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Saponins from *Tribulus terrestris* L. protect human keratinocytes from UVB-induced damage

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ABSTRACT

Chronic exposure to solar UVB radiation damages skin, increasing the risk to develop cancer. Hence the identification of compounds with a photoprotective efficacy is essential. This study examined the role of saponins derived from *Tribulus terrestris* L. (TT) on the modulation of apoptosis in normal human keratinocytes (NHEK) exposed to physiological doses of UVB and to evaluate their antitumoral properties. In NHEK, TT saponins attenuate UVB-induced programmed cell death through inhibition of intrinsic apoptotic pathway. In squamous cell carcinomas (SCC) TT saponins do not make the malignant keratinocytes more resistant to UVB and determine an enhanced apoptotic response. The photoprotective effect of TT saponins is tightly correlated to the enhancement of NER genes expression and the block of UVB-mediated NF-κB activation. Collectively, our study shows experimental evidence that TT has a preventive efficacy against UVB-induced carcinogenesis and the molecular knowledge on the mechanisms through which TT saponins regulate cell death suggests great potential for TT to be developed into a new medicine for cancer patients.

1. Introduction

There is a growing scientific interest in herbal medicine, and the promising results obtained in herbal anti-cancer therapy have stimulated further research into the medicinal use of plants. Plant saponins are widespread distributed and have been commonly employed in medical practice for their potential health benefits. Saponins, the active components in *Tribulus terrestris* L. (referred hereafter as TT), have long been used for their androgenic activity, but new evidence has shown that saponins also have a cytostatic activity against cancer cells [1–3]. Nevertheless, the anti-cancer potential of TT saponins in humans is still largely unstudied.

The B portion of ultraviolet (UV) light has long been recognized as the most prominent risk factor for the development of skin cancer, one of the commonest malignancies in the Caucasian population. At the cellular level, UVB signal transduction regulates the expression of genes involved in DNA repair and, when the damage is beyond repair, provokes apoptotic cell death to protect the host against the accumulation of potentially mutagenic keratinocytes [4,5]. Thus an understanding of the molecular mechanisms underlying the process of apoptotic cell death in UVB-exposed keratinocytes, is of the outmost importance to reveal defects in apoptotic pathways that can contribute to skin cancer. The regulation of the apoptotic response seems to be mediated by transcription factors such as AP-1, NF-kB, and p53, activated after UVB exposure [6–8]. It is now well-established that the disruption of apoptosis can foster premature aging of the skin, melanoma and non-melanoma skin cancers [9] and therefore, since apoptotic programs can be manipulated to produce massive changes in cell death, the genes and proteins controlling apoptosis are potential drug targets in cancer treatment [10].

In this context, the aim of the current research was to investigate the effect of TT saponins on the modulation of apoptosis induced in normal human epidermal keratinocytes (NHEK) and in malignant squamous cell carcinoma (SCC) by a physiological UVB dose, to individuate TT saponins' anti-cancer properties and to unveil, at least in part, the molecular mechanisms underlying the role of cell death in the progression of skin tumours.

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2. Materials and methods

2.1. Plant materials and purification of saponins

T. terrestris L. full plants including roots and fruits, were harvested in Italy, in different sites of Salento peninsula in July 2010. Plants were authenticated by Dr. Di Sansebastiano, from the Department of Biological and Environmental Sciences and Technologies, University of Salento, Italy.

Fruits were separated from plants, frozen in liquid nitrogen and lyophilised in a Christ Alpha 2-4 LSC freeze-dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). The lyophilised material was ground in a laboratory mill (Retsch GmbH, Haan, Germany) to obtain a homogeneous powder. Saponins and other soluble molecules were extracted with 70% ethanol. Saponins were precipitated adding two volumes of cold acetone and centrifuging at 20,000*g* for 30 min. The pellet, corresponding to about 2% of the starting material, was air dried for 30 min and suspended in distilled water to produce a final concentration of 2 g/ml TT extract. The TT extract was used at concentration of 50 μg/ml, that causes any toxicity to cells, in all experimental procedure (data not shown).

2.2. Cell culture and UVB irradiation

Human epithelial keratinocytes derived from normal skin tissue (NHEK) were purchased from Clonetics (Charlotte, NC, USA). The cells were grown in Minimum Essential Medium (MEM) (SIGMA, MO, USA) supplemented with 10% (v/v) foetal bovine serum (SIGMA), 1% (v/v) antibiotic solution (100 U/ml of penicillin, 100 μg/ml streptomycin) (SIGMA), 2 mM L-Glutamine (SIGMA), 1% (v/v) non essential aminoacid solution (SIGMA). The SCC (A253 cell line), derived from a head and neck squamous cell carcinoma of submandibular gland, were grown in DMEM enriched with 10% fetal calf serum and antibiotics. All cells were grown in incubator at 37 °C in 5% CO_2. The sub-confluent cells (70%) were treated with 50 μg/ml of saponins from TT prior to the UVB exposure.

For UVB-irradiation, NHEK and SCC were irradiated with UVB (312 nm; range, 0–400 mJ/cm^2) using UVB generator (Philips TL20W12, Anderlecht, Belgium) at a distance of 30 cm from the light source for 30 min. To minimize absorption of the radiation by the medium, a thin layer of phosphate-buffered saline (about 1.0 mm) was left above the cells during UVB exposure. The cells were exposed through the cover of the dish which filters out residual UVC.

2.3. Cell viability assay

NHEK and SCC were plated in 6-well plates at 1×10^6 cells/well, with opportune media and cultivated to sub-confluence (70–80%). NHEK and SCC were UVB exposed and TT was added at concentration of 50 μg/ml using the cultivating media as a solvent. Cells were incubated with TT for 24 h. Viability was monitored using the Trypan Blue Exclusion Test. The numbers of stained and unstained cells were counted with a hemocytometer. At least 500 cells were counted per experiment time point. The percentage of viability represents the ratio of unstained cells to total cells.

2.4. Apoptosis assessment

2.4.1. Fluorescent microscopy analysis of keratinocytes apoptosis

To detect apoptotic cells, the Vibrant Apoptosis Assay kit (Molecular Probes, Inc., OR, USA) was employed. This assay uses the green fluorescent non permanent nuclear dye YO-PRO-1 that is able to enter apoptotic cells. The details of experimental procedure are reported previously [11].

2.4.2. DNA fragmentation

Degradation of the NHEK and SCC DNA was used as an index of apoptosis. The description of the technique is reported in a previous work [11].

2.4.3. Caspases activation analysis

The enzymatic activity of caspase-3 in lysates of NHEK and SCC cells UVB-irradiated and TT saponins-treated was evaluated using the CaspACE™ colorimetric assay system (Promega Corporation, WI, USA) as reported previously [11].

The caspase-8 and -9 enzymatic activities were evaluated using specific colorimetric protease assay kits (BioSource International, Inc., CA, USA, as described previously [12].

2.4.4. Western blot analysis

Protein lysates, obtained from variously treated cells were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Membranes were incubated for 90 min with goat anti-caspase-3 pAb, rabbit anti-caspase-9, rabbit anti-caspase-8 (all pAbs from Santa Cruz Biotechnology) and with the relative secondary antibodies-HRP conjugate (Santa Cruz Biotechnology). Proteins recognized by the antibodies were revealed using the chemiluminescence luminal reagent (Santa Cruz Biotechnology) according to the protocol. β-actin was used as protein loading control. For the cytochrome c quantification, mitochondrial and cytosolic proteins were isolated using the cytochrome c Releasing Apoptosis Assay kit (Abcam, Cambridge UK) according to the manufacturer's instructions. Equal amounts of proteins (20 μg) were separated by 12% SDS–PAGE. The membranes were incubated with mouse anti-cytochrome c monoclonal antibody (1 μg/ml) and mouse anti-COXIV monoclonal antibody (0.5 mg/ml). An HRP-labeled anti-mouse was used as secondary antibody. The membranes incubated with a HRP-conjugated monoclonal anti-β-actin served as the control.

2.4.5. Analyses of nucleotide excision repair (NER) genes expression

Total RNA was isolated from NHEK and SCC cells using the TRI reagent (Sigma). 2 μg of RNA were treated with DNase I (GIBCO, Life Technologies, Carlsbad, CA, USA), reverse transcripted and used for each PCR. Analysis of genes expression was done using Real-Time PCR. The NHEK and the SCC were subjected to UVB exposure and/or TT saponins treatment. At time points cellular RNA was extracted and the mRNA expression of XPA, XPC NER genes, was determined. β-2 microglobulin (part no: 4326319E; β2M) (Applied Biosystems) was used as internal control gene. Real-Time quantitative PCR was performed with an ABI PRISM 7700 (Applied Biosystems). The threshold was determined as 10 times the SD of the baseline fluorescence signal. The cycle number at the threshold was used as the threshold cycle (Ct).

2.4.6. Immunodetection of thymine dimers

Genomic DNA of trypsinized cells was purified using a genomic DNA extraction kit (Qiagen, Crawley, UK). Polyvinylchloride 96-well flat-bottomed multiwell plates, precoated with 0.003% protamine sulfate, were coated with equal amounts (200 ng) of sample DNA. Using specific anti-monoclonal anti-thymine dimer antibody (Abcam) and ELISA assay was performed to quantify thymine dimers spectrometrically.

2.5. Assessment of NF-κB-p65 nuclear translocation

NHEK and SCC cells were harvested after irradiation and/or TT treatment. Cells were then lysed using the Compartmental Protein

Extraction Kit (Millipore, Billerica, MA, USA), to yield the purification and preparation of proteins from cytoplasmic and nuclear fraction. Samples were separated by 10% SDS–PAGE and proteins transferred to a nitrocellulose membrane (Amersham-Pharmacia, Amersham, UK) by electroblotting. Membranes were incubated for 1 h with anti-human NF-κB p65 subunit antibody (Santa Cruz Biotechnology) and the related secondary antibody. Membranes were incubated with ECL substrate (Amersham-Pharmacia, UK), followed by exposure to an autoradiographic film and subsequent semi-quantification of band intensity by densitometry.

2.6. Evaluation of NF-κB activity

Nuclear fractions were harvested from NHEK and SCC cells using the nuclear extraction kit following the manufacturer's suggestions (Active Motif, Carlsbad, CA, USA). The protein concentration was determined using the Bradford's method. NF-B activity was measured in nuclear protein extracts by the TransAM NF-κB p65 protein assay (Active Motif), an ELISA-based method designed to specifically detect and quantify NF-κB p65 subunit activation, with high sensitivity and reproducibility. The assay was performed according to the manufacturer's protocol and the absorbance was measured at 450 nm by the VERSAmax microplate reader (Molecular Devices Corp, Silicon Valley, CA, USA).

2.7. Statistics

Differences in means for paired observations were analyzed by student's *t*-test. Values of $p < 0.05$ were considered as statistically significant.

3. Results

3.1. T. terrestris L. saponins increase the cell viability of NHEK under UVB irradiation

NHEK were treated 1 h before and immediately after UVB irradiation with TT saponins (50 μg/ml). This low, non toxic dose of TT saponins significantly increased their resistance to UVB irradiation, as shown by significantly higher cell viability (Fig. 1A) up to 72 h after irradiation. The microscope images, shown in Fig. 1B, illustrates the decreased sensitivity of TT-treated cells (c) to a UVB dose that was lethal to the control cells (b).

3.2. TT saponins inhibit the apoptotic response of NHEK

UVB irradiation triggers apoptosis in NHEK within 24 h. As shown in Fig. 2A and B treatment with TT saponins before and immediately after irradiation with UVB inhibited UVB-induced cleavage of caspase-3. These data were confirmed and quantified by immunofluorescence analysis of YOPRO-1 stained NHEK (Fig. 2C and D), showing that the percentage of apoptosis in UVB-irradiated cells (87.9 ± 0.7) was significantly higher ($p < 0.01$) than the percentage of apoptotic cells observed in TT-treated cells (25.5 ± 0.7). In addition, UVB induce DNA fragmentation in NHEK, whereas this is prevented by TT treatment. No DNA fragmentation was observed in untreated control cells (Fig. 2E).

3.3. TT saponins modulate the intrinsic apoptotic pathway in NHEK

We investigated the effect of TT saponins treatment on caspase-8 and caspase-9 activation in apoptosis induced by UVB in NHEK. To understand which of the two major pro-apoptotic signalling pathways triggered by UVB irradiation, intrinsic and extrinsic, is sensitive to the effect of TT saponins, NHEK, treated or not with TT saponins, were exposed to UVB light, and the activities of caspase-8 and -9 were evaluated using fluorogenic substrates and western blotting (Fig. 3). As shown in panels A and B, caspase-9 activity was more strongly increased than caspase-8 activity after UVB irradiation, suggesting that the intrinsic pathway plays a predominant role in UVB-induced apoptosis, and that TT saponins treatment modulates this pathway but does not interfere with activation of the extrinsic apoptosis pathway. To confirm that mitochondria-dependent apoptosis was involved in UVB irradiation of NHEK and that TT saponins have a protective effect against UVB damage, we detected mitochondrial and cytosolic proteins by

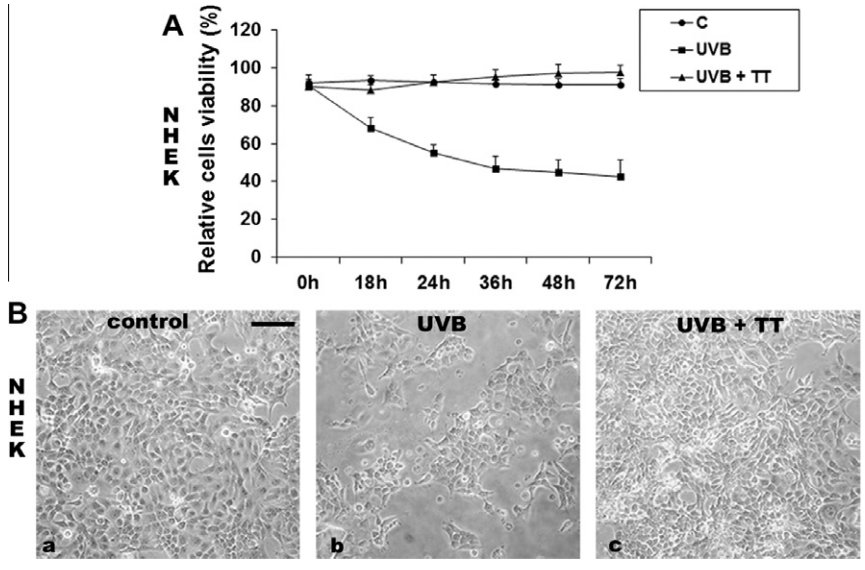

Fig. 1. Saponins from *T. terrestris* L. Significantly increased cell viability of UVB irradiated-human keratinocytes. (A) A time-dependent Trypan blue analysis was performed to evaluate the viability of NHEK irradiated with UVB (312 nm; range, 0e400 mJ/cm^2) after TT saponins treatment (50 μg/ml) (experiment performed twice in triplicate). Viability is presented as a percentage of living cells over total cells counted. (B) Microscopic observations of UVB-irradiated NHEK after TT saponins treatment. In standard conditions cultured NHEK show a typical epithelial morphology when confluent (a); after UVB exposure, the NHEK morphology changed and a progressive detachment of cells from wells was observed (b). In UVB-irradiated NHEK TT saponins treatment seemed to help NHEK preserve the typical epithelial morphology (c). (Scale bar = 10 μm, magnification 10×).

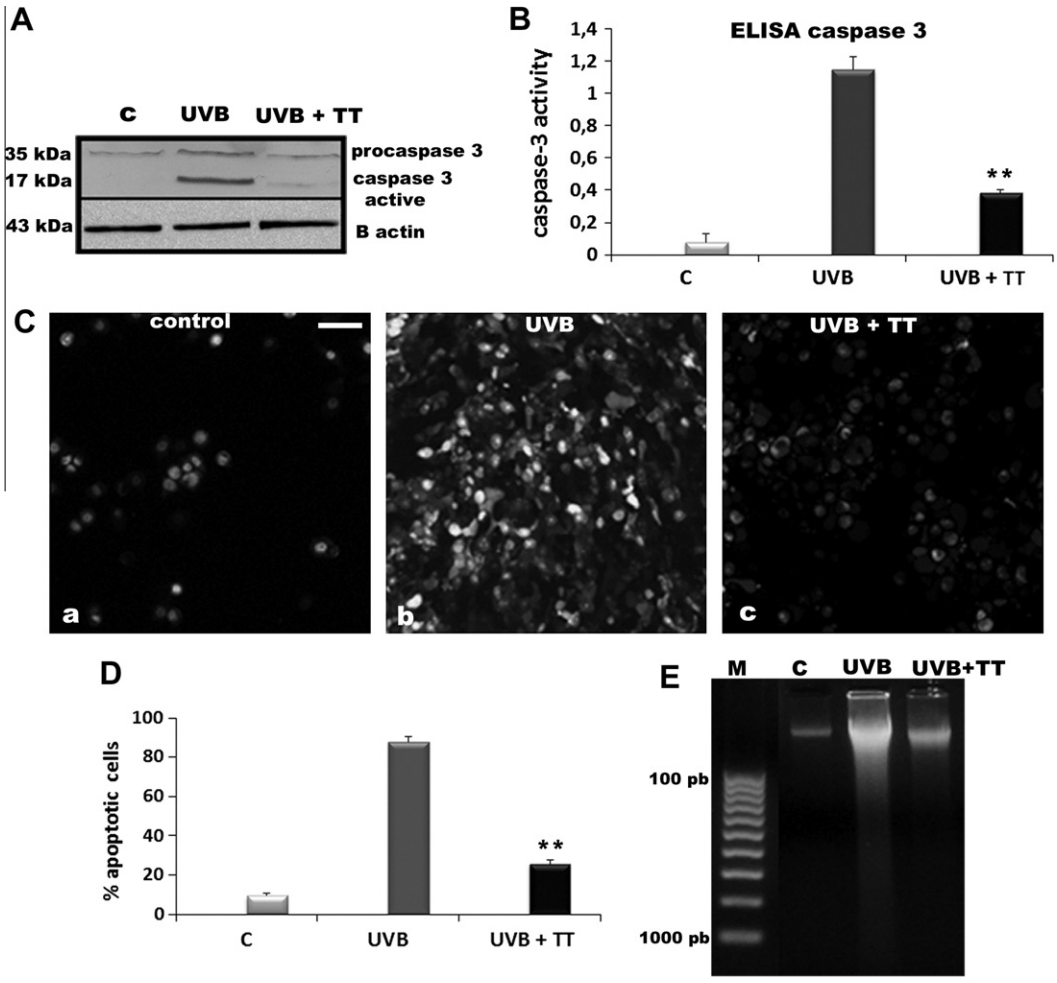

Fig. 2. TT saponins decreased apoptotic response in UVB-irradiated NHEK. Expression of caspase-3 activation was determined by Western blot (A) in UVB-irradiated NHEK after TT saponins treatment. β-actin was used as the loading control. (B) ELISA of Caspase-3 activity in NHEK similarly treated. Untreated cells (C) were used as controls. The results are expressed as arbitrary units. (** = $p < 0.01$). (C) YOPRO-1 staining of apoptosis in UVB-irradiated TT saponins-treated NHEK. (D) The percentage of apoptotic cells was assessed by fluorescence microscopy using the YOPRO-1 assay kit. (Means ± SE of four independent experiments). (** = $p < 0.01$). (E) NHEK were assayed for DNA fragmentation induced by UVB and inhibited by TT saponins treatment.

western blotting. COXIV, a mitochondrial protein whose localization does not change on disruption of the mitochondrial outer membrane, was used to determine the purity of the cytoplasmic fraction and as a control for equal mitochondrial protein loading. β-actin was used as the cytosolic loading control. Western blot analysis (Fig. 3, panel C) showed that TT saponins inhibited the release of cytochrome c after UVB irradiation. Compared to the untreated control cells, leakage of the caspase-9 activator cytochrome c from the mitochondria to the cytosol increased after UVB radiation of NHEK, while TT saponins decreased the release of cytochrome c from the mitochondria.

3.4. Saponins from TT influence the survival of UVB-exposed SCC

We then investigated the effect of TT saponins on apoptosis observed in UVB-exposed SCC. Assessing cellular viability (Fig. 4A), we found a decreased amount of UVB-irradiated malignant viable cells under TT saponins treatment in comparison with treatment with medium alone. In order to determine the time-dependent cell death induced by TT saponins (50 μg/ml), SCC were UVB irradiated and monitored for 18–72 h, and the Trypan Blue Exclusion Test was performed. As shown, the anti-viability effects of TT saponins occurred in a treatment time-dependent manner and, after UVB irradiation, SCC viability decreased at different times of incubation. A classical laddering pattern of internucleosomal DNA fragmentation was observed in UVB-irradiated cancer cells after TT saponins treatment, indicating that irreversible death had been induced by TT saponins treatment (Fig. 4B). YOPRO-1 staining demonstrated that a large increase in the percentage of cells taking up the DNA dye was observed in UVB-irradiated malignant cells treated with TT saponins in comparison with UVB-irradiated cells (Fig. 4C and D). The expression of pro-caspase-3 in UVB-irradiated SCC was reduced after TT-saponins treatment, with the accumulation of its cleaved active 17 kDa subunit, data confirmed by enzymatic activity analysis. These findings indicate that under UVB irradiation, the TT saponins-induced apoptosis is caspase-3-dependent. Furthermore, ELISA assays and western blot analysis of caspases-8, -9 showed that only caspase-9 is markedly activated in TT saponins-treated SCC irradiated with UVB in comparison with cells exposed to UVB alone (Fig. 4E and F). The above results suggest that these plant extracts possess an anti-tumor potential, affecting UVB-induced apoptotic intrinsic pathway in malignant SCC.

3.5. TT saponins differentially regulate the levels of NER genes in UVB-exposed NHEK and SCC

To determine the protective effect of TT saponins against UVB radiation-induced cell DNA damage in NHEK, we assessed whether

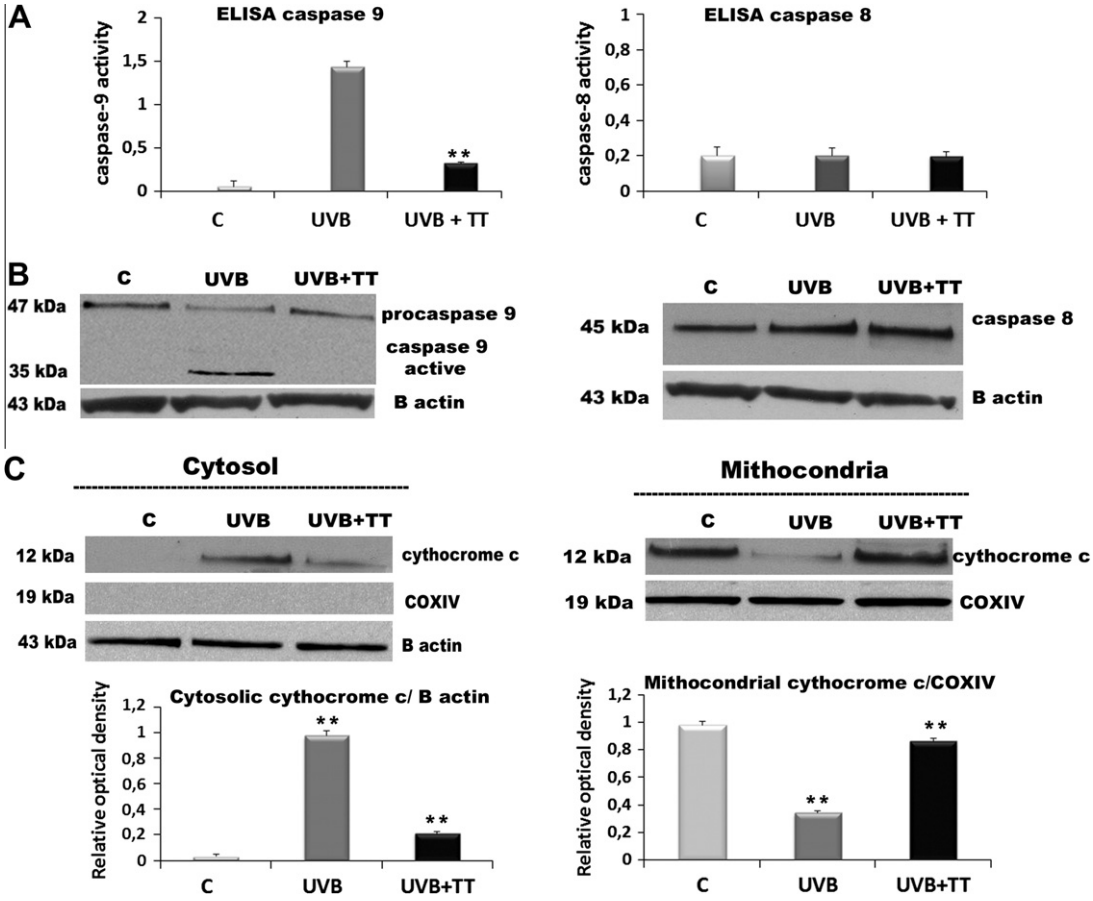

Fig. 3. TT saponins protect NHEK through the apoptotic intrinsic pathway inhibition. (A) The enzymatic activity of caspase-8 and caspase-9 was measured in lysates of UVB-irradiated NHEK after TT saponins treatment. Untreated cells (C) were used as controls. The *y*-axes shows the amount of free p-nitroaniline (pNA) released from the caspase-8 specific substrate IETD-pNA and the amount of free p-nitroaniline (pNA) released from the caspase-9 specific substrate LEHD-pNA. The results are expressed in arbitrary units; (** = $p < 0.01$). (B) Cell lysates of UVB-irradiated TT saponins-treated NHEK were separated by SDS–PAGE and transferred to a nitrocellulose membrane for western blot analysis. Blots were probed with rabbit anti-caspase-9 (1:500) and rabbit anti-caspase-8 (1:500). β-actin was used as protein loading control. (C) Western blot analysis of cytochrome c distribution. Densitometric analysis was performed and COXIV and β-actin detection were used to determine the purity of the cytoplasmic fraction and to control for equal protein loading. (** = $p < 0.01$).

NER genes were activated to repair damaged DNA. To evaluate this hypothesis, NHEK were exposed to UVB and treated or not with TT saponins. We analyzed the expression of NER genes mRNA (XPA, XPC) using real-time PCR, since XPA and XPC are key genes in the NER pathway [13]. UVB exposure of the NHEK increased the levels of NER genes, significantly as compared to non-UVB-exposed control NHEK ($p < 0.05$). As shown in Fig. 5, panel A, TT saponins treatment determined an highly significant enhancement of the expression of the XPA and XPC mRNA levels ($p < 0.01$) in the UVB-exposed TT saponins treated NHEK as compared with UVB-exposed untreated NHEK and, in accordance with our expectations, the protective effect of TT saponins against UVB irradiation resulted in a selective DNA damage response. Analyzing the expression of XPA and XPC genes in UVB-irradiated TT saponins-treated SCC, we demonstrated that the expression of XPA and XPC mRNA remained unchanged in TT saponins-treated UVB-irradiated SCC as compared with only UVB-irradiated SCC (0.876 ± 0.021 versus 0.861 ± 0.011 for XPA and 0.760 ± 0.012 versus 0.775 ± 0.009 for XPC). The levels of the XPA and XPC mRNA expression resulted slightly decreased but not significantly altered in SCC following UVB irradiation alone in comparison with non-irradiated SCC (Fig. 5A). This novel finding that TT saponins increase the abundance of NER transcripts in UVB-irradiated healthy keratinocytes, but not in malignant transformed cells, highlights a possible anti-tumor property of these plant extracts suggesting, indeed, that they may have a cancer preventive action.

3.6. TT saponins prevent the thymine dimers formation in UVB-exposed NHEK

As direct DNA damage is one of the triggers leading to apoptosis, we checked the effect of TT saponins on the induction and repair of thymine dimers after UVB irradiation (Fig. 5, panel B). In UVB irradiated TT saponins-treated NHEK a large part of the thymine dimers was removed in comparison with UVB-irradiated NHEK. UVB exposure of SCC resulted in an increased detection of thymine dimers. Application of TT saponins to UVB exposure, determined a more marked increase of thymine dimers production. These data suggest that the preventive efficacy of TT saponins against UVB-caused tumoral transformation could be, in part, via an inhibition in UVB-induced DNA damage.

3.7. Inhibition of NF-κB activity by TT saponins protects from carcinogenesis

We specifically compared the effects of TT saponins treatment on NF-κB activity in UVB-irradiated NHEK and SCC. To determine whether treatment with TT saponins induced an inhibition of cytoplasm-to-nucleus translocation of NF-κB in UVB-irradiated NHEK, western blotting was used to specifically measure the amount of p65 subunit protein in the cytoplasmic and nuclear fractions (Fig. 6). We demonstrated an inhibition of the NF-κB pathway

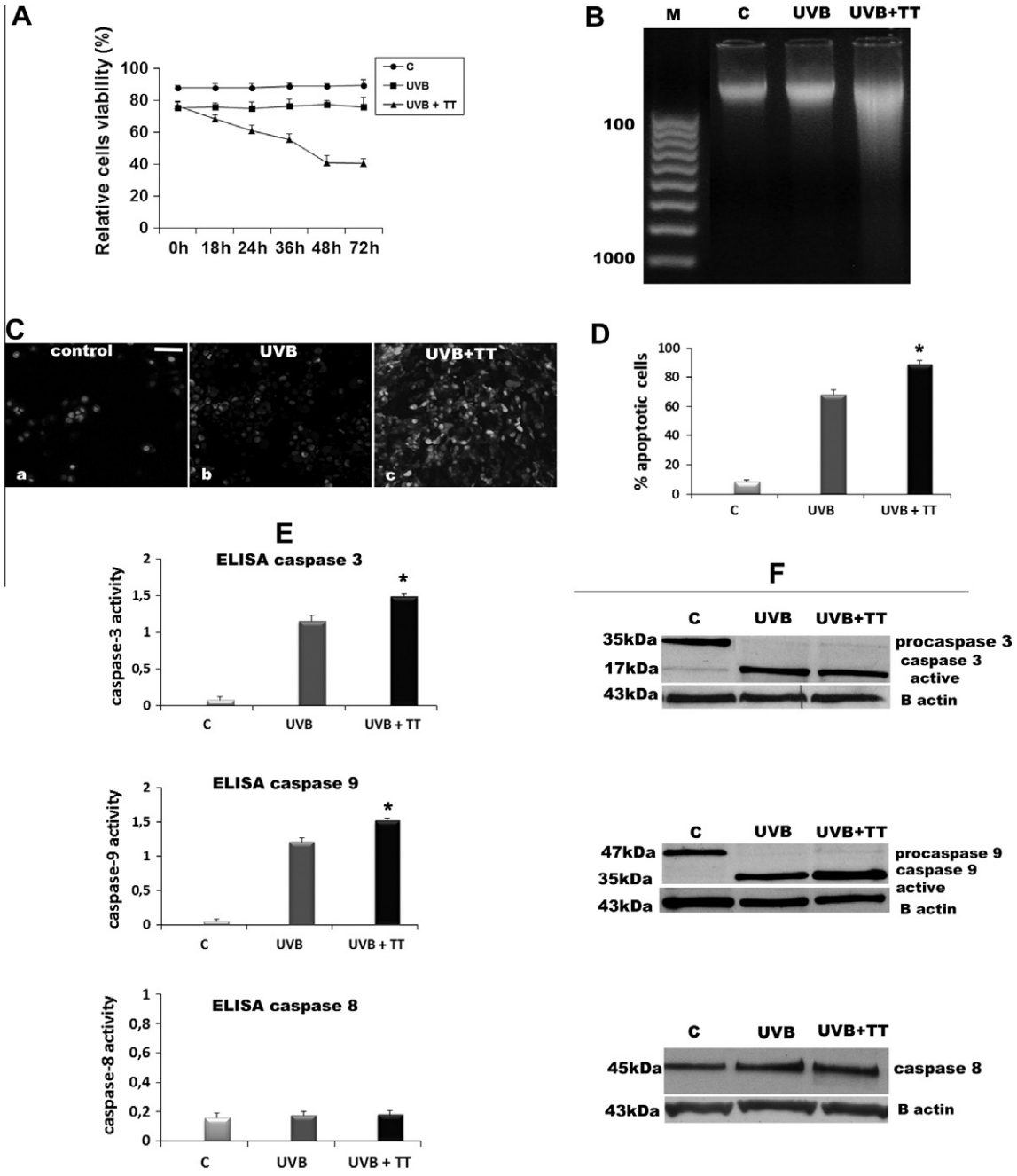

Fig. 4. TT saponins induce apoptosis in squamous cell carcinoma. (A) SCC viability was determined after the UVB exposure and TT saponins treatment using trypan blue stain for various periods of time as indicated in graph. Cell viability was quantified as percent viable cells. (B) Illustration of DNA fragmentation induced by TT saponins in UVB-irradiated SCC. (C and D): YOPRO-1 staining in UVB-irradiated TT saponins-treated SCC and percentage of apoptotic cells assessed by fluorescence microscopy. (Means ± SE of four independent experiments). (∗ = $p < 0.05$). (E) Enzymatic activities of caspases-3,-8,-9 in UVB-irradiated TT saponins treated SCC. (∗ = $p < 0.05$). In the figure, C represents untreated control cells. (Means ± SE of four independent experiments). (F): UVB-irradiated TT saponins-treated SCC lysates were assayed by Western blotting for caspases-3, -8,-9 activation and β-actin expression.

activation in UVB-irradiated TT saponins-treated NHEK and in malignant SCC, similarly stimulated.

As shown in Fig. 6A, of the cytoplasmic fraction that corresponds to inactive NF-κB, one major band was detected at 65 kDa in UVB-irradiated NHEK treated with TT saponins, demonstrating that, through inhibiting NF-κB, TT saponins protect the keratinocytes against malignant transformation. This was confirmed by analysis of the NF-κB p65 subunit expression in the nuclear fraction of irradiated NHEK, treated or not with TT saponins, in which UVB determine an increased nuclear localization of the NF-κB 65 kDa subunit. In contrast to normal human keratinocytes, irradiated SCC lysates contained substantially higher levels of active NF-κB (Fig. 6B). Like in normal human keratinocytes, UVB irradiation of SCC led to an increased nuclear translocation of NF-κB. In UVB-irradiated SCC, an inhibition of NF-κB activation occurs following TT saponins treatment, demonstrating the anti-carcinogenic activity of *Tribulus*.

Finally, to confirm the inhibitory effect of TT saponins, we demonstrated, using the TransAM NF-κB p65 protein assay, that unstimulated normal human keratinocytes have little active NF-κB DNA-binding activity. However, after UVB irradiation, NF-κB DNA-binding activity increased, and was reduced after TT saponins

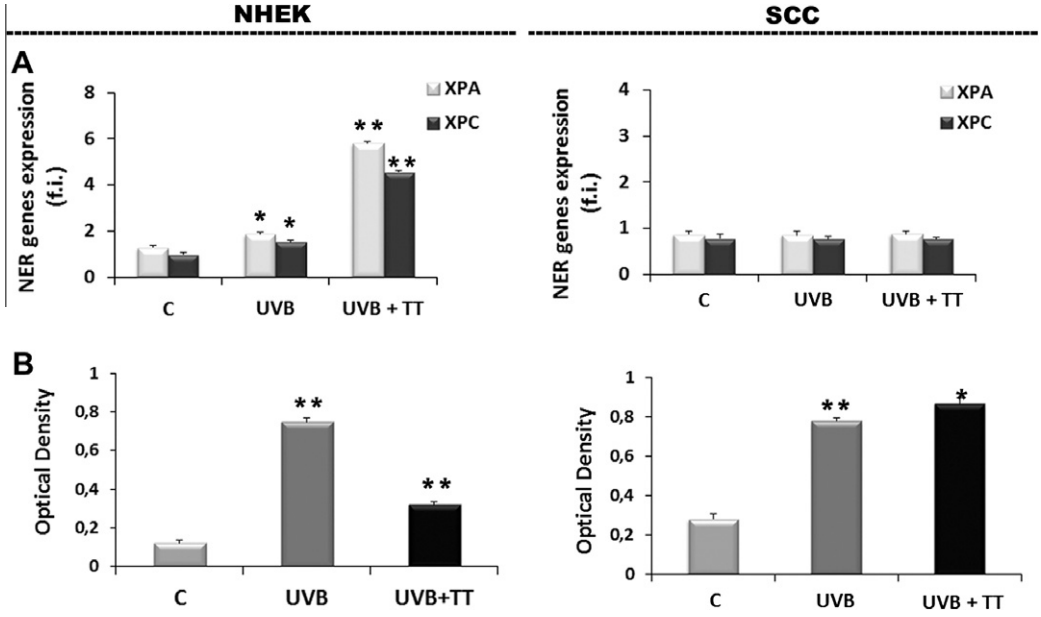

Fig. 5. TT saponins repair damaged DNA in healthy keratinocytes but not in cancer cells. Panel A: Real-time quantitative RT-PCR analysis for differentially expressed NER genes in NHEK and SCC. Transcript levels were quantified in cDNAs obtained from normal keratinocytes and squamous cell carcinoma after UVB exposure and TT saponins treatment. Quantitative Real-time RT-PCR was performed in triplicate on the ABI PRISM 7700 Sequence Detection System. The β-2 microglobulin cDNA probe was used as a control for loading of RNA samples. (∗ = $p < 0.05$, ∗∗ = $p < 0.01$). Panel B: immunodetection of thymine dimers in genomic DNA of UVB-irradiated NHEK and SCC treated or not with TT saponins (representative experiment, (∗ = $p < 0.05$, ∗∗ = $p < 0.01$).

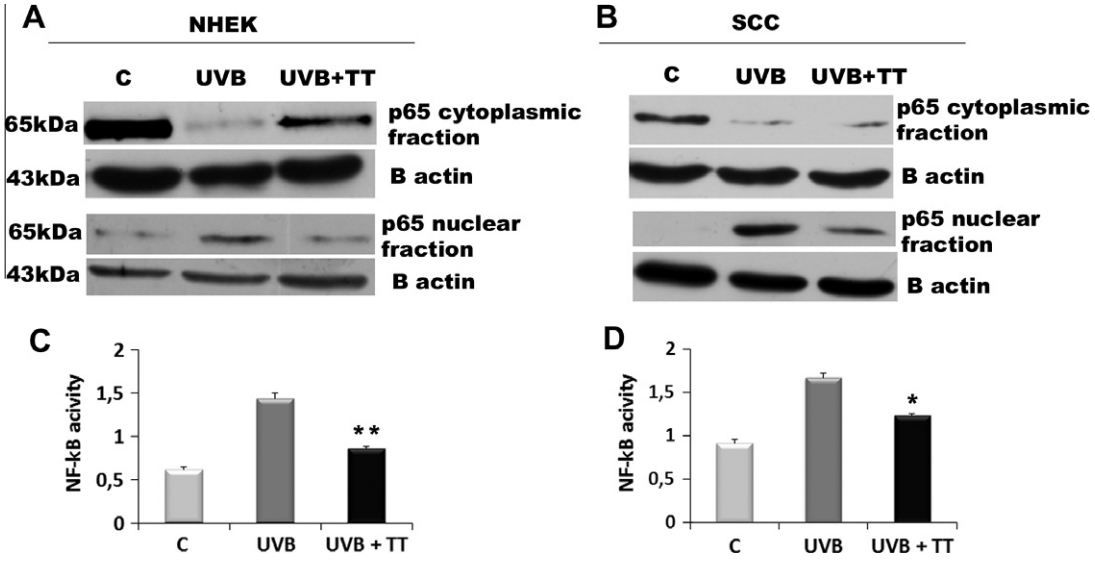

Fig. 6. TT saponins effects on the NF-κB pathway activation. (A) Anti-NF-κB p65 subunit western blot analysis performed on cytoplasmic and nuclear extract of NHEK and SCC UVB exposed and treated with TT saponins. Analysis of the NF-κB p65 subunit expression in the nuclear fraction of irradiated NHEK demonstrated that TT saponins inhibited NF-κB activation preventing malignant transformation of the cells. The increased nuclear translocation of NF-κB induced by UVB irradiation was inhibited by TT saponins treatment. (B) DNA binding activity of NF-κB was examined in UVB-irradiated NHEK and SCC after TT saponins treatment using TransAM assay as described in Section 2. (∗ = $p < 0.05$, ∗∗ = $p < 0.01$).

treatment. As shown in the same figure, in SCC the anti-tumoral effect of TT saponins was demonstrated by the reduction of NF-κB activation (Fig. 6C and D).

4. Discussion

In this study, we show that saponins from *T. terrestris* L. increase the survival of normal keratinocytes after UVB irradiation, by inhibiting the intrinsic apoptotic pathway. Moreover, TT saponins affect UVB-induced apoptosis in squamous cell carcinoma showing a highly specific sunburn protection mechanism. TT saponins increase the NER genes expression, repairing UVB-induced DNA damage in NHEK. This protective property occurs through an inhibition of the NF-κB pathway activation, highlighting a possible anti-oncogenic activity of *T. terrestris* L.

It has been amply demonstrated that human keratinocytes undergo programmed cell death following UVB exposure. The induction of apoptosis is considered to be a protective function against skin cancer, and the intrinsic apoptosis pathway has been shown to be crucial in this protective mechanism, ensuring the removal of UVB-damaged human keratinocytes and potentially transformed cells [5].

The saponin constituents from TT are well known to exhibit antimicrobial and cytotoxic effects [14–16], to induce apoptosis in liver cancer cells [17], as well as having antihyperlipidemic properties [18] and improving reproductive function, libido and ovulation [19].

Nevertheless, the effects of TT saponins on the molecular aspects of the sunburn response in human normal skin cells and in transformed cells has not previously been analyzed and few studies have yet been made of the biological activities of these compounds.

To determine the protective effect of TT saponins, we investigated cell survival of NHEK after UVB irradiation demonstrating that TT saponins significantly increased their resistance to UVB. Since a controlled apoptotic response is vital for skin cells, as it prevents the replication of cells containing damaged DNA, we expected that TT saponins would determine cellular survival by inhibiting apoptosis. Indeed, in the present study, TT saponins decreased the levels of caspases involved in the intrinsic apoptotic pathway induced by UVB irradiation and prevented both the leakage of cytochrome c from mitochondria and UVB-induced DNA fragmentation. To test the differential effect of TT saponins treatment in normal keratinocytes and malignant cells after UVB irradiation, we tested the effects of TT saponins on the apoptotic mechanism in human squamous cell carcinoma. Interestingly, TT saponins inhibited SCC cell viability and increased the DNA ladder formation induced by UVB. Furthermore, results suggest that in the apoptosis induced in UVB irradiated-TT saponins-treated SCC it is the intrinsic pathway that is activated. Taken together, these data suggest that saponins have differential effects in normal versus malignant cells, and the potential to inhibit tumor growth by triggering a selective apoptotic process.

The NER system is a major mechanism of defence against the carcinogenic effects of ultraviolet light and there are many reports in the literature supporting the opinion that in cancer cells the NER system and apoptosis are the pathways that suffer the most severe impairment [20]. In recent years, NER proteins have been isolated, and their roles have been analyzed in vivo and in vitro [21–23]. After we had verified the photoprotective effect of TT saponins on UVB-induced cellular damage, in terms of a decreased apoptotic response in normal keratinocytes, we addressed the issue whether these plant components repair DNA damage through an enhanced NER genes transcription. Our data revealed that TT saponins enhanced the levels of XPA and XPC genes and then contributed to a rapid repair of damaged DNA. Therefore, we focused our study on gaining a better understanding of the effects of TT saponins on the impaired DNA repair mechanisms, which are a factor of susceptibility for skin cancer induced by sunlight exposure. We investigated the expression of the same selected NER genes in UVB-irradiated SCC, and demonstrated that the NER pathway, that is significantly down-regulated in malignant as opposed to normal keratinocytes, was not susceptible to TT saponins treatment. In fact, our data demonstrate, for the first time, that TT saponins do not significantly influence the levels of XPA and XPC genes expression, suggesting that the NER system, a molecular target for saponins, may have a preventive action on the risk of developing UVB-induced skin cancer. The fact that TT saponins prevent the induction of cell death in NHEK, allowing the cells more able to repair the photodimers through nucleotide excision repair, was additionally confirmed detecting a significant reduction of thymine dimers because of TT saponins treatment in UVB-irradiated NHEK.

In many cell types, the induction of the NF-κB signalling cascade serves to protect cells from a variety of cellular stresses [24,25]. Many lines of evidence support this possibility; in fact, disturbances of the NF-κB signalling pathway lead to changes in keratinocytes cell growth and epidermal thickness [26], causing a homeostatic breakdown, resulting in a loss of function. Furthermore, a variety of agents which promote tumorigenesis (such as UV radiation, phorbol esters and TNF-α), are known to activate NF-κB [27,28], and data derived from experimental model systems suggest that NF-κB plays a role in suppressing carcinogenesis [29–33]. Furthermore, NF-κB regulates the transcription of several genes involved in malignant transformation and inflammation processes [27,34]. Elevated levels of active NF-κB are detected in many human diseases including breast cancers, multiple myeloma, as well as in many other cancers [35,36]. It is also important to mention that TT saponins have been shown to block the proliferation of human liver cancer cells through inhibiting NF-κB signalling [37]. Then, we hypothesized that inhibition of the NF-κB pathway could be responsible for the chemopreventive effects of TT saponins against UVB-mediated skin carcinogenesis, and investigated the effect of TT saponins on UVB-mediated modulation of NF-κB. We used multiple methods to achieve our goal and demonstrated that in NHEK, saponins blocked UVB-mediated NF-κB activation, suggesting that they may have a protective role against UVB-mediated damage. Additionally, in both normal and malignant cells, exposure to UVB irradiation evokes an activation of NF-κB, as measured by an increase in specific DNA binding and, like in normal cells, also in malignant cells, an inhibition of NF-κB activation occurs after TT saponins treatment demonstrating an anti-carcinogenic activity of *T. Terrestris* L. Interestingly, comparing the levels of NF-κB activation in the non-irradiated NHEK and SCC, it notes that NF-κB resulted more active in malignant cells. This supports data available in the literature demonstrating that NF-κB is constitutively activated in several human cancers [35,36].

The results of this study show great promise for the future. We believe that TT saponins are well worth investigating for the purposes of devising therapeutic treatments to be adopted in the fight against skin photocarcinogenesis.

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