

18 β -Glycyrrhetic Acid and Glabridin Prevent Oxidative DNA Fragmentation in UVB-irradiated Human Keratinocyte Cultures

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Abstract. *Background: UVB radiation is the major etiological factor in the pathogenesis of skin aging and cancer development. New approaches to prevent and reverse UVB damage are needed to reduce sunlight-induced skin cancer. This study aimed to investigate a possible protective activity of liquorice root extracts glycyrrhizin (GL), 18 β -glycyrrhetic acid (18 β -GA) and glabridin (GLB) against UVB radiation damage in human keratinocyte cultures. Materials and Methods: The MTT test was performed to assess cell viability. DNA damage was evaluated by comet assay, whereas generation of intracellular reactive oxygen species (ROS) was measured by fluorescent 2',7'-dichlorodihydrofluorescein diacetate assay. In addition, the activation of p53, regulation of BCL-2 and PARP cleavage were analyzed by Western blot analysis. Results: The treatment of human keratinocytes with 18 β -GA and GLB prevented direct and indirect DNA damage avoiding apoptosis activation. Conclusion: 18 β -glycyrrhetic acid and glabridin are potent antioxidants that prevent oxidative DNA fragmentation and the activation of apoptosis-associated proteins in human keratinocytes.*

The UV component of sunlight is now recognized as a major environmental factor deleterious to human health. In particular, UVB activates various signal transduction pathways and induces the expression of several specific genes (1). Such radiation may cause tumour initiation by inducing chromosome alterations and DNA damage and its promoting activity includes the transcriptional modulation of

genes involved in tumour promotion (2). UVB induces direct damage to DNA and proteins by interaction with aromatic amino acids (3). Indirect DNA damage is also caused by enhanced production of reactive oxygen species (ROS) that facilitate DNA oxidation (4). Skin UV exposure generates ROS in excessive quantities which, in turn, may cause oxidative damage to cells (5-7) and consequently apoptotic cell death through a variety of mechanisms, including the activation of stress kinases and the induction of apoptosis (8, 9). Whereas low doses of UVB cause DNA mutation leading to tumour initiation, high doses result in irreparable DNA damage, which leads to apoptosis and eventually cell deletion. In addition, the formation of sunburn cells (SBC) is linked to the severity of UVB-induced DNA damage (10). Human skin mainly consists of epidermis, which is a highly regenerative tissue characterized by a fine balance between keratinocyte proliferation and apoptosis, responsible for epidermal homeostasis (11). UVB radiation damages the DNA in human keratinocytes (the most numerous cells) with the consequence of cell cycle arrest by activation of the nucleotide excision repair program. When the repair process fails, cells can be eliminated *via* apoptosis (formation of the so-called SBC) or acquire mutations leading to the development of skin cancer (12, 13).

Active principles of licorice root have found extensive therapeutic use all over the world (14-16), and have been identified by the National Cancer Institute as possessing cancer preventive activity (17, 18). The hydrophobic fraction of licorice root extract is rich in glabridin (GLB), a flavone that exerts an inhibitory effect on tyrosinase activity, thus producing beneficial effects on the skin, while the main constituents of the hydrophilic fraction are glycyrrhizin (GL) and glycyrrhetic acid (GA). A number of triterpenoids, exemplified by GL and GA and isoflavones, primarily represented by GLB, isolated from liquorice root display antioxidant and free radical-scavenging activities. Flavonoids, such as GLB, are recognized as potent antioxidants; GL and

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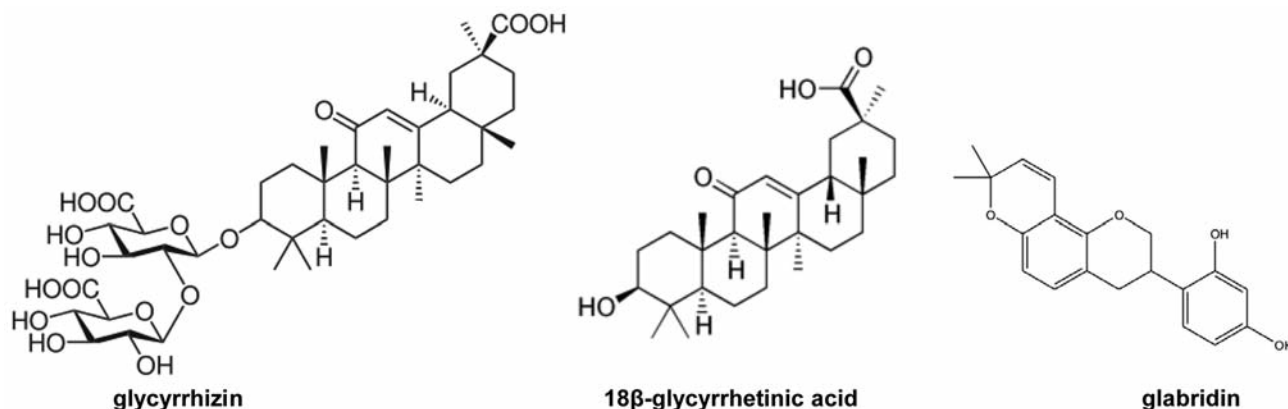


Figure 1. Chemical structure of glycyrrhizin, 18β-glycyrrhetic acid and glabridin.

its aglycone, 18β-GA, have a wide range of pharmacological activities including anti-allergenic, anti-carcinogenic and anti-immune-mediated cytotoxicity (19). On the basis of these activities, the possibility that GL, 18β-GA and GLB can protect human keratinocytes from UVB damage thus acting as protective agents against both SBC formation and skin cancer induction was investigated.

Materials and Methods

Chemicals. GL (Sigma, St. Louis, MO, USA), 18β-GA (Sigma) and GLB (Chromadex Inc, Irvine, CA, USA) were tested (Figure 1). All the substances were dissolved in dimethylsulphoxide (DMSO) (final concentration 0.01%) and the solutions of each compound sterilized by filtration. Final concentrations were obtained in keratinocyte basal medium (KBM, Lonza, Basel, Switzerland).

Cells and cell cultures. Normal human keratinocytes (NHK) were obtained after excision of skin specimens from plastic surgery procedures in accordance with consent procedures approved by the Internal Review Boards of the General Hospital of Modena and Reggio Emilia. NHK cultures were prepared as described by Rheinwald and Green (20). The skin samples were minced and trypsinized (0.25% trypsin, 0.02% EDTA) at 37°C for 30 minutes and the NHK were grown in 75 cm² culture flasks (Costar, Cambridge, MA, USA) with 10 mg/ml mitomycin-treated (Sigma) 3T3 fibroblasts for 2 hours at 37°C. NHK were cultured in Dulbecco's modified Eagle's medium/Ham's F12 medium (DMEM/F12, 3:1) (Seromed-Biochrom KG, Berlin, Germany) containing insulin 5 µg/ml, transferrin 5 µg/ml, triiodothyronine 2 nM, hydrocortisone 0.4 µg/ml, adenine 180 mM, mouse epidermal growth factors (EGF) 10 ng/ml (all from Sigma), 10% foetal calf serum and 100 U/ml penicillin-100 µg/ml streptomycin-amphotericin B (PSA) (Lonza). Sub-confluent primary cultures were transferred to secondary culture in keratinocyte growth medium (KGM; Lonza) containing bovine pituitary extract (BPE; Lonza) until near confluence. Once cell growth was well established, the cells were plated at 10⁴/cm² into disposable 96-multiwell plates (Falcon; Becton Dickinson, Franklin Lakes, NJ, USA) for the MTT test and ROS assay and at 1.5×10⁴/cm² in P60 culture dishes (Costar) for the comet and Western blot tests.

Cell viability assay. Cell viability was measured by the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide test (MTT) according to the method described by Mossman (21). The MTT test was performed in triplicate on 12 and 24 hour cultures of NHK, added with equimolecular increasing concentrations of GL, 18β-GA or GLB (upper concentration: 120 µM). Control cells were maintained in KBM. The reaction was carried out *in situ* in the multiwell plates and the reaction product was measured colorimetrically at 540 nm using a Bio-Rad 550 microplate reader (Bio-Rad, Monaco, Germany). The results were expressed as the percentage of cell viability with respect to the control (plates without test molecules).

UVB treatment. GL (30 µM), 18β-GA (30 µM) or GLB (15 µM) were added to the KBM and NHK were treated with the compounds for 12 hours. After this period, KBM was removed and replaced with PBS+ (with Ca²⁺ and Mg²⁺; Lonza) (22) added with GL, 18β-GA or GLB. The cells were immediately exposed to UVB radiation (50 mJ/cm², 75 mJ/cm² or 100 mJ/cm²) using a UVB TL-20 W/12 RS lamp. After UVB irradiation, PBS+ was removed and the cells provided with fresh KBM added with GL, 18β-GA or GLB and left for an additional 12 hours. Two controls were prepared: the first was UVB irradiated, but without GL, 18β-GA or GLB; the second was with GL, 18β-GA or GLB, but without UVB irradiation. The effects of GL, 18β-GA and GLB after UVB treatment were evaluated by the comet assay, the measurement of intracellular ROS levels and Western blot analysis.

Comet assay. The comet assay was performed as described by Alapetite *et al.* (23). The treated cells were trypsinized and 100 µl of the re-suspended pellet, mixed with a 1% low-melting point agarose, were transferred onto a pre-coated microscope slide. The slide with solidified gel was placed into pH10 lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM TRIS, 1% Triton X-100 and 10% DMSO), at 4°C for at least 1 hour. The slides were then incubated in alkaline unwinding electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) and electrophoresis was performed at 20 V, 300 mA for 30 minutes. The gels were stained with 2 µg/ml ethidium bromide and examined using an Axioskope 40 epifluorescence microscope (Zeiss, Jena, Germany).

Measurement of intracellular ROS levels. The generation of ROS was measured using a ROS-detecting probe, 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA, Invitrogen, Carlsbad, CA, USA) (24).

Briefly, after UVB treatment, the cells were loaded with H2DCFDA for 30 minutes (10 µg/ml in PBS+). The production of intracellular ROS was measured spectrofluorometrically. Fluorescence was quantified with a Shimadzu RF5301 PC spectrofluorophotometer (Shimadzu, Duisburg, Germany), set at an excitation of 485 nm and an emission of 535 nm. The test was carried out in triplicate.

Western blotting assay. Control and treated cells were washed in cold PBS then were lysed in 70 µl of lysis buffer pH 7.4 (50 mM Tris-HCl, 150 mM NaCl, 1% Na deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulphate, 0.2% NaN₃) with protease inhibitor cocktail tablets (Roche, Penzberg, Germany). Equal quantities of protein were separated by SDS polyacrylamide gel electrophoresis (PAGE). The separated proteins were transferred onto a nitrocellulose membrane and blocked with 5% non-fat dry milk in PBS. Red Ponceau was used to verify equal loading of the total protein. The blot was incubated with the following primary antibodies: anti-BCL-2 (Dako, Glostrup, Denmark), anti-poly(ADP-ribose)polymerase (PARP) (Biomol, Hamburg, Germany) and anti-p53 (Calbiochem, Darmstadt, Germany). Subsequently, the blots were incubated with horseradish peroxidase-conjugated goat antimouse IgG (Bio-Rad) at 1:3000 dilution at room temperature. Immunoblotted bands were visualized by an ECL chemiluminescent system (Amersham, Glattbrugg, Switzerland). β-Actin antibody (Sigma) was included as a loading control. Relative density of each band was measured as arbitrary units by MacBiophotonics ImageJ software (MacBiophotonics; Hamilton, ON, Canada).

Statistical analysis. Differences between control and treated samples were determined by Student's *t*-test. Values of $p \leq 0.01$ and $p \leq 0.001$ were considered statistically significant. The results are expressed as the mean value \pm standard deviation (SD).

Results

MTT test. As summarised in Figure 2, the molecules under study were characterised by low cytotoxicity in the NHK cultures since the percentage of cell survival was around 80% when GL, 18β-GA or GLB were added at concentrations lower than 30 µM. In contrast, NHK survival was greatly affected by GLB at higher concentrations (120 µM), while 18β-GA was confirmed as being the most active compound of liquorice root, showing marked cytotoxic effects on the cell cultures at 60 µM both at 12 and 24 hours. No severe effect was observed in the NHK cultures treated with GL.

UVB treatment and DNA damage. On the basis of the results obtained from the MTT test, non-toxic GL, 18β-GA and GLB concentrations were used to evaluate the protective effect on UVB-exposed cells. As in our previous work (25), GL and 18β-GA were used at the concentration of 30 µM, while GLB was added to the culture cells at 15 µM as reported by

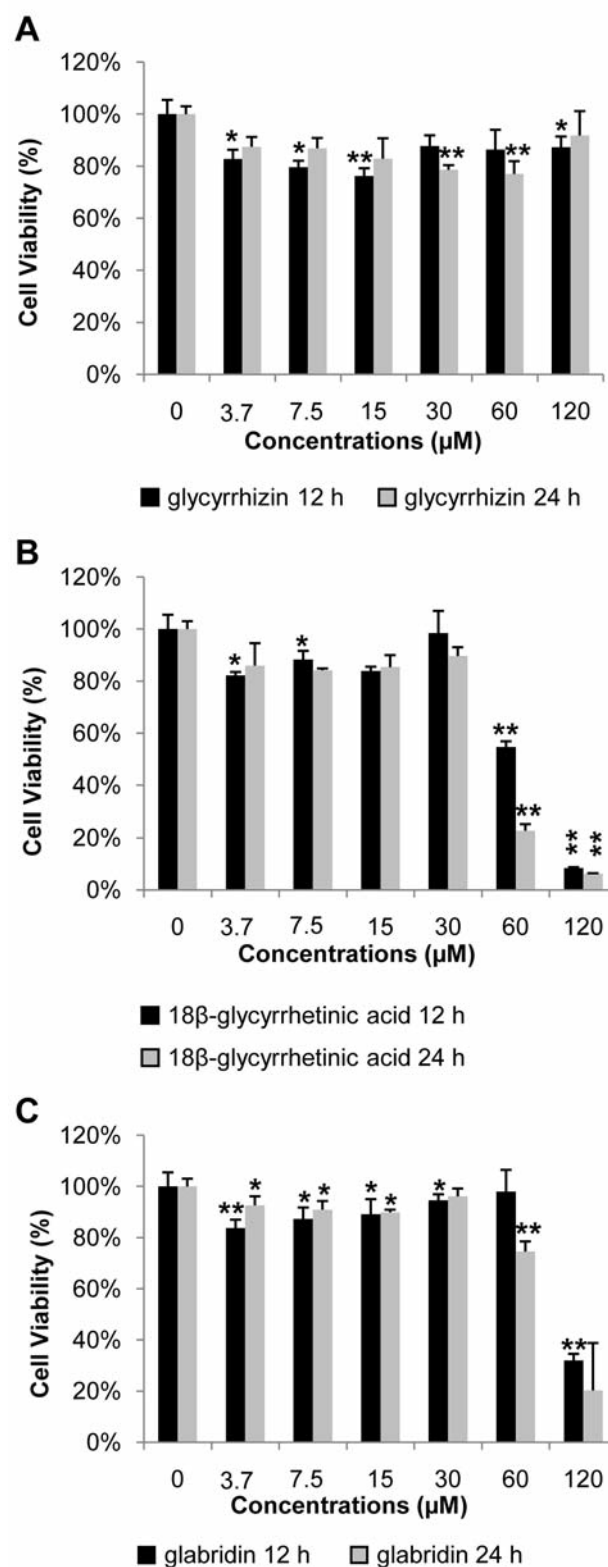


Figure 2. Cell viability assay (MTT) of keratinocyte cultures after glycyrrhizin (A), 18β-glycyrrhetinic acid (B) or glabridin (C) treatment. * $p \leq 0.01$ and ** $p \leq 0.001$ by *t*-test with respect to control (100% growth).

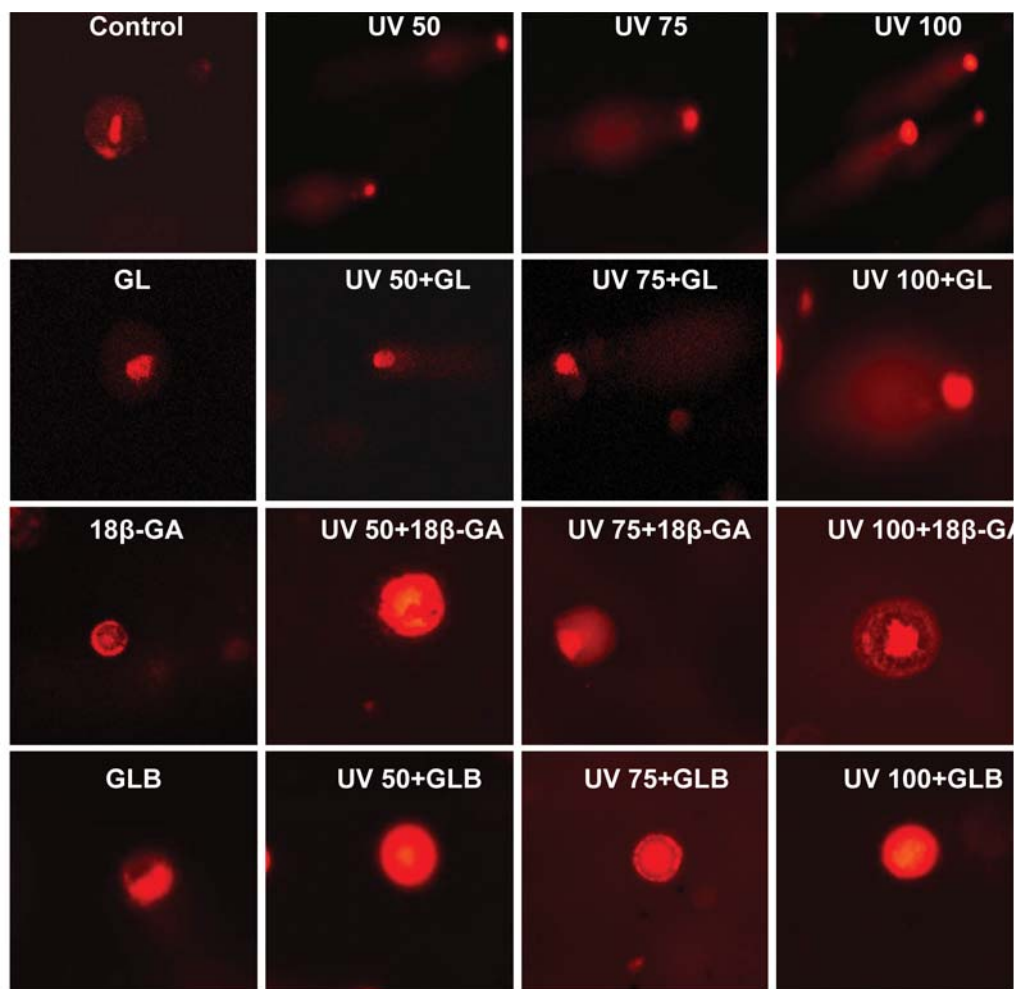


Figure 3. Comet assay of UVB-irradiated keratinocytes in the absence or presence of glycyrrhizin (GL), 18 β -glycyrrhetic acid (18 β -GA) or glabridin (GLB). Images from epifluorescence microscopy. UVB treatment: 50 mJ/cm², 75 mJ/cm², 100 mJ/cm². Untreated cells (control): fluorescence confined to the nucleus. DNA fragmentation: fragments migrate from the nucleus in typical comet tails.

scientific literature (26). The comet assay result is shown in Figure 3; from the first row of the images, it is evident that UVB treatment produced DNA fragmentation and the presence of the tail in the slides of the second row shows that GL treatment was unable to prevent DNA damage. In contrast, in the remaining slides (bottom two rows), the absence of a tail demonstrated that UVB damage was prevented by 18 β -GA at 30 μ M and GLB at 15 μ M.

ROS production. The intracellular antioxidant activity after 24-hour treatment with GL, 18 β -GA or GLB in NHK cultures is shown in Figure 4A, where the ROS production is expressed by optical density (OD) measurement. The most interesting result in comparison with control wells (without treatment) was the significant reduction of intracellular ROS ($p < 0.001$) induced by 18 β -GA. This potent antioxidant activity was confirmed by the subsequent experiments shown in Figure 4B.

As expected, ROS levels increased in a dose-dependent manner after UVB treatment, but when the cells were treated with GLB at 15 μ M, or even more so with 18 β -GA at 30 μ M, the ROS production was significantly reduced. In contrast, GL at 30 μ M seemed to act as an oxidative agent.

Apoptosis in UVB-irradiated NHK. Western blot analysis was employed in the NHKs irradiated with three increasing doses of UVB to investigate the possible antiapoptotic action of GL, 18 β -GA and GLB. The data showed the up-regulation of p53 expression levels after UVB exposure in the control cells, and GL, 18 β -GA and GLB treatment seemed to reduce the UVB-induced p53 levels in the irradiated keratinocytes (Figure 5). Moreover, UVB irradiation was responsible for the down-regulation of BCL-2 (Figure 5) and PARP cleavage (Figure 6). 18 β -GA and GLB treatment in the UVB-irradiated keratinocytes induced a noticeable decrease of bcl-

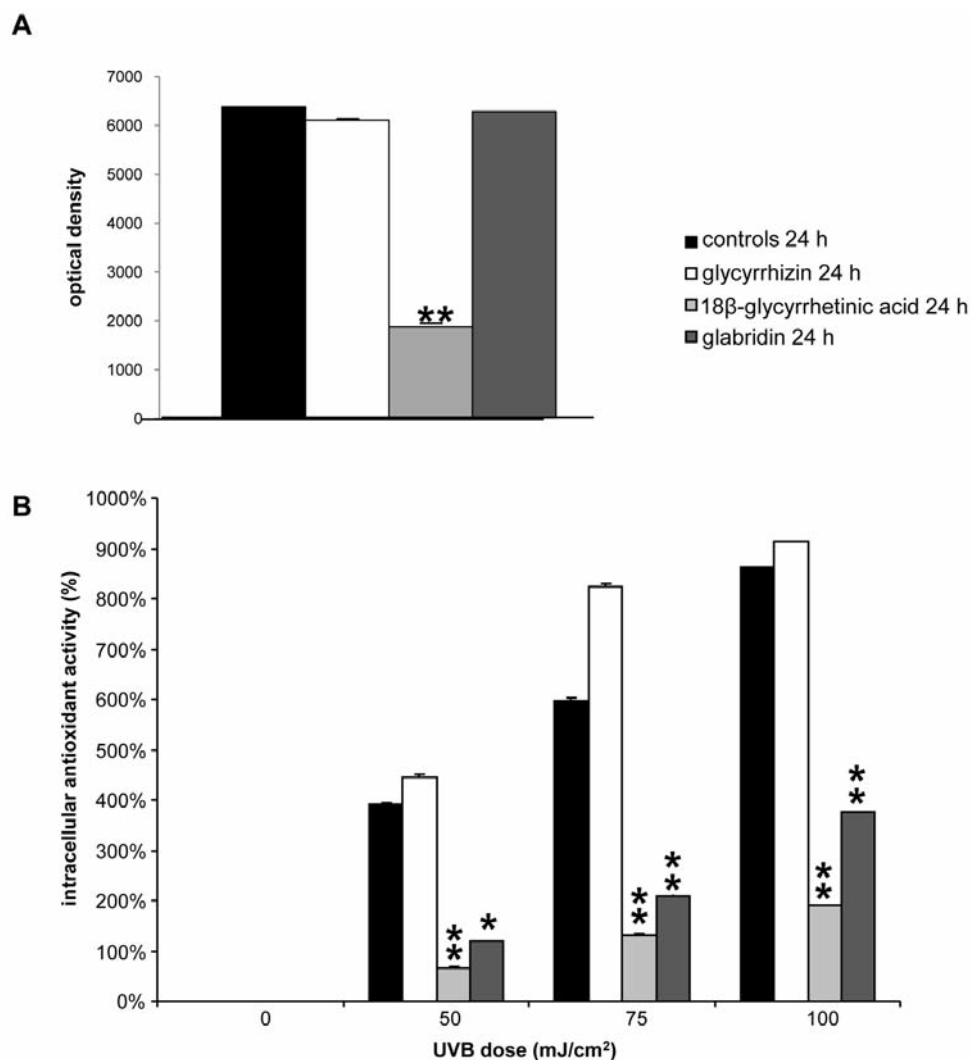


Figure 4. ROS assay. (A) Human keratinocytes treated for 24 hours. (B) Human keratinocytes irradiated (UVB 50 mJ/cm², 75 mJ/cm² or 100 mJ/cm²) for 24 hours and treated with glycyrrhizin 30 μ M, 18 β -glycyrrhetinic acid 30 μ M or glabridin 15 μ M. ROS production expressed as intracellular antioxidant activity. * $p \leq 0.01$, ** $p \leq 0.001$ by t-test with respect to control.

2 down-regulation (Figure 5) in addition to the inhibition of proteolytic cleavage of PARP (Figure 6). These results were not observed in the GL treatment of UVB-irradiated NHK.

Discussion

In this study 18 β -GA and GLB but not GL prevented cell apoptosis thus demonstrating protection of the NHK from DNA damage induced by UVB radiation. The beneficial effects of 18 β -GA and GLB may have been due to the regulation of UVB-mediated ROS production.

18 β -GA was shown to be a potent antioxidant in basal conditions and reduced ROS production after UVB treatment as did GLB.

UVB-induced apoptosis in keratinocytes has been reported to be related to several pathways, including the extrinsic pathway, involving death receptor signalling, and intrinsic pathway involving the mitochondrial cascade (27-29). GA can trigger the proapoptotic pathway by inducing mitochondrial permeability transition, G1 cell cycle arrest and this property may be useful in inducing apoptosis of tumor cells (15, 16).

Solar UV radiation, a major cause of oxidative stress in skin, also influences these pathways. The effects of ROS and solar UVA/UVB on cell signalling in skin may be involved in the pathogenesis of various skin diseases. We demonstrated that GL treatment protects human melanoma cell cultures from UVB-radiation (25). In the present study, after 18 β -GA and GLB treatment (in the presence of UVB)

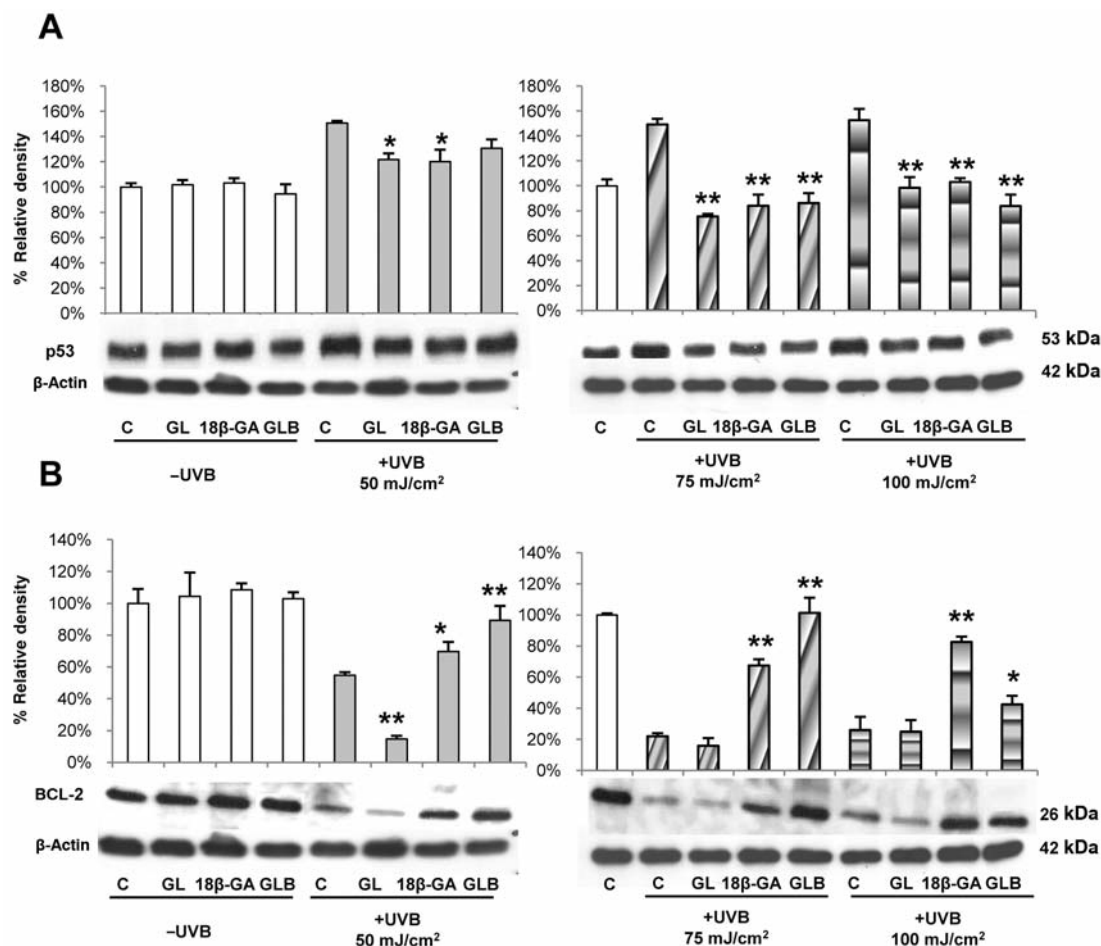


Figure 5. Effect of GL 30 μ M, 18 β -GA 30 μ M and GLB 15 μ M on p53 (A) and bcl-2 (B) expression, by Western blot analysis. Protein levels were determined in NHK in the presence or absence of three different UVB doses. * $p \leq 0.01$; ** $p \leq 0.001$ by t-test with respect to UVB-irradiated control.

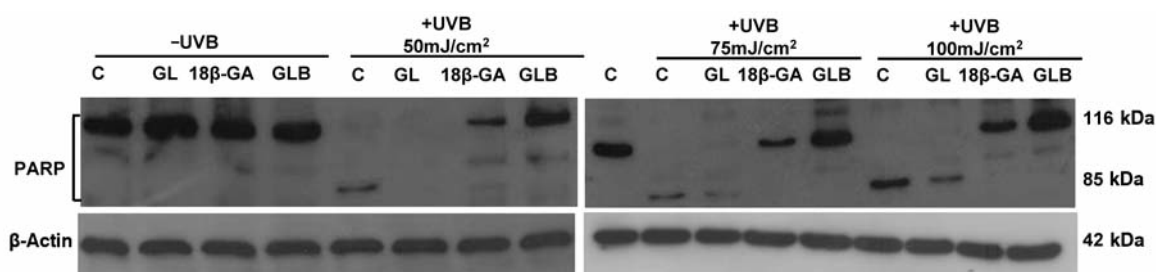


Figure 6. Western blot analysis. PARP cleavage was compared in treated and non-treated NHKs (glycyrrhizin, GL, 30 μ M; 18 β -glycyrrhetinic acid 18 β -GA 30 μ M or glabridin, GLB, 15 μ M) in the presence or absence of three different UVB doses.

p53 activation was inhibited (the activation of p53 represents a key event of apoptosis), BCL-2 protein (a negative regulator of the mitochondrial apoptotic pathway) was up-regulated and PARP cleavage was inhibited (may lead to skin cell damage).

All the effects of 18 β -GA and GLB were observed at non-toxic concentrations, therefore 18 β -GA and GLB must be considered as important natural active principles to include among skin photoprotective agents with promising applications in dermatological clinical research.

Moreover, we observed that the antioxidant action of 18 β -GA and GLB increased with increasing intensity of UVB radiation in a dose-dependent manner, thus exerting its protective effect mainly when the damage was greater.

In conclusion, 18 β -GA and GLB are potent antioxidants, able to prevent oxidative DNA fragmentation and to reduce ROS production and activation of apoptotic pathway proteins in UVB-irradiated NHKs. Furthermore, we hypothesize that the strong antioxidant activity expressed at high UV dose may be due to a photoactivation of 18 β -GA and GLB.

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