

Potential of silymarin and its polyphenols to affect Nrf2 signalling pathway in human skin cells

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Introduction. Human skin is constantly exposed to various agents causing oxidative stress. In response, skin cells can activate transcriptional nuclear factor erythroid 2-related factor 2 (Nrf2), which influences the expression of several protective genes. However, accumulating evidence suggests severe adverse effects of chronic Nrf2 activation. Phytochemicals are popular for supporting skin health. Some are potent Nrf2 inducers and their long-term use may be hazardous. Silymarin (SM), an extract from *Silybum marianum* fruits, and its major flavonolignan silybin (SB) are used in cosmetic formulations for their protective and regenerative activities. However, knowledge about their potential to modulate Nrf2-driven pathway in skin cells or tissue is lacking.

Methods. Here we evaluated effects of SM, SB, silychristin (SC), silydianin (SD), isosilybin (ISB) and 2,3-dehydrosilybin (DHSB) and taxifolin (TA) on Nrf2 nuclear translocation, the level of Nrf2-control proteins (Keap1 and Bach1) and selected Nrf2-driven genes (NAD(P)H:quinone oxidoreductase 1 and heme oxygenase 1) in human primary fibroblasts and keratinocytes.

Results. SM and its components significantly stimulated Nrf2 nuclear translocation; SM and SB were most potent on both cell types. The effect of SM and compounds was more pronounced in fibroblasts than on keratinocytes. Regardless, effects of SM and studied compounds on the Nrf2 regulating and Nrf2-controlled proteins were non-significant.

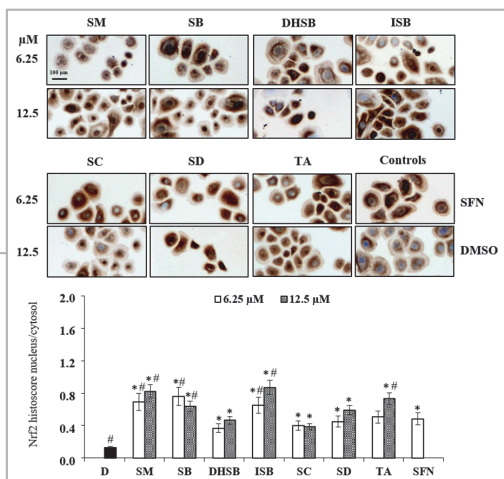
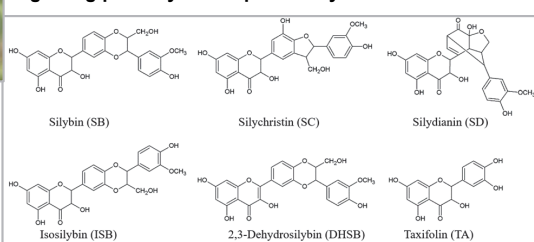
Conclusion. SM and its components do not have a significant effect on the studied proteins, and seem to be safe with respect to possible negative effects associated with the Nrf2 signalling pathway activation during dermal application. However, we cannot give a definitive answer on their effect after repeated long-term application to the whole skin. Further experiments on human skin *ex vivo* or clinical studies on volunteers are necessary.

POTENTIAL OF SILYMARIN AND ITS POLYPHENOLS TO AFFECT NRF2 SIGNALLING PATHWAY IN HUMAN SKIN CELLS



Silybum marianum L.
Gaertner (Asteraceae)

There is accumulating evidence for severe adverse effects of long-term Nrf2 activation in the skin tissue. Silymarin and its components has become attractive for dermal applications. Since studies evaluating the effect of silymarin and/or its components on the Nrf2 transcription factor in human skin tissue or cells are lacking, we investigated their potential to affect the Nrf2 signaling pathway in this pilot study.



Silymarin and its flavonolignans differentially stimulated Nrf2 translocation to the nucleus, but the levels of the Nrf2-regulated proteins Bach1, Keap1, HO1 and NQO1 were not significantly affected.

Rysava A. et al., doi: 10.5507/bp.2025.020

Graphical Abstract

Biomedical Papers

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Key words: flavonolignan, flavonoid, nuclear factor erythroid 2-related factor 2, primary fibroblasts, primary keratinocytes, silymarin

Received: February 14, 2025; Revised: May 18, 2025; Accepted: June 27, 2025; Available online: July 25, 2025

<https://doi.org/10.5507/bp.2025.020>

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INTRODUCTION

Skin represents the first barrier protecting the body against environmental assaults. Many agents cause reactive oxygen species generation and redox imbalance. Therefore, the skin developed protective mechanisms against oxidative damage. A major antioxidant defence regulator is transcription factor Nrf2 that controls the expression of several genes involved in the cytoprotection¹. Therefore, Nrf2 level is strictly controlled and under normal homeostatic conditions, Nrf2 level is kept low by continuous proteasomal degradation, which is mediated by a strong specific inhibitor Kelch-like ECH-associated protein 1 (Keap1). In addition to Keap1, transcription factor BTB domain and CNC homolog 1 (Bach1) regulates Nrf2 activity. Bach1 is nuclear protein that makes heterodimers with Nrf2's transcription partner sMaf and thus blocks the transcription activity of Nrf2 (ref.²). Pharmacological Nrf2 activation seems to be a promising strategy for skin protection against stress condition. A variety of Nrf2 activators have been discovered, among them also naturally occurring compounds such as sulforaphane (SFN), curcumin or resveratrol³. These molecules represent attractive ingredients for various cosmetic products. However, there is accumulating evidence for severe adverse effects of long-term Nrf2 activation, including namely keratinocyte hyperproliferation, impaired desquamation, hyperthickening of the cornified layer and impaired skin barrier function^{3,4}. These findings need to be considered when Nrf2-activating compounds are used for human skin treatment, even for skin protection or regeneration.

One plant that has become interesting for the cosmetic and dermatological industry is the milk thistle (*Silybum marianum* L. Gaertner) from the *Asteraceae* family. *S. marianum* has been used in traditional European medi-

cine for almost 2 000 years⁵. A standardized polyphenols mixture from its seeds is known as silymarin (SM). The major SM's component is the flavonolignan silybin (SB). Other less abundant components include flavonolignans silychristin (SC), silydianin (SD), isosilybin (ISB) and 2,3-dehydrosilybin (DHSB) and a flavonoid taxifolin (TA), see Fig. 1 (ref.⁶). SM and SB have antioxidant and anti-inflammatory properties and modulate various signalling pathways^{5,7}. These properties make SM and its components attractive for dermal application in cosmetics. For example, SM (0.5%) containing serum improved acne's clinical severity and related skin biophysical parameters in patients with mild or moderate form of acne vulgaris⁸. Several commercial dermatological preparations containing SM or SB as the active ingredients are currently available on the market. Besides a daily cream (Olive⁹) or sunscreens (Banobagi¹⁰; PCA skin¹¹), SM/SB is found in preparations for the treatment of acne (SynCare¹²), for the suppression of wrinkle formation and skin aging (Eucerin¹³, Banobagi⁹) or the treatment of rosacea (RosaCure+ (ref.¹²)). All these preparations are for long-term use and thus potentially influence Nrf2-driven genes. Modulation of Nrf2 nuclear translocation or expression and the Nrf2-controlled genes expression by SM or SB was shown in several experimental models such as in indomethacin-induced gastric injury in rats¹⁴, in paraquat-induced lung injury in rats¹⁵, in arsenic-induced oxidative stress in heart of rats¹⁶ or in acrylamide-induced neurotoxicity *in vitro*¹⁷. However, these studies used some agents capable of inducing oxidative stress and the purpose of SM application was to suppress oxidative damage. To our knowledge, no study has evaluated the effects of SM/SB on human skin cells/tissues, either normal (without induced damage) or exposed to oxidative stress stimulating agents. Recently we found stimulation of Nrf2 nuclear translocation, mRNA and/or the protein

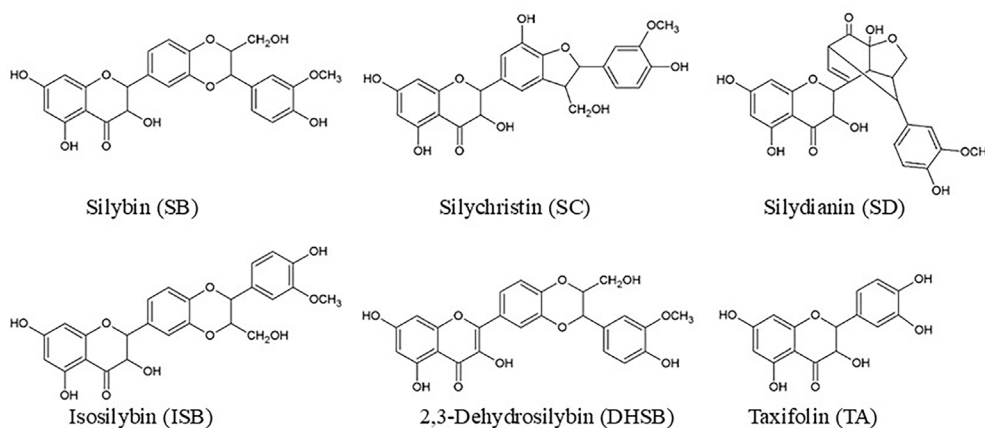


Fig. 1. Chemical structures of studied polyphenols from silymarin.

level of NAD(P)H:quinone oxidoreductase 1 (NQO1), heme oxygenase-1 (HO-1) and catalase by flavonoids TA and quercetin (QE), structurally-related to SB and DHSB, respectively, in normal human epidermal keratinocytes (NHEK) (ref.¹⁸). Therefore, in this pilot study we investigated effect of SM and its selected components (Fig. 1) on the Nrf2 signalling pathway in NHEK and normal human skin fibroblasts (NHDF).

MATERIALS AND METHODS

Chemicals

Dulbecco's modified Eagle's medium (high glucose 4.5 g/L; DMEM), Ham's F12 nutrient mixture, stabilised penicillin-streptomycin solution, amphotericin B, hydrocortisone, adenine, insulin, epidermal growth factor, 3,3',5-triiod-L-thyronin, ampicillin, trypsin-EDTA (0.25%), N,N,N',N'-tetramethylethylenediamine (TEMED), sodium dodecyl sulphate (SDS), Ponceau S in acetic acid (0.1%, w/v), Carestream Biomax Light film, Carestream Kodak autoradiography GBX developer, fixer and replenisher, and dimethyl sulfoxide (DMSO) were supplied by Sigma-Aldrich (Czech Republic). Keratinocyte basal medium-2 (KBM-2), KBM-2 supplements kit KGM™-2 SingleQuots™, EpiLife® medium, human keratinocyte growth supplement kit and heat-inactivated fetal bovine serum (FBS, HyCylone™) were from Life Technologies (Czech Republic). Acrylamide-bisacrylamide mixture (40%, v/v) was from Merck (Czech Republic). Ammonium peroxodisulfate was from BioChemika Fluka (Czech Republic). Protease inhibitors Complete™ tablets were from Roche Diagnostics (Germany). The Pierce™ BCA protein assay kit was supplied by Thermo Fisher Scientific (Czech Republic). Albumin bovine fraction V (BSA) was from Serva (Germany) and non-fat milk was from Samantha (Czech Republic). Western blotting luminol reagent (ImmunoCruz™) for chemiluminiscent horseradish peroxidase (HRP) detection, Nrf2 rabbit polyclonal IgG (C-20, sc-722; 1:500), NQO1 goat polyclonal IgG (R-20, sc-16463; 1:500), HO-1 rabbit polyclonal IgG (H-105, sc-10789; 1:500), actin goat polyclonal IgG (I-19, sc-1616; 1:500), goat anti-rabbit and rabbit anti-goat HRP-conjugated secondary antibody (1:5000) were purchased from Santa Cruz Biotechnology Inc. (USA). Keap1 rabbit (ab227828, EPR 22664-26; 1:500), Bach1/Brip1 rabbit (ab151509; 1:1000), TATA-binding protein (TBP) rabbit (ab74222; 1:2000) from Abcam (UK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) rabbit (5174S, D16H11XP, 1:1000) were from Cell Signaling Technology (USA). Solution 1 for primary and Solution 2 for secondary antibodies were from Calbiochem (USA). Protein block serum-free, peroxidase and diaminobenzidine (DAB), Dako REAL™ antibody diluent, secondary antibody DAKO REAL EnVision™ HRP Mouse/Rabbit were supplied by DAKO (Denmark).

Test Compounds

SM (batch 17306S/089) and silybin (batch 120692; purity 98%; natural mixture of diastereomers ca. 1:1)

were kindly provided by IVAX Pharmaceuticals (Czech Republic). SM contained 67.6% flavonolignans and 2.1% TA. The most abundant component was SB (37.98%). Details of the SM analysis were published earlier¹⁹.

ISB, SC, SD and DHSB were prepared at the Institute of Microbiology, Czech Academy of Sciences (Prague, Czech Republic). ISB (natural mixture of diastereomers ca. 95:5), SC (natural mixture of diastereomers ca. 9:1) and SD were isolated from SM as described previously²⁰. DHSB was prepared as described Gažák et al.²¹. TA and SFN were from Sigma-Aldrich (Czech Republic). The stock solutions of SM and compounds were prepared in DMSO.

Cell culture

NHDF and NHEK were isolated from superfluous skin of healthy adult donors. The skin fragments were from patients undergoing plastic surgery (Department of Plastic and Aesthetic Surgery, University Hospital in Olomouc). The tissue acquisition protocol adhered to the requirements of the Ethics Committee of the University Hospital in Olomouc and Faculty of Medicine and Dentistry, Palacký University in Olomouc (ref. number 41/09). All volunteers gave their written informed consent. NHDF (ref.²²) and NHEK (ref.^{23,24}) were isolated and cultivated as described earlier. Cells from 3 donors were used. For western blot (WB) analysis, cells were seeded on Petri dishes; for immunocytochemical analysis, cells were seeded in a drop of appropriate culture medium on sterile SuperFrost® Plus Menzel-Glaser slides (Thermo Scientific, Czech Republic), embedded in sterile Petri dishes. The concentrations of 50 000 cells/mL (NHDF) and 100 000 cells/mL (NHEK) were used.

Treatment with compounds

After reaching 90–100% confluence (WB) or 24 h after seeding (immunocytochemistry), cells were treated with the selected polyphenols SB, DHSB, SC, SD, ISB, TA (6.25 and 12.5 µM) or SM (3.015 and 6.031 mg/L ~ the concentrations correspond to the flavonolignans molar concentrations) in appropriate serum-free medium for 1 h. The final DMSO concentration in the medium was 0.5%. Cells treated with serum-free medium with DMSO (0.5%) and with SFN (6.25 µM) were used as a negative and a positive control, respectively. Then cells were fixed (immunocytochemical analysis) or incubated in fresh serum-free medium, see below.

Western blot analysis

At 6 or 24 h after treatment with the compounds (NHEK or NHDF, respectively) cell were harvested and total cellular lysates²⁵ or nuclear and cytosolic extracts²⁶ were prepared. The protein amount was quantified by the bicinchoninic acid assay. Proteins were separated in 10% SDS-polyacrylamide gel and then transferred on a PVDF membrane. Membranes were blocked with 5% non-fat milk in Tris-buffered saline with 0.05% Tween-20 (TBS/T) for 2 h and incubated (overnight, 4 °C) with primary antibodies (Table 1). Incubation with the respective secondary antibody (Table 1) followed for 1 h. Proteins were

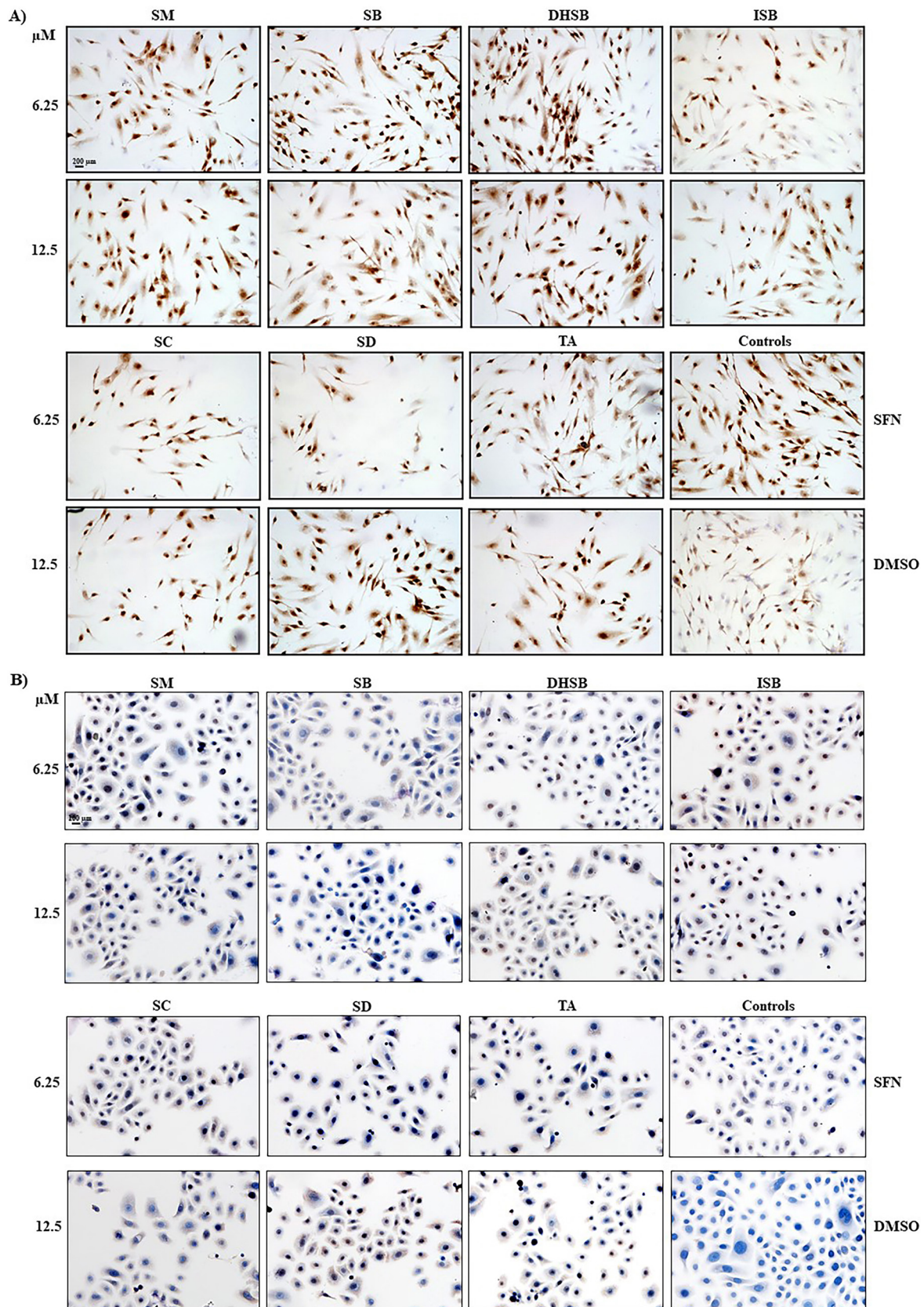


Fig. 2. Effect of SM and polyphenols on Nrf2 intracellular localization.

Nrf2 protein was evaluated in the cytosol and nucleus (brown) by immunocytochemistry after 1 h of treatment with test compounds in **A**) NHDF or after 1 h of treatment with test compounds and 6 h of additional incubation in serum-free medium in **B**) NHEK. Microscopy images of SM and selected compounds are representative from three independent experiments. Haematoxylin was used for nuclei staining (blue). Medium with DMSO (DMSO) was the negative control and SFN (6.25 μ M) the positive control. The microphotographs are at 100-fold magnification (NHDF) or 200-fold magnification (NHEK).

detected by chemiluminescence and autoradiography. The quantification was performed by a densitometric analysis using the ImageJ software.

Immunocytochemical analysis

The Nrf2 translocation was evaluated immediately after treatment with the compounds (NHDF) or after 6 h of additional incubation in serum-free medium (NHEK). Immunostaining was performed as described previously¹⁸. The slides were scanned in a light microscope (CK2 with a camera DP71, Olympus, Japan). The Nrf2 protein localization and amount was analysed visually at a magnification of 100/200× (NHDF/NHEK). The staining intensity was assessed semi-quantitatively by dividing into four categories using the QuPath software²⁷: negative (C1), weakly positive (C2), moderately positive (C3) and strongly positive (C4). Approximately 100 cells were evaluated in 3 fields of view (microscopic photographs). The histscore (range 0–300) was calculated according to the formula:

$$\text{Histscore} = (0 * \%C_1) + (1 * \%C_2) + (2 * \%C_3) + (3 * \%C_4)$$

Statistical analysis

Data were expressed as means $\pm$ standard deviation in MS Excel. The statistical comparison was performed using Tukey's HSD test by one-factor ANOVA in the program Statistica 12. Statistical significance was determined at $P < 0.05$.

RESULTS

For experiments, low non-cytotoxic concentrations were chosen according to our previous studies^{19,22}. The treatment and incubation periods for individual parameters (Nrf2 localization and level of selected proteins) were chosen according to the times used in our previous study¹⁸. Immunocytochemical analysis showed an increased Nrf2 amounts in the nucleus of NHDF treated with SM and pure compounds (Fig. 2A). In cells treated with ISB (6.25 μM), a lower Nrf2 translocation was found. SM and pure compounds at both concentrations exhibited a lower effect than the positive control SFN (6.25 μM) (Fig. 3A). In NHEK, SM and test compounds increased Nrf2 protein level not only in the nucleus, but also in the cytosol (a higher total intracellular content) (Fig. 2B). With SB, the Nrf2 translocation to the nucleus was more potent at the lower concentration (6.25 μM). SM and DHSB, ISB, SD and TA exhibited a concentration-dependent stimulation of the Nrf2 translocation. In addition, SM, SB and ISB demonstrated a higher effect on Nrf2 translocation than SFN at the same concentration (Fig. 3B).

Regulatory proteins levels were evaluated by WB. Keap1 level in the cytosol of NHDF was minimally affected by SM and most of pure compounds. Only DHSB and ISB non-significantly increased the protein level (Fig. 4A). The nuclear level of Bach1 protein was reduced in cells treated with most of compounds except for SM (12.5 μM) and TA (6.25 μM) (Fig. 4B). In keratinocytes, SM, SB,

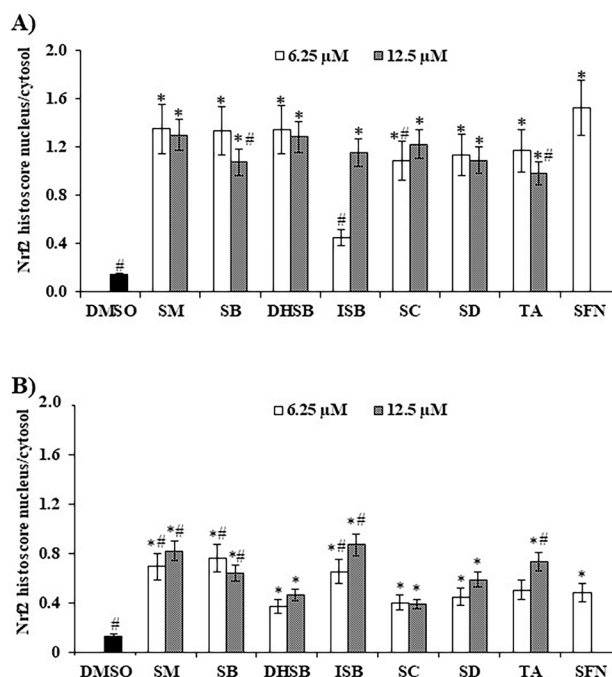


Fig. 3. Effect of SM and polyphenols on Nrf2 intracellular localization.

Semi-quantitative evaluation of Nrf2 protein translocation in **A)** NHDF and **B)** NHEK was evaluated using a light microscope and the program QuPath, and was processed as the ratio of Nrf2 protein in the nucleus/cytosol. Data were expressed as the mean $\pm$ SD of 3 independent experiments (primary cells from 3 donors). Statistical significance was determined at $P < 0.05$; * significantly different from the negative control (DMSO) and # significantly different from the positive control (SFN).

DHSB and ISB stimulated reduction in Keap1 content. The effect of ISB (12.5 μM) was most pronounced. TA showed a minimal change in Keap1 level, while SC and SD obviously increased its level (Fig. 4C). Regarding the Bach1 level, SM, SB and DHSB had minimal effect; ISB caused its decrease and SC, SD and TA stimulated its increase (Fig. 4D).

NQO1 and HO-1 protein level was evaluated by WB as well. Although SM and the tested compounds increased the nuclear Nrf2 level in fibroblasts (Fig. 3A), the NQO1 level was increased only in cells treated with SD (both concentrations) and TA (12.5 μM), but not significantly. Their effect was comparable to that of SFN. Only a minimal effect was observed with the other compounds (Fig. 5A). HO-1 levels were enhanced in fibroblasts treated with SC (12.5 μM) and SD (6.25 and 12.5 μM), and their effect was stronger than that of SFN (Fig. 5B). Even though all compounds and especially SM, SB, ISB and TA clearly stimulated Nrf2 translocation in NHEK (Fig. 3B), they had minimal effect on NQO1. SFN showed a moderate ($\sim 125\%$ of control), but non-significant effect (Fig. 5C). DHSB, ISB and TA increased potently the HO-1 protein level. However, their effect was lower than that of SFN at the same concentration (180% of control). The other compounds had a rather minimal effect (Fig. 5D). The variability in response among donor skin cells (mainly

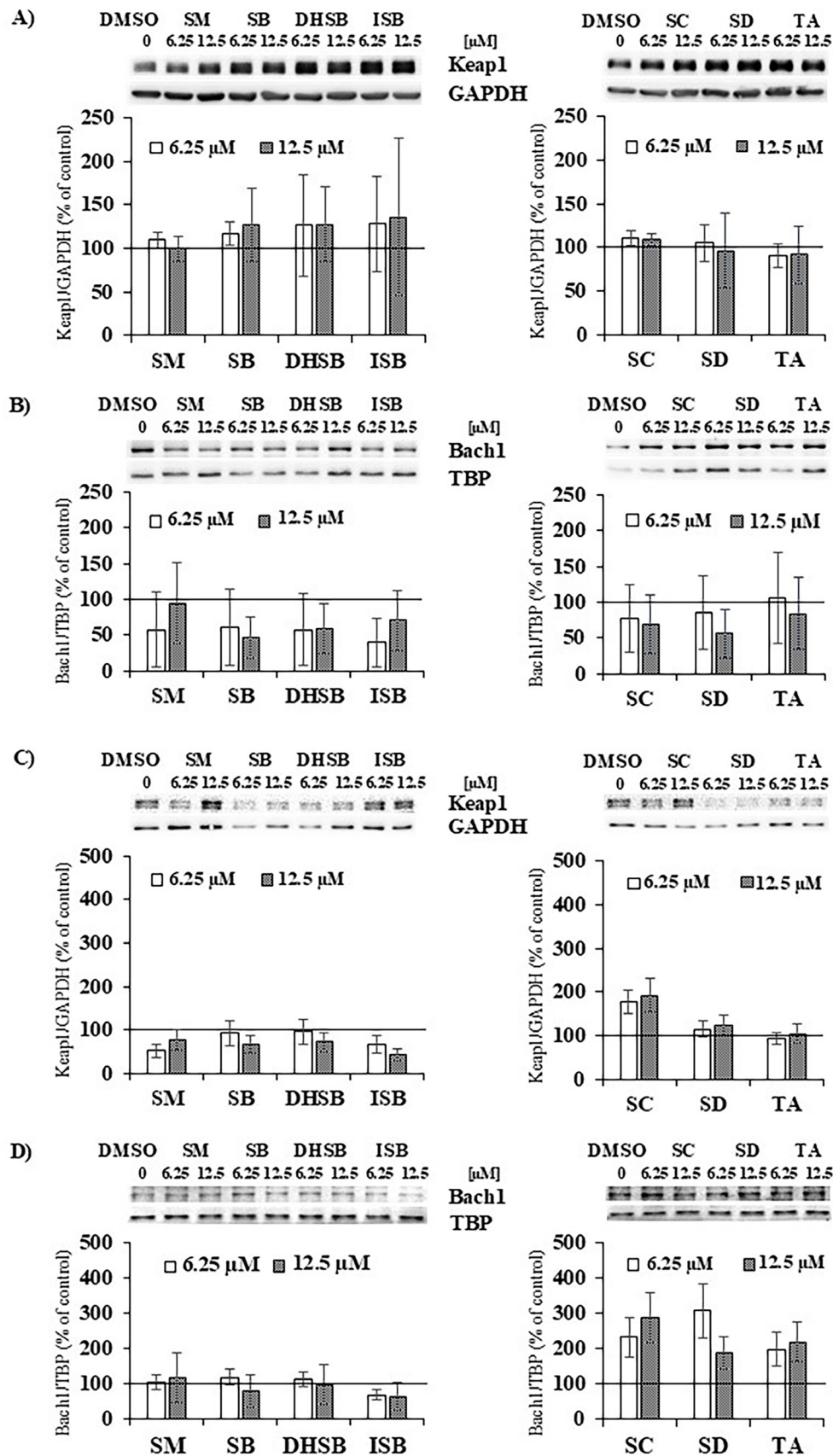


Fig. 4. Effect of SM and polyphenols on Keap1 and Bach1 level.

The levels of Keap1 in **A)** NHDF and **C)** NHEK were analysed in cytosolic lysates and of Bach1 in **B)** NHDF and **D)** NHEK in nuclear lysates by Western blot after 1 h of treatment with SM/polyphenols and 6 h (NHEK) or 24 h (NHDF) of additional incubation. Data were normalized to the reference protein in cytosol (GAPDH) and in nucleus (TBP). Results were expressed as mean $\pm$ SD from 3 independent experiments (primary cells from 3 donors). Statistical significance was determined at $P < 0.05$; * significantly different from the negative control (DMSO).

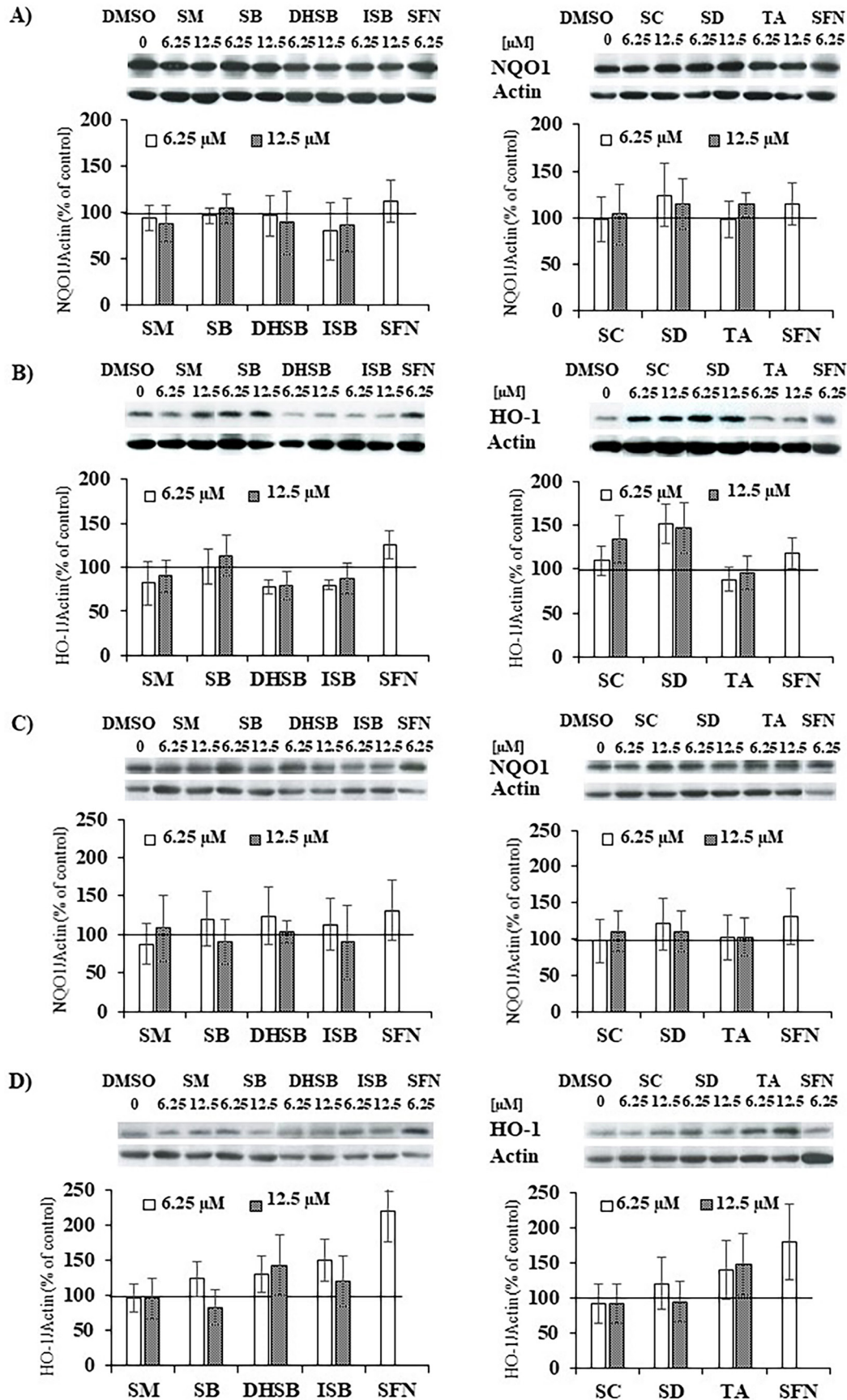


Fig. 5. Effect of SM and polyphenols on NQO1 and HO-1 level.

The levels of NQO1 in A) NHDF and C) NHEK and of HO-1 in B) NHDF and D) NHEK were analysed in whole-cell lysates by Western blot in after 1 h of treatment with SM/polyphenols and 6 h (NHEK) or 24 h (NHDF) of additional incubation. Data were normalized to the reference protein (actin). Results were expressed as mean $\pm$ SD from 3 independent experiments (primary cells from 3 donors). Statistical significance was determined at $P < 0.05$; * significantly different from the negative control (DMSO).

NHEK) increased standard deviation of the results obtained.

DISCUSSION

SM/SB showed beneficial effects on chemically²⁸ or UVB-induced²⁹ skin carcinogenesis, wound healing, dermatitides, psoriasis³⁰, acne³¹ or UVA-induced damage^{19,22,32}. Therefore, they represent attractive dermatological ingredients and are present several marketed products⁸⁻¹¹. Here we studied the potential of SM and its components to activate the Nrf2 signalling pathway in skin cells that has not been studied yet.

In NHDF and NHEK, SM and its polyphenols increased the nuclear Nrf2 level compared with the control (Fig. 2 and 3). The effect was stronger in NHDF compared with NHEK (Fig. 3). A previous study on SB showed no effect on the Nrf2 nuclear translocation in hepatic cells³³. But consistently with our data, the increased nuclear Nrf2 protein level was reported in HT-22 hippocampal cells treated with ISB (10 μ M) (ref.³⁴).

Although detected Nrf2 translocation, a surprisingly minimal effect on the level of NQO1 protein (Fig. 5), specifically controlled by the Nrf2 transcription factor, was found. The HO-1 protein expression was moderately increased in fibroblasts treated with SC and SD and in keratinocytes treated with DHSB, ISB and TA (Fig. 5). The different effect of the compounds on HO-1 compared to NQO1 could be related to HO-1 Nrf2-independent activation, e.g. by the forkhead-box protein O1 (ref.³⁵). Moreover, HO-1 expression is significantly affected by the nuclear protein Bach1, which blocks the Nrf2 activity³⁶. The Bach1 effect on HO-1 expression in keratinocytes is Nrf2-independent and (phyto)chemicals can stimulate Bach1 nuclear export and induce the Nrf2-target genes expression, with HO-1 being the most upregulated of them³⁷.

We further investigated the impacts on Nrf2 regulatory proteins. Keap1 causes sequestering Nrf2 in the cytoplasm and facilitating Nrf2 degradation. Bach1 forms a heterodimer with other proteins, binds to the antioxidant responsive element (ARE) and blocks the Nrf2 binding to DNA (ref.²). A recent computational study on Keap1 interaction with flavonolignans predicted that SB and DHSB might potentiate the disruption of the Nrf2-Keap1 complex³⁸. Here we found a minimal effect of SM and its components on Keap1 level in fibroblasts. In keratinocytes we detected reduced Keap1 level after treatment with SM, SB, ISB and DHSB and increased in cells treated with SC and SD (Fig. 4). This is largely consistent with Nrf2 translocation in NHEK (Fig. 3). Bach1 levels were reduced in fibroblasts treated with most of compounds except for SM (12.6 μ M), TA (6.25 and 12.5 μ M). In keratinocytes only ISB decreased Bach1 level. Treatment of human hepatocytes CON1 with SB (50 and 200 μ M) reduced Bach1 protein and mRNA expression³⁹, which agrees with our findings. Other study showed the different effect of SM (100 and 200 μ M) on Bach1 level in three human hepatoma cell lines. While no effect was found in Huh-7 cells, the higher concentration stimulated

Bach1 levels in CNS3 cells, and both SM concentrations increased Bach1 level in 9–13 cells⁴⁰.

The effect of SB and DHSB was significantly lower than that of structurally-related flavonoids TA and QE, respectively reported earlier¹⁸. The prominent effect of TA and especially QE is probably related to their stronger antioxidant activity compared to SB and DHSB. The QE antioxidant potential⁴¹⁻⁴³ is associated with a 3-hydroxy group coupled with the 2,3-double bond in the ring C. DHSB contains a similar structural moiety, but a larger substituent on the ring B (a coniferyl group instead of two hydroxyls). TA, a QE structural derivative without the 2,3-double bond (Fig. 1), was more potent than DHSB. There exists a correlation between flavonoids redox properties and their ARE-mediated response. The 3-hydroxy group strongly increases the ease with which a flavonoid donates electrons and the pro-oxidant action of flavonoids is essential for their ability to stimulate an ARE-mediated response⁴⁴. Therefore, also other flavonoids structurally-related to QE, kaempferol, myricetin⁴⁵ or luteolin⁴⁶ were reported as effective Nrf2 protein inducers.

CONCLUSION

In summary, SM and SB stimulated Nrf2 nuclear translocation and their effect is possibly associated with the reduction of Keap1 in keratinocytes and Bach1 in fibroblasts. However, the NQO1 and HO-1 protein expression was only slightly affected. Significantly less abundant SM's components also increased the Nrf2 translocation; some of them also affected the levels of HO-1 and regulatory proteins, but they showed a minimal effect on NQO1, directly regulated by Nrf2. Based on the low bio-availability of SM's components and the observed non-significant effects of SM and SB, they both appear to be safe in term of potential adverse effects associated with long-term Nrf2 activation. The effects of flavonoid TA on the Nrf2 signalling pathway (translocation, level of Bach1 and HO-1) were more apparent compared to most of flavonolignans, mainly namely in human keratinocytes. TA is more abundant in the plant kingdom compared to flavonolignans and thus as a pure component or extract, it can be a component of many dermatological preparations, not only those containing SM where it is found in very low concentration. Our experiments on cell monolayer have limitations and provide results after only a single application. Therefore, we cannot give a definitive answer on their effect after repeated long-term treatment of the whole skin. Further experiments with human skin *ex vivo* or clinical studies on volunteers are necessary.

Acknowledgement

This study was financially supported by the grants IGA_LF_2025_008 and IGA_LF_2024_011, the institutional support of Palacky University Olomouc – RVO 61989592 and the institutional support of University Hospital Olomouc – RVO 98892. KV thanks the Czech Science Foundation (23-04654S). The authors thank to donors of skin tissue, to Iveta Hatalová (University

Hospital in Olomouc) for support with skin tissue donor recruitment and doc. Jana Franková, Ph.D. (Department of Medical Chemistry and Biochemistry), for the isolation of NHEK.

Authors contribution: AR: investigation, visualization, writing original draft; KV: resources, writing review and editing; JV: conceptualization, methodology, writing original draft; BZ: resources, writing review and editing; KC: investigation, writing review and editing; ARS: conceptualization, visualization, writing original draft. All authors have read and agreed to the final version of the manuscript.

Conflict of interest statement: The authors state that there are no conflicts of interest regarding the publication of this article.

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