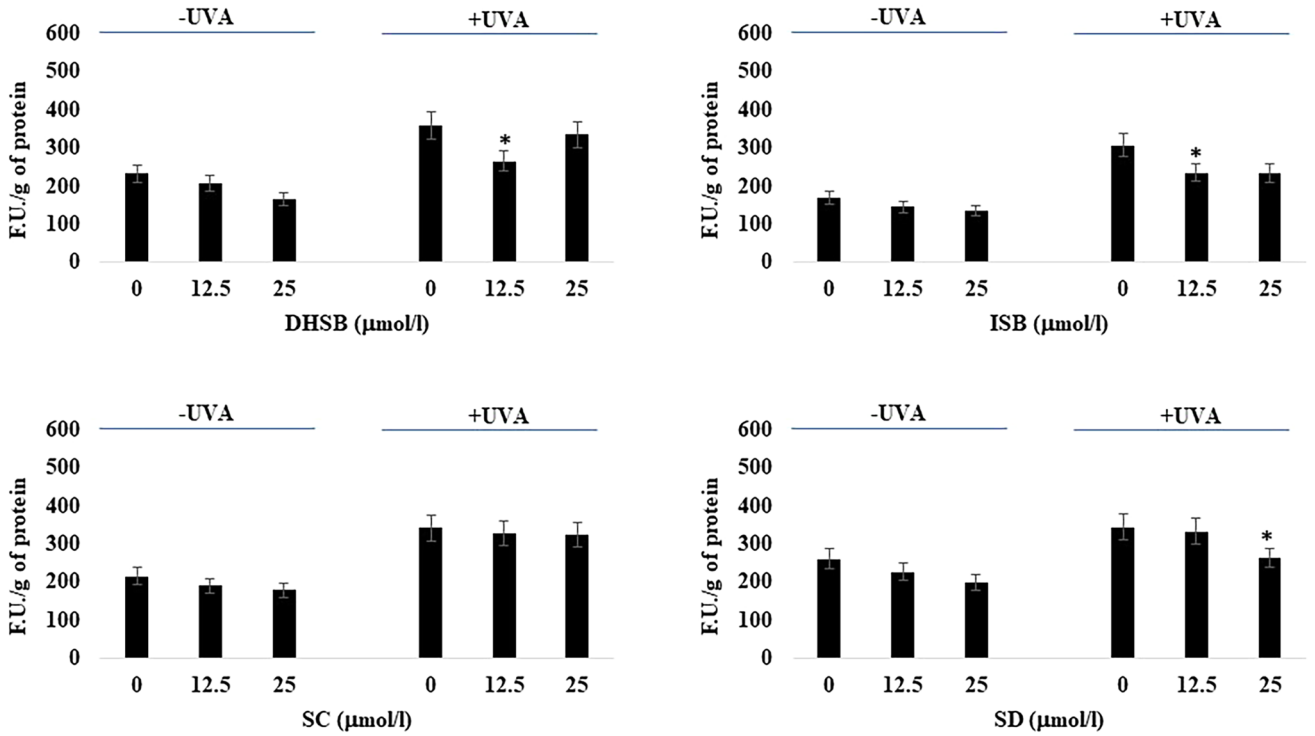

Fig. 8 Effect of flavonolignans on UVA-induced carbonylated protein level. Cells pre-treated with DHSB, ISB, SC, or SD were non-irradiated (-UVA) or exposed to 10 J/cm^2 (+UVA), and after 24 h of incubation, protein oxidation was evaluated as described in "Materials and Methods". Results are mean ± SD of four experiments employing cells from four donors. *Significantly different from irradiated cells at $p = 0.05$

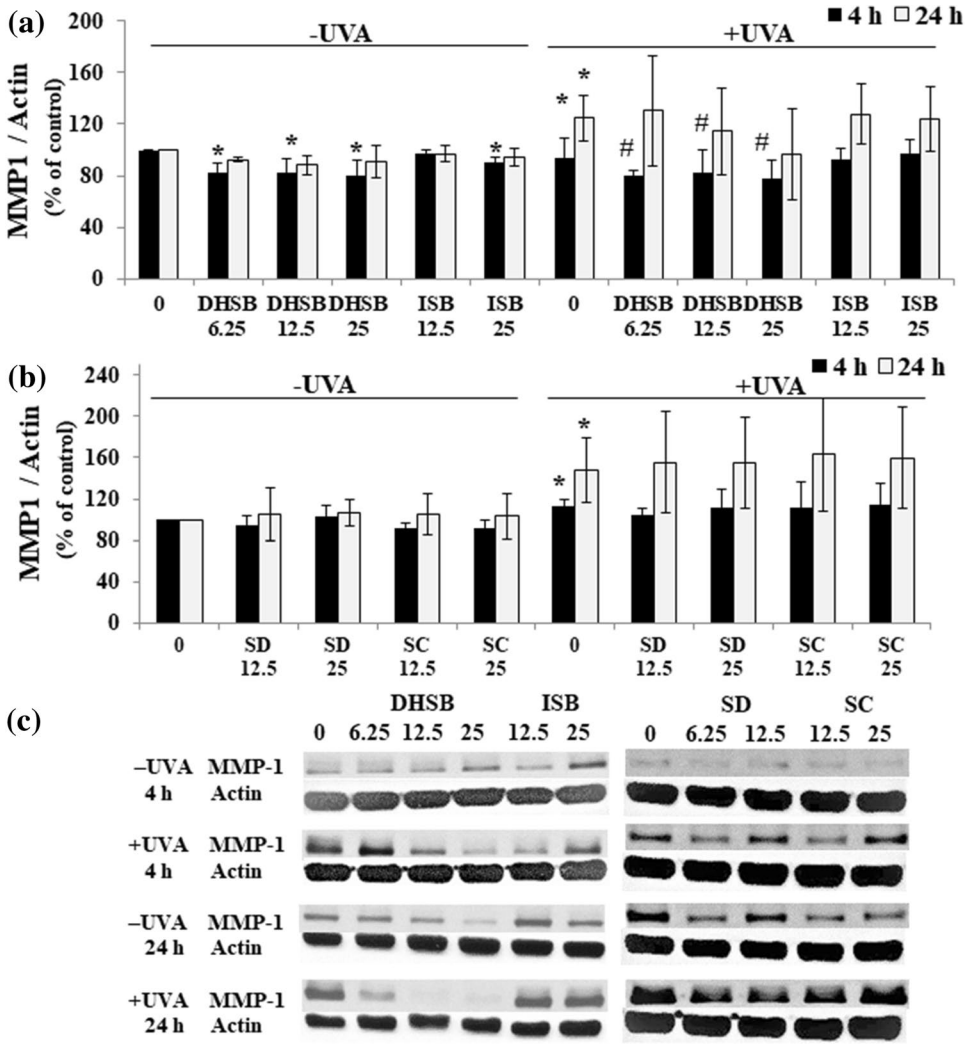

Fig. 9 Effect of flavonolignans on UVA-affected MMP-1 level. Cells pre-treated with DHSB, ISB, SC, or SD were non-irradiated (-UVA) or exposed to 7.5 J/cm^2 (+UVA), and after 4 or 24 h of incubation, expression of MMP-1 was evaluated by Western blot as described in "Materials and Methods" (**a**) and (**b**). Results are mean ± SD of four experiments employing cells from four donors. * Significantly different from non-irradiated cells at $p = 0.05$. #Significantly different from irradiated cells at $p = 0.05$. **c** Data show representative examples of four independent experiments

(Fig. 8). Effect of DHSB also corresponds with our previous study demonstrating its ability to reduce UVA-induced oxidative damage to keratinocytes HaCaT [22].

The dermis, which is significantly affected by longwave UVA radiation, contains, besides cellular elements (mainly fibroblasts), extracellular matrix components including the proteins collagen and elastin. These proteins are essential for maintaining structural integrity and elasticity and for physiological functions of the skin [5]. The degradation of dermal proteins has been directly linked to wrinkle formation, one of the signs of skin aging. The degradation correlates with an increase in the activity of certain enzymes that degrade extracellular matrix proteins (EMP), for example MMP-1. The amount of EMP-degrading enzymes also grows in skin exposed repeatedly to solar (UV) light, leading to premature skin aging [17]. Our data showed that the pre-treatment of human fibroblasts with DHSB reduced the protein level of MMP-1, a major enzyme of collagen degradation, in both irradiated and non-irradiated cells. The other three flavonolignans had only a non-significant effect on MMP-1 level. These results agree with our recent study on the effect of flavonolignans on collagenase activity measured by a tube test. DHSB was the most potent compound with $IC_{50} = 23.4 \pm 2.9$ µmol/l. Other flavonolignans, including SB, had the IC_{50} value higher than 50 µM [26].

HSP are stress proteins and their upregulation, especially that of HSP70, is considered to be an early repair mechanism that prevents irreversible cell damage. HSP70 are responsible for the re-folding or degradation of denatured proteins produced due to stress. HSP70 are induced by many different agents including UV radiation [18]. The exposure of NHDF-to-UVA radiation caused a moderate increase in HSP70 protein level. The pre-treatment of NHDF with flavonolignans only had a non-significant effect on the HSP70 protein level (Fig. 11). Another stress-related protein, HSP32, is more commonly known as HO-1. HO-1 is stimulated by similar factors as HSP70, but also by many other stimuli; for example, hydrogen peroxide, lipopolysaccharide, inflammatory cytokines, and other antioxidants [27]. This may be the reason for the more pronounced effect of UVA radiation on

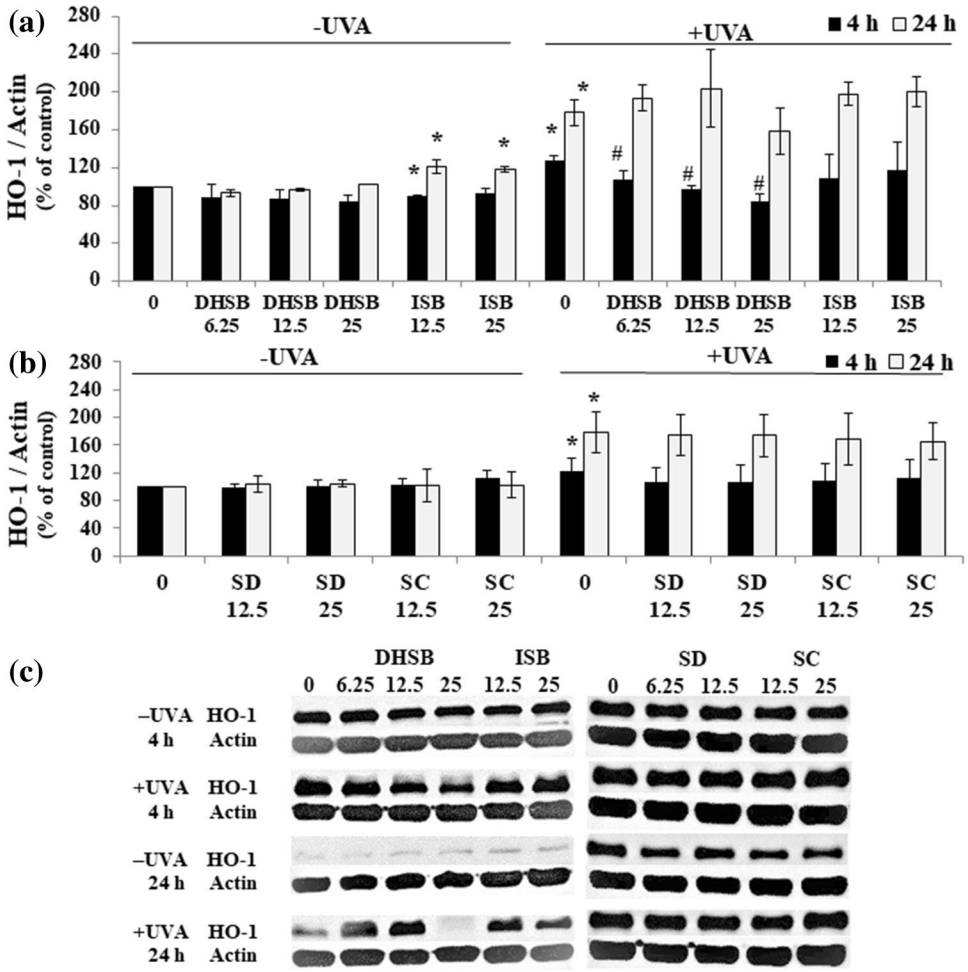

Fig. 10 Effect of flavonolignans on UVA-affected HO-1 level. Cells pre-treated with DHSB, ISB, SC, or SD were non-irradiated (-UVA) or exposed to 7.5 J/cm^2 (+UVA) and after 4 or 24 h of incubation, expression of HO-1 was evaluated by Western blot as described in "Materials and Methods" (**a**) and (**b**). Results are mean ± SD of four experiments employing cells from four donors. *Significantly different from non-irradiated cells at $p = 0.05$. #Significantly different from irradiated cells at $p = 0.05$. **c** Data show representative examples of four independent experiments

HSP32 compared to HSP70 (see Figs. 10 and 11). DHSB significantly reduced HO-1 level in both irradiated and non-irradiated fibroblasts at the earlier time-point and increased HO-1 level after 24 h, but only non-significantly. HO-1 increase was also stimulated by ISB (after 24 h). The other flavonolignans had a minimal effect on the protein level. The non-significant effects of flavonolignans on the protein levels, particularly MMP-1, HO-1, and HSP70, may be connected to the large variability between four donors of fibroblasts (shown by the large SD). Moreover, in the NHDF from two donors, we found more pronounced harmful effects of UVA as well as protective effect of flavonolignans compared to NHDF from the other two donors (Figs. 9, 10, 11).

To summarize the overall potential of the studied flavonolignans to suppress UVA-induced damage according to the obtained data, the most potent seems to be DHSB followed by SC. These two compounds had significant effects on most of the studied parameters. Moreover, here, we demonstrated that DHSB was effective at the two lowest concentrations tested (6.25 and 12.5 µmol/l). This fact increases its biological relevance—a potential practical application. On the other hand, DHSB exhibited phototoxic effects that we reported previously in a separate study [16] and demonstrated here as increased GSH depletion, carbonyl proteins formation, and activation of pro-caspase-3 at higher DHSB concentrations (25 and/or 75 µM). In this respect, SC (the second most abundant flavonolignan in silymarin) was completely without phototoxic potential, as demonstrated here and previously [16]; meanwhile, its photoprotective effect was primarily found at double the concentration of DHSB. SC was also slightly less effective than SB, the major SM component (described elsewhere) [15]. The higher biological activity, e.g., photoprotectivity, but also photoreactivity of DHSB is linked to its structure which explains its high antioxidant properties. Compared to SB and the other flavonolignans studied here, there is a 2,3-double bond in the ring B of DHSB (see Fig. 1) that significantly influences both its protective and adverse properties [7, 24]. Thus, contrasting data suggest that DHSB could be a useful agent for UV-protective dermatological preparations. However, due to its observed phototoxic effect in vitro, further studies are needed to exclude phototoxicity for humans as well as to

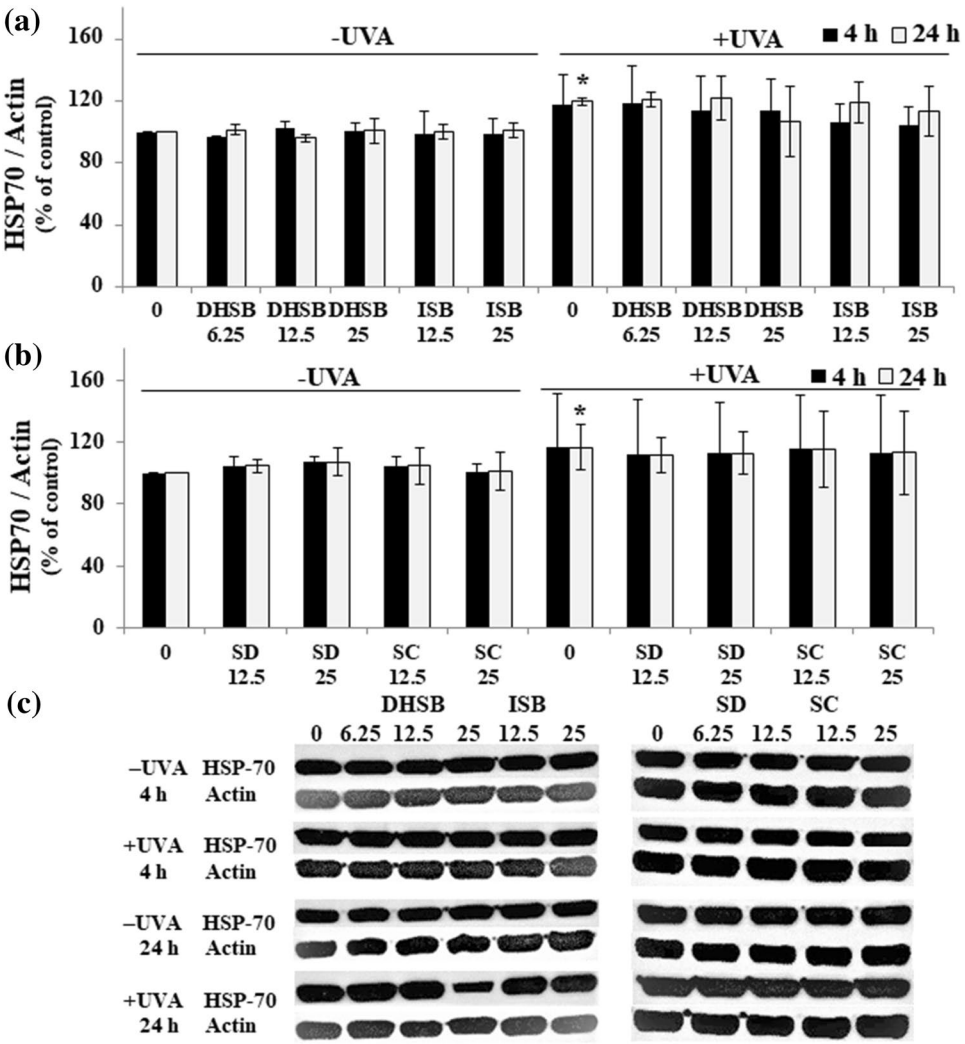

Fig. 11 Effect of flavonolignans on UVA-affected HSP-70 level. Cells pre-treated with DHSB, ISB, SC, or SD were non-irradiated (-UVA) or exposed to 7.5 J/cm^2 (+UVA), and after 4 or 24 h of incubation, expression of HSP-70 was evaluated by Western blot as described in "Materials and Methods" (**a**) and (**b**). Results are mean ± SD of four experiments employing cells from four donors. *Significantly different from non-irradiated cells at $p = 0.05$. **c** Data show representative examples of four independent experiments

confirm our results on more sophisticated models such as human skin ex vivo and in vivo.

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