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RESEARCH ARTICLE

Apigenin, a Bioactive Flavonoid from *Lycopodium clavatum*, Stimulates Nucleotide Excision Repair Genes to Protect Skin Keratinocytes from Ultraviolet B-Induced Reactive Oxygen Species and DNA Damage

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Abstract
In this study, we examined the antioxidative and the DNA protective potentials of apigenin, a flavonoid polyphenol isolated from *Lycopodium clavatum*, in both *in-vitro* (HaCaT skin keratinocytes) and *in-vivo* (mice) models against UV-B radiation. We used DAPI staining in UV-B-irradiated HaCaT skin keratinocytes pre-treated with and without apigenin to assess DNA damage. We also used a flow-cytometric analysis in mice exposed to UV-B radiation with or without topical application of apigenin to assess, through a comet assay, chromosomal aberrations and quanta from reactive oxygen species (ROS) generation. Data from the stability curves for the Gibb's free energy determined from a melting-temperature profile study indicated that apigenin increased the stability of calf thymus DNA. Immunofluorescence studies revealed that apigenin caused a reduction in the number of cyclobutane pyrimidine dimers (CPDs) after 24 h, the time at which the nucleotide

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excision repair (NER) genes were activated. Thus, apigenin accelerated reversal of UV-B-induced CPDs through up-regulation of NER genes, removal of cyclobutane rings, inhibition of ROS generation, and down-regulation of NF-κB and MAPK, thereby revealing the precise mechanism of DNA repair.

1. Introduction

Exposure to solar ultraviolet (UV) light, especially to the UV-B spectrum (290—320 nm), is a major etiological factor for skin damage. Acute exposure to UV-B causes sunburn, DNA damage, and immune suppression [1]. The molecular pathways leading to UV-B radiation-induced skin and DNA damage generally include the formation of cyclobutane pyrimidine dimers (CPDs), (6-4)-photoproducts, DNA single strand breaks, and double strand breaks (DDBs) [2]. UV-B exposure also causes generation of reactive oxygen species (ROS), which induces damage to cell structure, DNA, proteins, and lipids, resulting in oxidative stress when the antioxidant defense mechanisms of the body are overwhelmed [3].

UV-B-induced skin damage may be prevented by avoidance of intense sun exposure [2] as well as topical and systemic administration of antioxidants to reduce free radical production [4]. The use of natural products showing a wide range of biological activities such as antioxidant, anti-inflammatory, immunomodulatory, and antitumor, is of great interest in UV skin protection. There are several drugs of plant origin containing substantial amounts of alkaloids, glycosides, and flavonoids bearing strong antioxidant properties, which have been described in ancient literature [5]. These drugs have proved to be mostly effective in long-term treatment and so often lose their importance when compared to the faster onset of action of some orthodox medicines. Therefore, not only do these drugs need proper scientific validation, but efforts are also needed to enhance their action and increase their bioavailability to targeted organs/organ system [5].

Lycopodium clavatum (LC), commonly known as "club moss," is a creeping perennial plant found in almost all parts of the world and is considered a great medicinal plant used in many traditional and alternative medicines including homeopathy. The spores are of major medicinal value, and their alcoholic extract is commonly used as homeopathic mother tincture [6]. Recently, we have reported that apigenin is the major flavonoid component present in the crude ethanolic extract of LC [7]. Briefly, homeopathic mother tincture of LC (Boiron laboratories, Lyon, France) was first evaporated; the dried residue was used for column chromatography and eluted by chloroform, chloroform/methanol, and chloroform/acetonitrile. Four fractions (F1—F4) were pooled from 16 fractions, based on thin layer chromatography (TLC). F2 was eluted by chloroform/methanol (9:1) to obtain four subfractions (F2.1—F2.4). These were evaluated by TLC using chloroform/methanol (19:1). F2.2 and F2.3 showed one clear spot each. These two subfractions were pooled, molecular weight was confirmed with electrospray ionization-mass spectrometry (ES-MS), and repeated TLC of these two subfractions yielded characteristic nuclear magnetic resonance ^{1}H and Fourier transform infrared spectroscopic features typical of apigenin. Several lines of evidence from cell culture and animal experiments, as well as epidemiological studies, suggest that flavonoids from plant origin are able to protect human skin from UV radiations [8]. It has been shown earlier that topical application of apigenin inhibits UV-induced tumor formation in a mouse model of skin carcinogenesis [9]. However, it is not yet known if apigenin acts by repairing damaged DNA and/or through any anti-oxidative mechanisms when skin is exposed to UV-B radiation.

Therefore, the hypotheses to be tested in the present study include: (1) whether apigenin extracted from the crude ethanolic extract of LC had any protective effect in human keratinocytes *in vitro* and in a mouse model *in vivo* when exposed to UV-B radiation; (2) if apigenin could render protection to UV-induced DNA damage, and if it did, whether it was mediated through the repair of DNA and reduction of ROS generation; (3) whether apigenin could reduce the formation of CPDs in human keratinocytes (HaCaT) and mice exposed to UV-B radiation; and (5) if it could, then whether it could also repair and retrieve UV-B-induced DNA damage through enhanced activity of nucleotide excision repair (NER) genes; and finally, (5) whether apigenin had any significant antioxidant activity.

2. Materials and methods

2.1. Antibodies and reagents

All chemicals and reagents used were of analytical grade. DAPI (4′,6′-diamidino-2 phenyl indole), MTT [3-(4,5-Dimethyl-thiazol-2-yl)-2, S-diphenyltetrazolium bromide] and all other chemicals used were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Apigenin, anti-NF-kB. anti-p38MAPK, anti-mouse and anti-rabbit secondary antibodies from Santa Cruz Biotechnology, Texas, USA;DMSO (Dimethyl sulphoxide) from Merck Ltd., Mumbai, India;MTT [3-4, 5-Dimethyl-thiazol-2-yl)-2, S-diphenyltetrazolium bromide] and all other chemicals were purchased from Sigma–Aldrich Inc. (St-Louis, MO, USA).

2.2. Stability curve for Gibb's free energy

The melting temperature profile data of CT-DNA and CT-DNA plus apigenin had already been obtained in our previous studies [7]. Changes in the temperature profiles for denaturation of CT-DNA and CT-DNA plus apigenin were deduced from the stability curve by deploying the equation: $y = mx + c$, where m denotes the slope that is equivalent to the equilibrium constant K. Then from this K, changes in Gibbs free energy were calculated from the equation $\delta G = -RT \ln K$, where R is Rydberg's constant

($10.097 \times 10^7\ m^{-1}$) and T denotes the change in temperature (here, $T = 90\ ^\circ C$ for all series). The stability and changes in the temperature for melting of DNA were then ascertained from the equation $\delta G = \delta H - T\delta S$, where δH denotes enthalpy (which was constant for all sets) and δS is entropy.

2.3. Cell culture in vitro

The human keratinocyte cell line HaCaT [procured from the National Centre for Cell Science (NCCS), Pune, India] was cultured in Dulbecco's modified Eagle's medium (DMEM; HiMedia) containing 10% fetal bovine serum (HiMedia Mumbai, India) at 37 °C in a humidified atmosphere with 5% CO_2. DMEM, fetal bovine serum (FBS), penicillin, streptomycin, and neomycin (PSN) antibiotic, trypsin and ethylenediaminetetraaceticacid (EDTA) were purchased from Gibco BRL (Grand Island, NY, USA). In all *in vitro* treatments, apigenin (Santa Cruz Biotechnology, Texas, USA) was dissolved in cell culture medium.

2.4. UV-B exposure of the cells

HaCaT cells were seeded in 96-well plates or 40-mm Petri dishes and grown to 80% confluence. Subsequently, DMEM was replaced with phosphate-buffered saline (PBS) (1X PBS: NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM, pH 7.4), pretreated with apigenin, and incubated for 1 hour. Cells were irradiated from UV-B lamps (TL 20 W/12 RS SLV) through PBS, at a distance of 20 cm. Initially, the UV-B dose kinetics experiment was performed and a dose of 60 mJ/cm^2 was selected for further experiments. Cells were irradiated from UV-B lamps (TL 20 W/12 RS SLV) through PBS, at a distance of 20 cm. Initially, the UV-B dose kinetics experiment was performed, and a dose of 60 mJ/cm^2 was selected for further experiments.

2.5. Animals

A total of 24 healthy inbred strain of Swiss albino mice (*Mus musculus*) purchased from Reeta Ghosh, Kolkata-700092, India (6—8 weeks; ~25 g), housed for at least 14 days in an environmentally controlled room (temperature $24 - 26 \pm 2\ ^\circ C$; humidity, $55 \pm 5\%$, 12-hour light/dark cycle) with access to food and water *ad libitum*, served as materials for the present study. The experiments were approved by the Animal Ethics Committee, University of Kalyani, (Vide: Certificate for Proposal No. KU/IAEC/Z-11/07, dated 18.5.2007.) and conducted under the supervision of the Animal Welfare Committee, Department of Zoology, University of Kalyani. For each fixation interval, six mice were used for each series. Mice were randomized and subdivided into the following subsets: Group 1, untreated control; Group 2, UV-B irradiated group; drug administered subgroups of mice—Group 3, UV-B + AP1 (1.5 mg/cm^2); Group 4, UV-B + AP2 (3 mg/cm^2).

2.6. UV-B irradiation of mice

Mice were irradiated with UV-B, as previously described [10]. Briefly, the shaved dorsal skin of mice were exposed to UV-B radiation from a UV-B lamp (TL 20 W/12 RS SLV; Philips, (Kolkata, India)) emitting between 290 nm and 315 nm, at a dose of 240 mJ/cm^2. The narrow waveband of the lamp is between 305 nm and 315 nm with its peak at 315 nm, which is the most solicited waveband for studying skin diseases.

2.7. Cell viability assay in vitro

HaCaT cells (80—90%) were treated with either of two concentrations (15 and 25 μg/mL) of apigenin or vehicle alone (media) that served as a control. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed to determine the percentage of viable cells following the protocol of Samadder et al [11]. Because apigenin was earlier shown not to have any influence on MTT reduction [12], we opted for the MTT assay. Briefly, cells were seeded in 96-well microtiter plates (Axygen, Union city, CA, USA). A 10-μL MTT solution Sigma Aldrich Inc. (St. Louis, MO, USA) was added to each well and incubated for 4 hours at 37 °C. To achieve solubilization of the formazan crystals formed in viable cells, the medium was discarded and 100 μL dimethylsulfoxide (Merck, Mumbai, India) was added to each well. Optical density was measured at 595 nm (Multiskan ELISA; Thermo Scientific, Waltham, USA). The absorbance correlates linearly to the number of living cells in culture.

The dorsal skin of different groups of mice was exposed to UV-B (240 mJ/cm^2) with or without pretreatment of skin with apigenin (1, 1.5, 3, and 3.5 mg/cm^2), sacrificed at 24 hours after UV-B irradiation. The skin was removed aseptically and perfused in DMEM. This was centrifuged at 500× *g* for 2 minutes. The intact and homogeneous population of skin cells in the supernatant was collected, then incubated at 37 °C, and the MTT assay was performed as described above.

2.8. DNA damage assay by fluorescence microscopy

The extent of apoptosis in untreated control, UV-B-treated (60 mJ/cm^2), and apigenin pretreated cells 1 hour before UV-B irradiation was checked after 24 hours of irradiation by staining with 4′,6-diamidino-2-phenylindole (DAPI; 10 mg/mL), and the extent of fluorescence was assessed and visualized under fluorescence microscope (Leica, Wetzlar, Germany).

2.9. Comet assay

Skin cells were isolated from different groups of experimental mice, and the extent of DNA damage in different groups was determined using single cell gel electrophoresis [11]. Briefly, after 24 hours of UV-B irradiation, skin cells from mice were isolated in Hanks' balanced salt solution (Sigma Aldrich Inc., St. Louis, MO, USA), mixed with an equal amount of 1% low melting agarose (HiMedia, Mumbai, India), and kept at 37 °C. One hundred microliters of the suspension was pipetted onto the frosted slide, covered with cover slip and placed on a glass tray on ice. Then the slides were immersed in cold lysis solution followed by incubation at 4 °C for 1 hour. Electrophoresis was performed in weak alkali at 1 V/cm and 30 mA for 15 minutes preceded by a 20-minute immersion of the slides in electrophoresis buffer to promote chromatin unwinding.

After electrophoresis, the slides were neutralized in 0.05 M Tris buffer. Then, DNA was stained with ethidium bromide (50 μg/mL) (SRL India, Mumbai, India) for 10 minutes, washed in distilled water, and examined under a fluorescence microscope (Leica, Wetzlar, Germany).

2.10. Cytogenetical parameters

Standard cytogenetical protocols for chromosome aberrations (CAs) and micronuclei (MN) were followed [13] to analyze the changes in chromosome morphology in untreated control, UV-B-treated, and apigenin-pretreated experimental sets of mice. Briefly, mice were injected with 0.03% colchicine Sigma Aldrich Inc. (St. Louis, MO, USA) intraperitoneally at the rate of 1 mL per 100 g of body weight 1 hour and 30 minutes before they were sacrificed. Bone marrow cells were collected from the femur, flushed in 1% sodium citrate (hypotonic) solution (Merck, Mumbai, India), brought into suspension by repeated flushing with a pipette, and incubated for 7—10 minutes at 37 °C. The materials were centrifuged for 6—8 minutes at 700× *g*, and the supernatant was discarded. By centrifugation and decantation, the fixative was changed twice at an interval of 20 minutes. Materials were dropped with the aid of a pipette from a distance of 20 cm on clean grease-free slides prechilled in 50% alcohol. The slides were air-dried and the alcohol and fixative were allowed to burn out. Images for CAs were taken by staining with Hoechst (10 mg/mL) Sigma Aldrich Inc. (St. Louis, MO, USA) and visualized under a fluorescence microscope (Motic Image, Xiamen, China).

2.11. Immunofluorescence

To visualize CPD *in vitro*, HaCaT cells were incubated with 25 μg/mL apigenin for 1 hour before UV-B irradiation. The effect of apigenin on the reduction of CPD formation was assessed at 1, 8, 16, and 24 hours after UV-B irradiation, and the immunofluorescence study was performed according to the manufacturer's instructions [Oxiselect Cellular UV-Induced DNA Damage Staining Kit (CPD), Cell Biolabs, San Diego, CA, USA.]. Images were taken under a fluorescence microscope (Motic Image, Xiamen, China.).

Different sets of experimental mice with or without pretreatment of apigenin were UV-B exposed and sacrificed after 24 hours. Skin tissues from different groups were collected and fixed in 10% normal buffered formalin (for 1 litre: formalin 100 ml, H_20 900 ml, NaH_2PO_4 4 g, Na_2HPO_4 6.5 g) and dehydrated in ascending concentrations of ethanol, cleared in xylene (Merck, Mumbai, India), and embedded in paraffin (Merck, Mumbai, India) to prepare the block. Tissues were sectioned into 6 μm, and immunofluorescence staining against CPD antibody was done according to the manufacturer's instructions (Cell Biolabs, San Diego, CA, USA).

2.12. Quantitative analysis of expression of NER genes using reverse transcriptase polymerase chain reaction

Total RNA was extracted from isolated skin cells of mice using Trizol reagent, according to the manufacturer's instructions, and then converted to cDNA by reverse transcriptase (RT). This cDNA served as the template for polymerase chain reaction (PCR) amplification by *Taq* polymerase, as per the standard practice [14]. Sequences (Bioserve Biotech, Hyderabad, India) of the primers used for specific amplifications were as follows: G3PDH (FP 5′-ATGGGGAAGGTGAAGGTCGG-3′ and RP 5′-GGATGCTAAGCAGTTGGT-3′); XPA (FP 5′-TTCCCTTCCTACGGAGTGTG-3′ and RP 5′-AGCCACCTTTGGTGTCAATC-3′); DDB2 (FP 5′-CTCCTCAATGGAGGGAACAA-3′ and RP 5′-CAATCACAGCATGGGTTC-3′); XPC (FP 5′-GAGGAGAGTGAGAGCGATGG and RP 5′-TGGGTCTTGGAAGCACTCTT-3′). After PCR amplification, the DNA bands were photographed and densitometrically analyzed using Image J software (Madison, Wisconsin, USA.).

2.13. Intracellular ROS generation

Generation of ROS in isolated skin cells from different groups of experimental mice was quantified. After 24 hours of UV-B irradiation, skin cells were washed with PBS and further incubated with 10 μM cell permeable fluorescent probe, 2′,7′-dichloro-dihydrofluorescein diacetate acetyl ester (H_2DCF-DA) for 30 minutes at 37 °C in the dark. After incubation, cells were washed twice in PBS at room temperature for 5 minutes each. The fluorescence measured at excitation and emission wavelengths of the oxidized form at 504 and 529 nm, respectively, and was analyzed in FACS (BD Bioscience, San Jose, CA, USA).

2.14. Analyses of protein expression by immunoblotting

Different sets of experimental mice were UV-B irradiated and sacrificed after 24 hours. To analyze the expressions of different proteins, immunoblot was conducted. The isolated mice skin pieces were homogenized and centrifuged at 500× *g* for 45 minutes at 4 °C. The supernatant was collected, and the protein concentration was measured using bovine serum albumin (BSA) as a standard. Aliquots of equal amounts protein (40 μg) were loaded, and samples were denatured in 12% SDS—PAGE for nuclear factor kappa-B (NF-κB), p38 mitogen-activated protein kinase (MAPK), and β-actin. The separated proteins were transferred separately onto polyvinylidene fluoride (PVDF) membranes and were probed with antimouse NF-κB (1:500) and antimouse p38 MAPK (1:1000) primary antibodies overnight at 4 °C followed by a 1-hour incubation with alkaline phosphatase-conjugated secondary antibody (1:500; Santa Cruz Biotechnology, Texas, USA) [7]. For the quantitative analysis of each band, band intensities were determined using the Total Lab software (Ultra Lum, Berlin, USA).

2.15. Statistical analysis

All experimental data are presented as the mean values of three independent experiments and statistically analyzed by Student *t* test and one-way analysis of variance using SPSS 14 software (SPSS Inc., Chicago, IL, USA). A probability of 0.05 or less was considered significant.

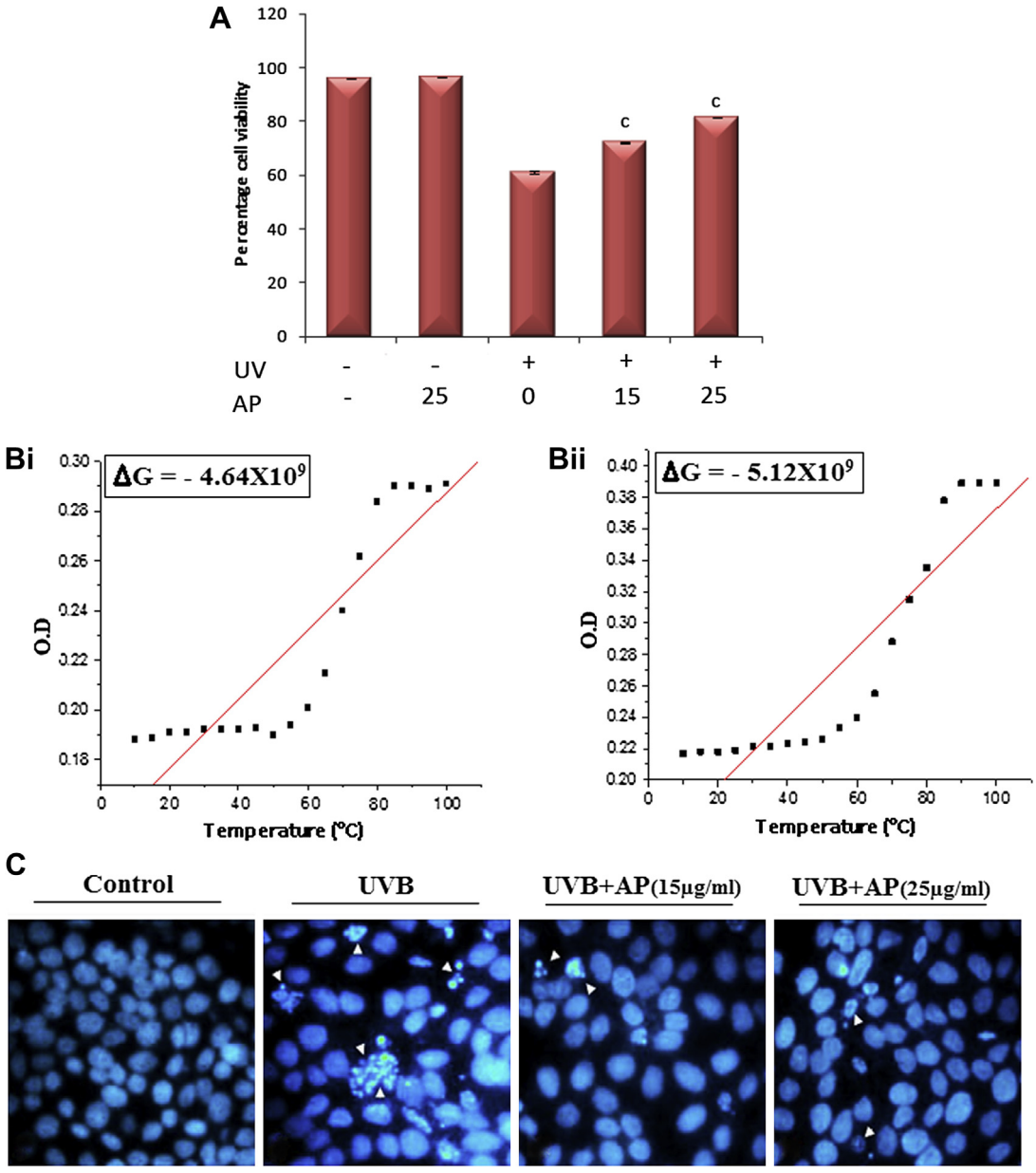

Figure 1 (A) Graphical representation of cell viability assessment in HaCaT keratinocytes. The difference between apigenin (AP)-treated groups versus the non-AP-treated UV-B exposed control was determined, and a probability of 0.05 or less was considered statistically significant. (B) The stability curve for Gibbs free energy in relation to temperature for denaturation of (i) CT-DNA and (ii) CT-DNA in the presence of apigenin. (C) Fluorescence microscopic analysis of UV-B-induced DNA damage in HaCaT keratinocytes by 4′,6-diamidino-2-phenylindole (DAPI) staining.

3. Results

3.1. Cell viability assay in vitro

Treatment of HaCaT cells with UV-B resulted in a significant reduction in percentage of viable cells (61.3%) compared with non-UV-B irradiated cells (95.7%). Cells pretreated with apigenin (15 and 25 μg/mL) caused a dose-dependent increase in the percentage of viable cells (72.1% and 81.5%, respectively) (Fig. 1A).

From the results of a pilot study of cell viability assay (data not shown), the two doses of apigenin, 1.5 (AP1) and 3.5 mg/cm^2 (AP2), were selected to be used for further *in vivo* experiments.

3.2. Stability of apigenin—CT-DNA interaction by evaluation of Gibb's free energy

The results of the stability curve for determination of melting temperature/temperature for denaturation of DNA, revealed that the δG value calculated for CT-

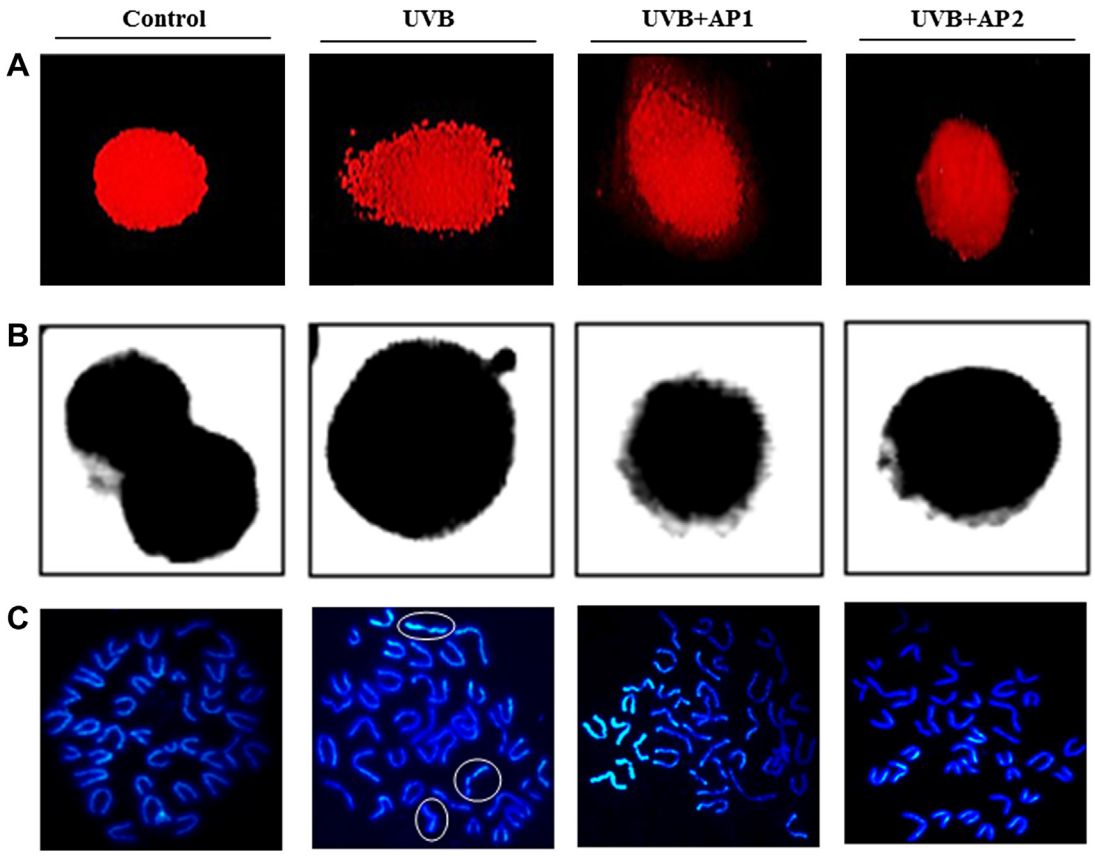

Figure 2 (A) DNA damage assessment by comet assay in different groups of experimental mice. (B) Chromosomal study of bone marrow smears showing normal/micronucleated erythrocytes (×200 magnification). (C) Chromosomal studies of metaphase complements in different groups of mice (×1000 magnification).

DNA + apigenin (-5.12×10^9) was more negative than that of the δG value calculated for CT-DNA alone (-4.64×10^9). The proportionality values were found to indicate that the entropy (δS) for CT-DNA + apigenin was more positive than that of CT-DNA alone, which would suggest that introduction of apigenin to CT-DNA brought an increase in the melting temperature of the naked DNA, thereby bringing about a more stable conformation in its structure as compared to that of CT-DNA alone.

3.3. Effect of apigenin against DNA damage

Apigenin was found to have a protective effect against UV-B-induced nuclear DNA damage in HaCaT cells in a dose-dependent manner when compared to untreated control (Fig. 1C).

The results of the comet assay showed that there were distinguishable changes in comet structure (decrease in tail length) in AP1- and AP2-treated mice compared to UV-B-treated ones (showing increased tail length) (Fig. 2A).

3.4. Cytogenetical parameters

We observed various types of aberrations (CA) with chromosome breaks, stretching, and MN in UV-B exposed groups (Fig. 2B and 2C). However, much less incidence of CA and MN was found in AP1 and AP2 pretreated groups, indicating suppression of CA and MN. Percentages of CN, MN, mitotic index,

Table 1 The differences between the apigenin (AP)-treated groups versus the non-AP-treated ultraviolet (UV)-B-exposed control.

Group	% of micronucleus	% of chromosomal aberrations	Mitotic index	Comet assay	
				% of normal cells	% of damaged cells
Control	0.113 ± 0.066	0.006 ± 0.006	0.313 ± 0.066	91.8 ± 1.4	7.9 ± 0.4
UV-B	0.604 ± 0.063	0.106 ± 0.016	0.343 ± 0.110	64.5 ± 1.9*	33.9 ± 1.4*
AP (15 μg/mL)	0.375 ± 0.105*	0.044 ± 0.012*	0.388 ± 0.066	74.0 ± 2.3*	29.0 ± 1.9*
AP (25 μg/mL)	0.250 ± 0.108*	0.025 ± 0.010*	0.310 ± 0.013	84.5 ± 1.7*	14.6 ± 0.4*

All data are presented as mean values of three independent experiments. All data were presented as mean values of three independent experiments. A probability of 0.05 or less (*$p < 0.05$) was considered statistically significant.

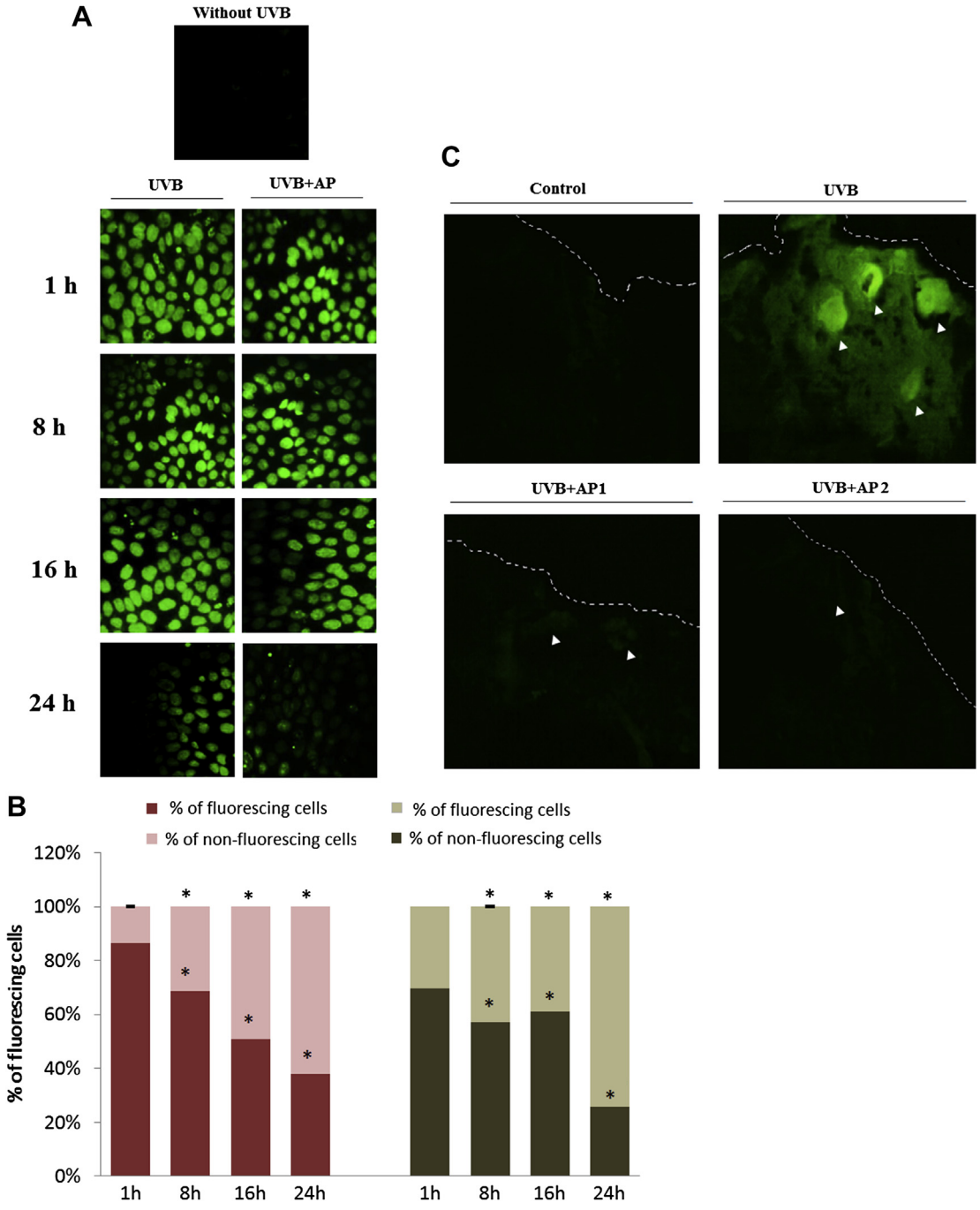

Figure 3 (A) Immunofluorescence determination of formation of cyclobutane pyrimidine dimmers (CPDs) at different time intervals after UV-B irradiation in HaCaT keratinocytes. (B) Graphical representation of percentage of fluorescing and nonfluorescing HaCaT cells at each period. All data were presented as mean values of three independent experiments. A probability of 0.05 or less was considered statistically significant. (C) Immunofluorescence studies of CPD formation in mice skin tissues in different groups of experimental mice.

and percentage of normal and damaged cells (increased comet tail length) in each group are shown in Table 1.

3.5. Inhibition of CPD formation by apigenin

CPD formation was found to have significantly reduced at 24 hours after UV-B induction as compared to UV-B induced apigenin untreated cells as shown in Fig. 3A. Percentages of fluorescing and nonfluorescing cells have been quantified as shown in Fig. 3B.

Mice skin cells pretreated with AP1 and AP2 showed decrease in green fluorescence in apigenin-treated groups in a dose-dependent manner (Fig. 3C) when compared with untreated normal and UV-B irradiated ones.

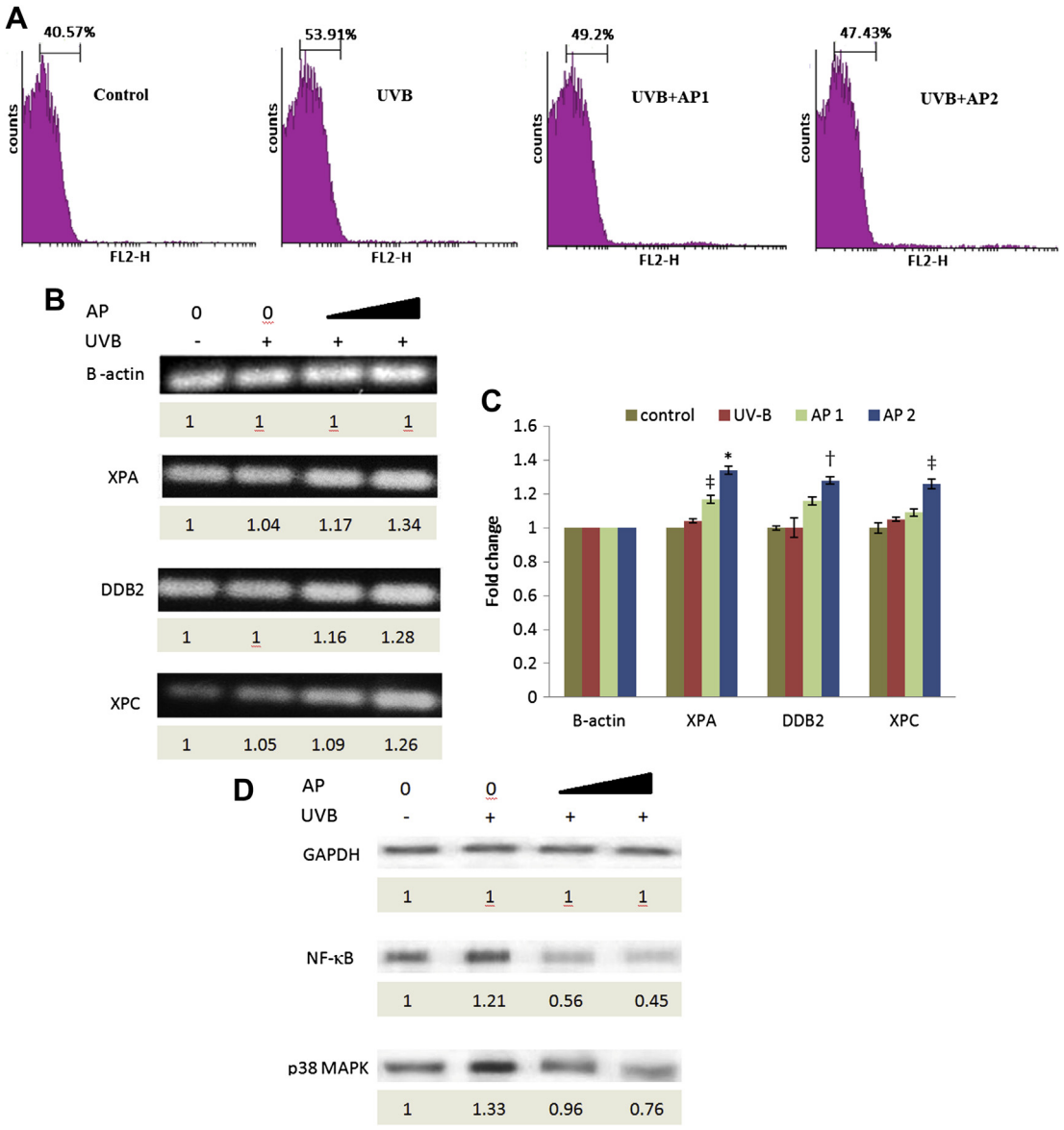

Figure 4 (A) Flow cytometric analysis of generation of reactive oxygen species (ROS). (B) Expression of mRNA of *G3PDH* (housekeeping gene), XPA, DDB, and XPC in different sets of experimental mice skin tissues. (C) Graphical representation of quantitative expression of mRNA fold change. All data were presented as mean values of three independent experiments. $*p < 0.001$, $\dagger p < 0.05$, and $\ddagger p < 0.01$ versus UV-B-treated group was considered significant. (D) Expression levels of B actin, NF-κB, and mitogen-activated protein kinase (MAPK) by immunoblot analysis in different sets of experimental mice skin tissues. Band intensities were expressed as mean of their relative density normalized to G3PDH (for RT-PCR) and b-actin (for immunoblot). The intensity of the control was normalized to 1.

3.6. Modulation of antioxidant activity of apigenin by inhibition of ROS

Pretreatment of UV-B irradiated mice skin cells pretreated with AP1 and AP2 significantly reduced UV-B induced ROS generation in a dose-dependent manner as shown in Fig. 4A.

3.7. Gene expression analysis (RT-PCR)

Results of RT-PCR revealed that NER genes *XPA*, *DDB2*, and *XPC* were overexpressed in AP1- and AP2-treated groups when compared to untreated control and UV-B-treated groups (Fig. 4B and 4C).

3.8. Immunoblot analysis

A decrease in NF-κB and p38 MAPK expression was also observed in AP1- and AP2-pretreated groups when compared to untreated control and UV-B-treated groups (Fig. 4D).

4. Discussion

UV light interacts with many types of cellular macromolecules, leading to the formation of photoproducts following energy absorption. Our study has shown that apigenin, a flavonoid from *L. clavatum*, protects keratinocytes and mice

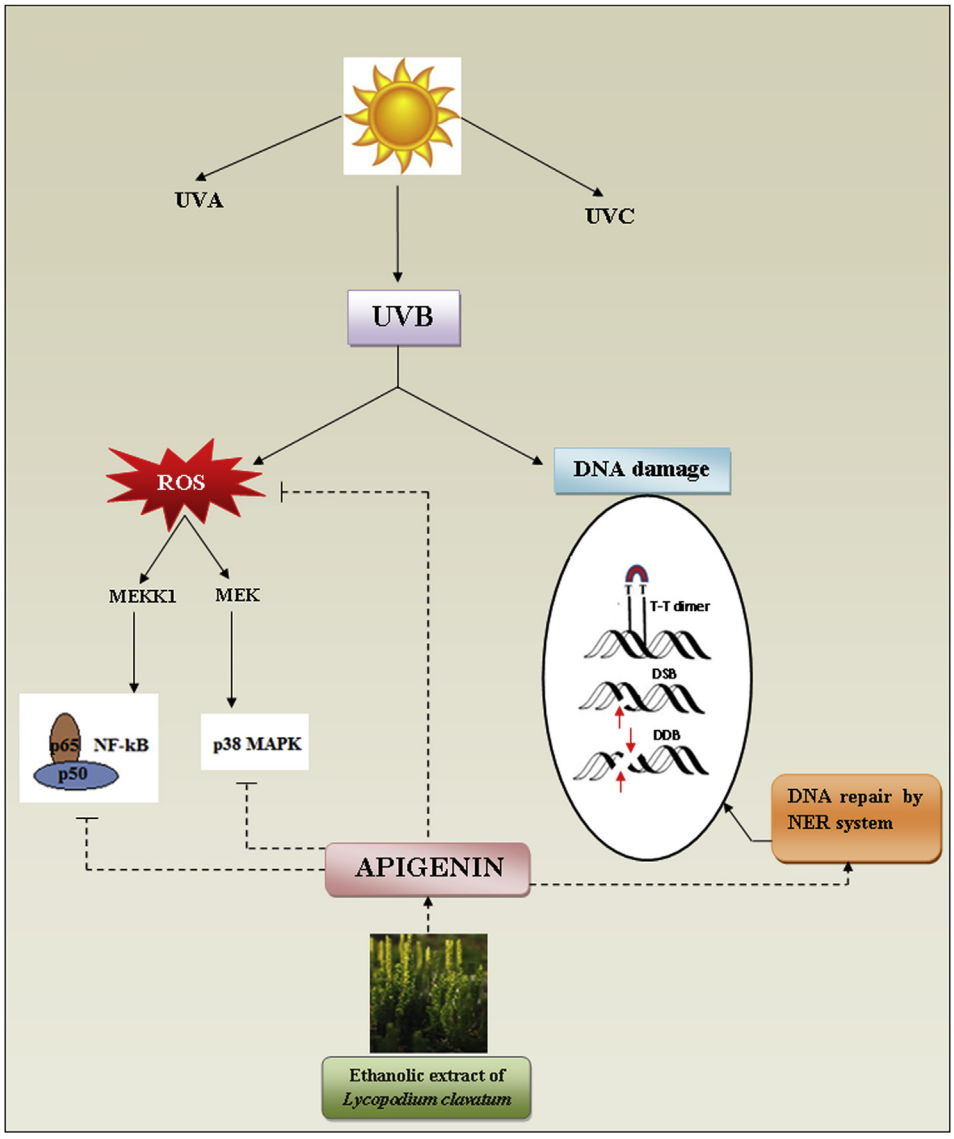

Figure 5 Schematic summary of skin-protecting properties of apigenin.

skin tissues from UV-B-induced DNA damage *in vitro* and *in vivo*, respectively (Fig. 5). Several modifications of DNA, such as CPDs, normally get removed by photoreactivation and NER systems. In addition to direct damage to DNA and proteins, perturbations of the cellular redox equilibrium also occur as a result of release of free radicals following UV-B irradiation [15] subjecting the cells to oxidative stress, inflammation, and especially DNA damage. To cope with the deleterious effects of UV-B radiation, both plants and animals are equipped with various protective molecules (e.g., antioxidants such as ascorbic acid [16] and proteins involved in DNA repair and detoxification of ROS). However, this endogenous protective system may be overwhelmed by prolonged and repeated UV exposure; therefore, additional protection may be required. The results of this study show that apigenin could significantly enhance the repair of UV-B-induced damaged DNA and CPD formation *in vitro* and *in vivo*, mainly through acceleration of the NER repair system. This correlates with the findings of Romanova et al [17], who investigated the DNA-protective effect of flavonoids such as luteolin and quercetin, *in vitro*. Our findings also revealed that apigenin could effectively reduce UV-B-induced oxidative stress in UV-B exposed mice skin cells, which was consistent with the findings of Seelinger et al [18].

We showed for the first time that binding of apigenin with CT-DNA increases the stability of DNA in terms of its entropy (δS). This would suggest that apigenin possibly plays an active role in the direct repair action on UV-B-induced damaged DNA.

CAs in living organisms are one of the most important biological consequences of exposure to UV radiation and other genotoxic agents. UV radiation causes DNA stretching and DNA breaks [19]. Although most of the earlier studies used oral or injection route for the drug treatment, we showed for the first time that even topical application of apigenin in UV-B irradiated mice could

significantly reduce the extent of CA and MN. Changes in comet structure with longer tail lengths were observed in the comet assay after UV-B treatment, whereas comet tail lengths were significantly shortened in apigenin pretreated groups. These findings are in agreement with the earlier reports where apigenin was found to reduce DNA damage and cytogenetic alterations caused by gamma radiation [19].

UV-B radiation generally induces DNA damage through the formation of CPDs in mammalian cells. Usually, CPD formation initiates from 1 hour after UV-B exposure. Therefore, we determined whether apigenin could prevent the immediate formation of CPDs. Our results revealed that no clear differences in the number of CPDs were observed in non-apigenin and apigenin pretreated UV-B exposed groups until 16 hours, but there were discernible changes at 24 hours showing reduced CPDs. This suggests that apigenin as such does not show preventive action on immediate CPD formation. Generally, the NER system is the major mechanism of DNA repair in mammalian cells that usually comes into action within 24 hours after UV-B irradiation. So we questioned whether the repair of UV-B induced CPDs by apigenin at 24 hours was mediated via induction and acceleration of NER. Our quantitative mRNA expression analysis of NER genes (*XPA*, *XPC*, *DDB2*) using the RT-PCR method revealed that apigenin-treated cells could significantly enhance the expression levels of NER genes in UV-B exposed mice, as compared to non-apigenin-treated mice, and this may have contributed to the rapid repair of damaged DNA in mice.

UV irradiation leads to the formation of ROS, which results in the subsequent activation of complex signaling pathways including NF-κB and p38 MAPK pathways [20]. We investigated if the protective effect of apigenin was through the attenuation of the oxidative stress induced by UV-B exposure in mice and tracked down the possible signaling pathways involved. The increase in generation of ROS on UV-B exposure to mice skin indicated ROS generation to be a critical event in the consequences of UV-B irradiation. We observed that topical application of apigenin was able to reduce ROS generation in UV-B-induced mice skin tissues in a dose-dependent manner, thereby showing a strong and effective antioxidant activity. The results were consistent with previous reports that demonstrated apigenin to have antioxidant activity in the skin of the carcinogenic mouse model [9].

NF-κB is one of the most ubiquitous nuclear transcription factors, commonly activated by agents that generate ROS such as UV radiation. Saliou et al [21] observed that antioxidants are able to induce UV-induced NF-κB activation in human keratinocytes. Oxidant production also contributes to the activation of p38 MAPK. Therefore, the signaling pathways involved in the regulation of NF-κB and p38 MAPK activities have become a focal point of drug discovery efforts. We observed that the topical application of apigenin attenuated NF-κB and p38 MAPK expressions in UV-B exposed mice. This was in agreement with earlier studies that had reported that apigenin was able to inhibit cell migration through MAPK pathways in human smooth muscle cells [22] and also inhibited NF-κB-dependent transcriptional activity in macrophage cells [23].

In summary, the present data suggest that apigenin protects human keratinocytes and mice skin tissues from the deleterious effects of UV-B radiation by adopting DNA protective measures, by reducing the formation of CPDs through the stimulation of NER genes and by antioxidative properties inhibiting the generation of ROS and interfering with NF-κB and MAPK pathways. These findings have important implications for identifying the precise mechanism by which apigenin enhances DNA repair in UV-B-exposed skin and therefore may also contribute in the process of suitable drug design for prevention of UV-induced skin tumor.

Disclosure statement

The author affirms there are no conflicts of interest and the author has no financial interest related to the material of this manuscript.

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