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Protective Effects of Standardized Pomegranate (*Punica granatum* L.) Polyphenolic Extract in Ultraviolet-Irradiated Human Skin Fibroblasts

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Exposure to ultraviolet (UV) radiation has been associated with several acute and chronic conditions, including sunburn, edema, hyperplasia, immunosuppression, photoaging, and skin cancer. The role of naturally occurring phytochemicals in the prevention of such UV-related conditions has captured increased interest. Pomegranate (*Punica granatum* L.) is a rich source of polyphenolics, which have been shown to exert anti-inflammatory, antioxidant, and anticarcinogenic activity in numerous in vivo and in vitro studies. This work investigated potential protective effects of a pomegranate fruit extract standardized to punicalagins against UVA- and UVB-induced damage in SKU-1064 human skin fibroblast cells. Pomegranate extract (PE), in a range from 5 to 60 mg/L, was effective at protecting human skin fibroblasts from cell death following UV exposure, likely related to a reduced activation of the pro-inflammatory transcription factor NF-κB, a downregulation of proapoptotic caspase-3, and an increased G0/G1 phase, associated with DNA repair. Higher polyphenolic concentrations (500−10000 mg/L) were needed to achieve a significant reduction in UV-induced reactive oxygen species levels and increased intracellular antioxidant capacity (from 1.9 to 8.6 μM Trolox equivalents/mL). Results from this study demonstrate the protective effects of PE against UVA- and UVB-induced cell damage and the potential use of pomegranate polyphenolics in topical applications.

KEYWORDS: UV; photoprotection; polyphenolic; pomegranate; ellagitannins; punicalagins

INTRODUCTION

Constant exposure to ultraviolet (UV) radiation has been identified as a cause of serious adverse effects to human skin, including oxidative stress, premature skin aging, sunburn, immunosuppression, and skin cancer (*1, 2*). Among these adverse effects, skin cancer and photoaging are of great concern (*3*−*5*), with an estimate of more than 1 million new cancer cases in the United States in 2007 (*6*). Solar UV radiation is classified by energy and wavelength levels, namely, UVA (320−400 nm), UVB (280−320 nm), and UVC (200−280 nm) (*7*). While UVA has been associated with some skin cancers and represents the majority (90−99%) of solar radiation reaching the surface of our planet (*8*), UVB radiation accounts for most of the carcinogenic dose of sunlight (*7*). The role of UVC radiation in human pathogenesis is minimal, mainly due to blockage by the ozone layer (*3*). UV exposure generally results in increased generation of reactive oxygen species (ROS) within the cells, which can result in oxidative stress and photodamage to proteins and other macromolecules in the skin (*1, 9*). Therefore, approaches aimed at reducing ROS production may be useful for the prevention of skin aging and related diseases (*3*).

In addition to increased oxidative stress, UV radiation is thought to be responsible for increased cell cycle arrest, inflammation, immunosuppression, oxidative DNA damage, and increased expression of metalloproteinases (MMPs) involved in collagen degradation (*10*). An increased inflammatory response is related to the activation of transcription factor nuclear factor-κB (NF-κB), which has been linked to epidermal hyper proliferation and accelerated collagen fiber breakdown, resulting in wrinkled, rough, dry, and pigmented skin (*11*). NF-κB is sequestered in the cytoplasm as an inactive complex with the inhibitory molecule IκB. Treatment of cells with UV results in degradation of IκB and nuclear localization of NF-κB dimers (p65/p50), followed by activation of target genes (*12*). Target genes include tumor necrosis factor α (TNFα) and interlukin-1 (IL-1), responsible for further inflammatory and immunosuppressive responses in the skin (*13, 14*), and the matrix MMPs, specifically MMP1 and MMP13, responsible for collagen

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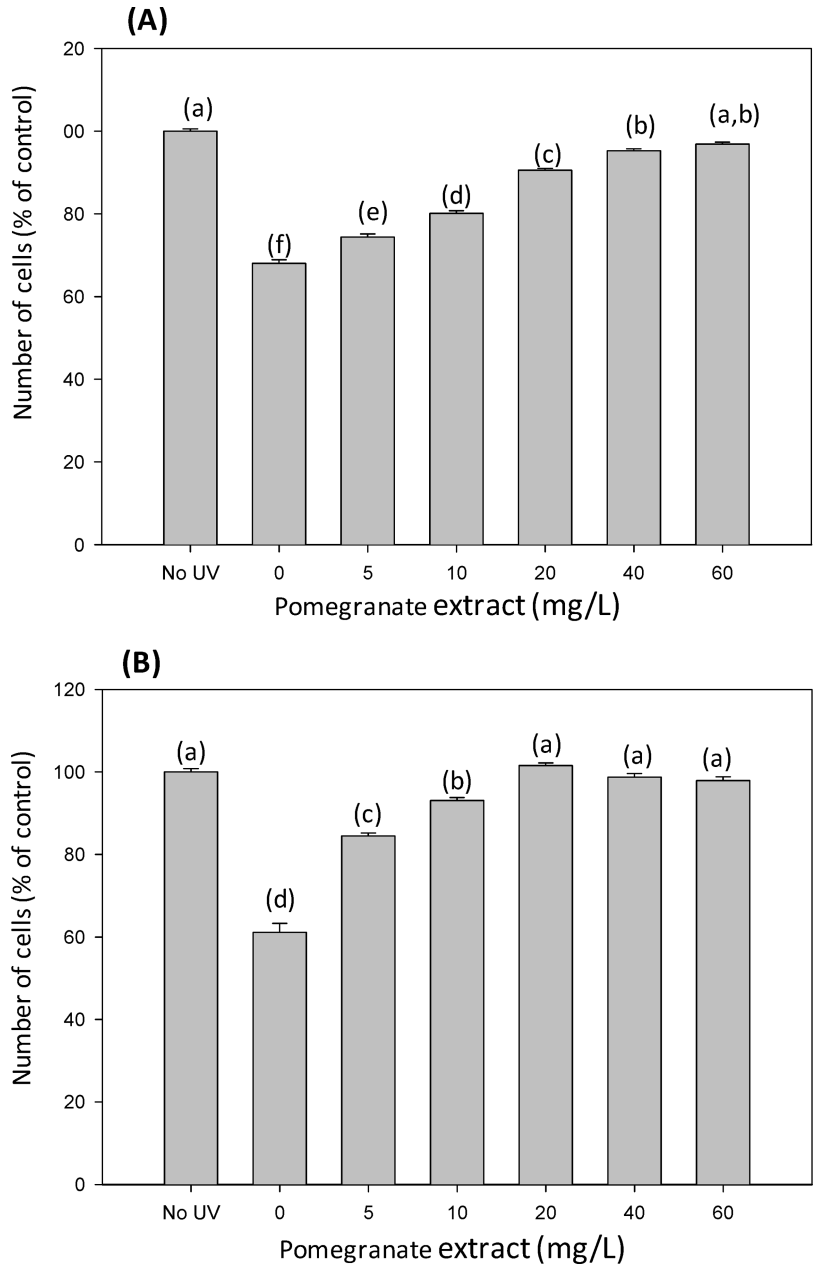

Figure 1. Cell proliferation of UVA- (**A**) and UVB (**B**)-treated SKU-1064 human skin fibroblasts treated with PE. Error bars represent the standard error of the mean ($n = 3$). Statistical significance of the differences between treatments was determined using ANOVA followed by paired-group comparisons. Different letters indicate significance at $P < 0.05$.

degradation (*15*), which is one of the hallmarks of photoaging (*16*). Moreover, MMPs oppose the activity of sirtuins or silent information regulators (SIRT), associated with genes that optimize cell functions, improve DNA resistance, and delay senescence (*17*). As a result, inhibition of hyper activated NF-κB may also be an effective strategy in the prevention of pathogenic changes induced by UV irradiation (*18*−*20*).

The relevance of antioxidant phytochemical preparations in topical applications used in the treatment and prevention of UV-induced skin damage and skin aging has increased within the past decade (*21*). Potential UV protective effects of polyphenolics have been of particular interest, and their effectiveness has been demonstrated using in vivo and in vitro models (*22*). Pomegranate (*Punica granatum* L.) is a rich source of polyphenolics, particularly punicalagins (ellagitannins), which have been shown to exert strong antioxidant, anti-inflammatory, and anticarcinogenic activities in several studies (*21, 23, 24*). Crude pomegranate extracts (PEs) have also been related to increased wound healing activity (*25*) and inhibition of mitogen-activated protein kinases (MAPK), MMPs, and NF-κB in human epidermal keratinocytes and chondrocytes (*22*).

Previous reports have suggested the potential use of PEs for skin applications; however, a lack of chemical characterization and standardization of the PEs used has led to inconclusive results. This study was designed to investigate the potential protective effects of a chemically characterized and standardized pomegranate polyphenolic extract against UVA- and UVB-induced damage in human skin fibroblasts.

MATERIALS AND METHODS

Pomegranate Phytochemical Extract. Standardized PE in capsule form was provided by Verdure Sciences (Noblesville, IN). The PE was high-performance liquid chromatography (HPLC)-standardized using validated standards and methods to 37.5% of the major ellagitannins (gallic acid, punicalagin α, and punicalagin β) and approximately 2.7%

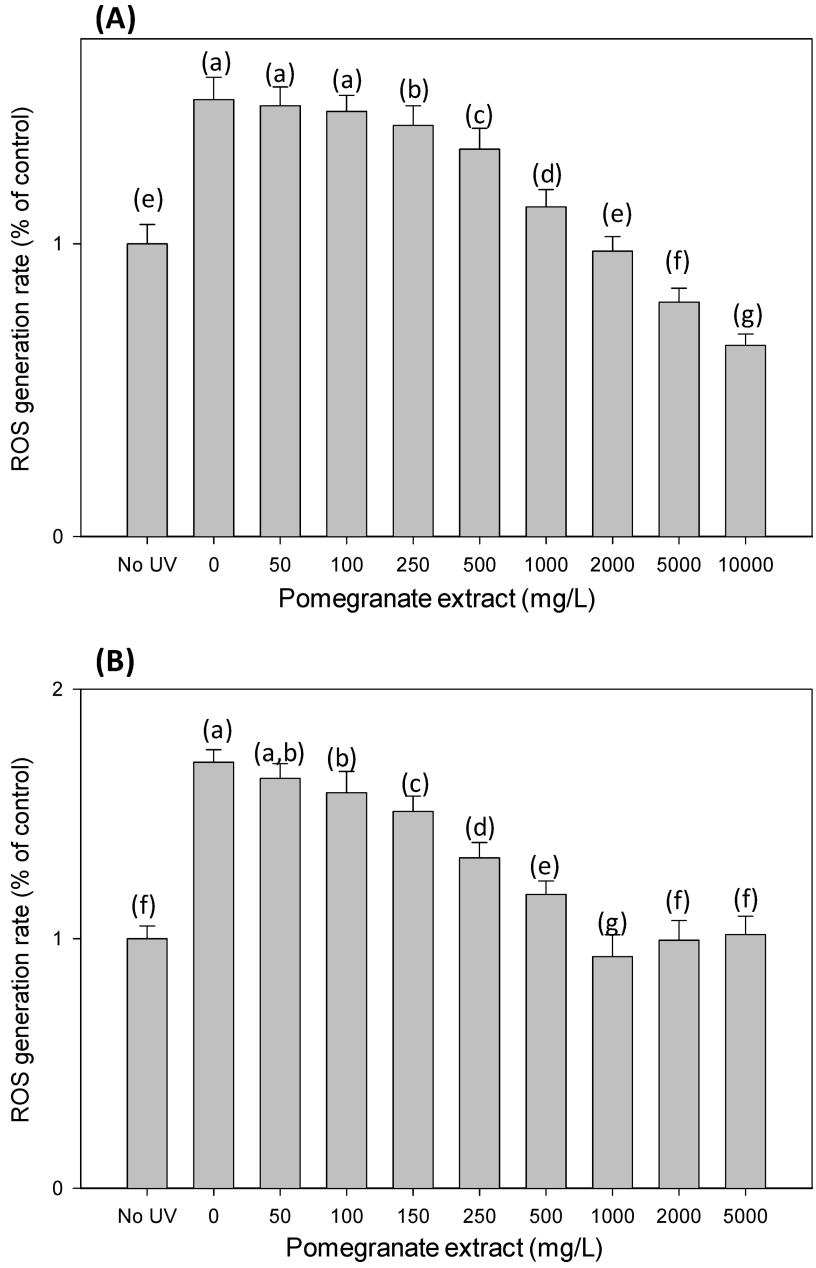

Figure 2. Generation of ROS in UVA- (**A**) and UVB (**B**)-treated SKU-1064 human skin fibroblast cells treated with PE. Error bars represent the standard error of the mean ($n = 3$). Statistical significance of the differences between treatments was determined using ANOVA followed by paired-group comparisons. Different letters indicate significance at the $P < 0.05$ level.

of ellagic acid as shown in the HPLC-photodiode array profile previously reported (*26*).

The extract was dissolved in dimethyl sulfoxide (DMSO) up to a concentration of 100,000 mg/L and diluted in a range from 0 to 10000 mg/L for use in cell culture experiments. Extracts were sterile-filtered prior to use in cell culture experiments and adjusted to a final concentration of 0.1% DMSO when applied to the cells. DMSO controls (0.1%) were included in all assays.

Cell Culture. SKU-1064 human skin fibroblast cells were obtained from American Type Culture Collection (ATCC, Manassas, VA), cultured in Dulbecco's modified Eagle's medium (1×) (DMEM) containing 10% fetal bovine serum, 1% nonessential amino acids, 100 units/mL penicillin G, 100 μg/mL streptomycin, 1.25 μg/mL amphotericin B, and 10 mM sodium pyruvate (Gibco BRL Life Technology, Grand Island, NY). Cells were incubated at 37 °C under 5% CO_2 and utilized between passages 15 and 30.

UV Treatment of Cells. SKU-1064 human fibroblast cells were exposed to a radiation dose of 60 mJ of UVA ($\lambda_{max} = 360$ nm) or UVB ($\lambda_{max} = 300$ nm) light for 1 min. Growth medium was removed just prior to UV exposure and immediately replaced with fresh medium after UV treatment. Cells were subsequently treated with different concentrations of PEs and incubated at 37 °C and 5% CO_2 atmosphere.

Cell Proliferation. SKU-1064 human skin fibroblast cells (5×10^4 cells/well) were seeded into each of a 12 well tissue culture plate. Following UV treatment, the growth medium was replaced with 1000 μL of media containing different concentrations of polyphenolic extracts (from 0 to 60 mg/L). Cells were incubated with the extracts for 2 h, after which culture medium was replaced with fresh medium and incubated for 24 h at 37 °C in a 5% CO_2 atmosphere. Cell numbers were determined and expressed as a percentage of control (UV-untreated, 0.1% DMSO medium) using a Beckman Coulter Particle Counter (Fullerton, CA).

Generation of ROS. The dichlorofluorescein (DCF) assay was used to assess the extent of ROS generation following UVA or UVB exposure (*41*). SKU-1064 human skin fibroblast cells (2×10^4/mL) were seeded in a 96 well plate and incubated for 24 h. Following UV exposure for 1 min, cells were washed twice with phosphate buffer

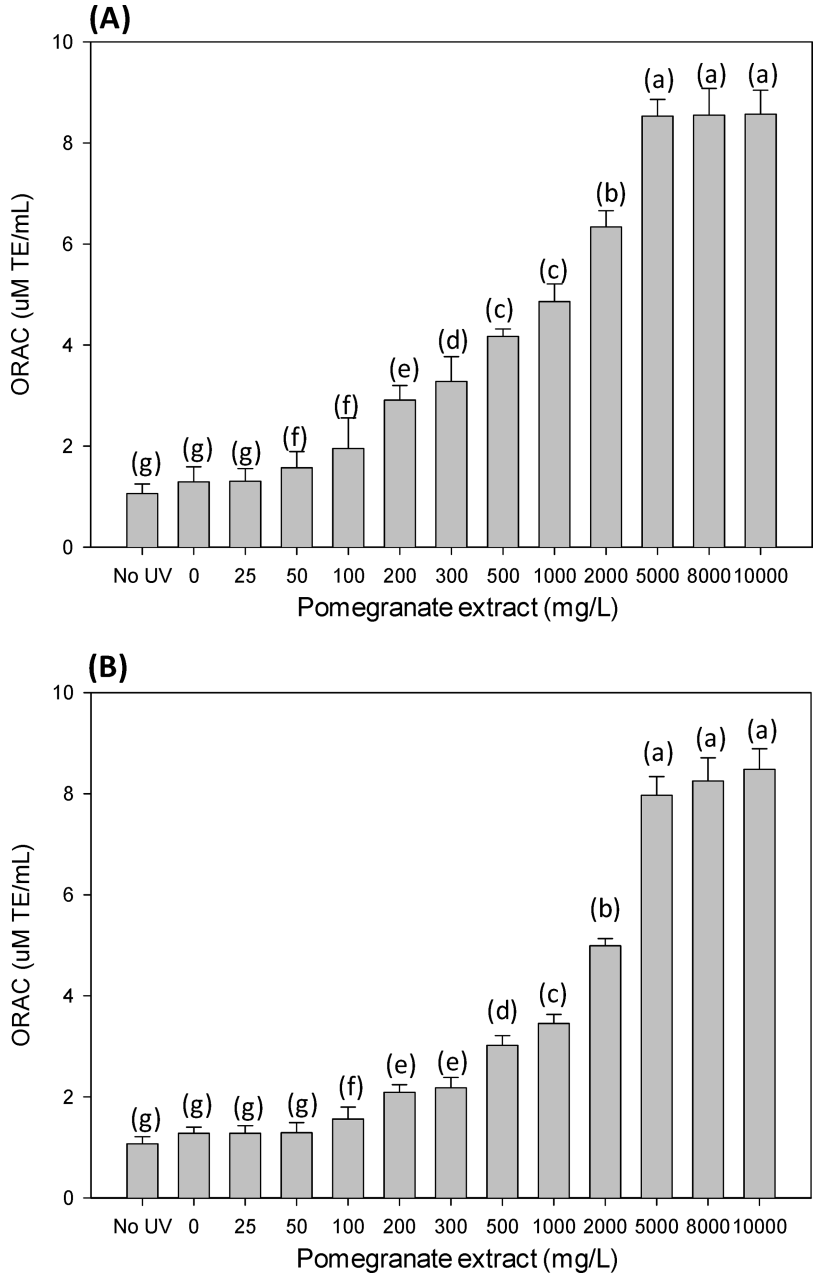

Figure 3. Antioxidant capacity following UVA (**A**) and UVB (**B**) exposure as influenced treatment with PE. Error bars represent the standard error of the mean ($n = 3$). Statistical significance of the differences between treatments was determined using ANOVA followed by paired-group comparisons. Different letters indicate significance at the $P < 0.05$ level.

saline (PBS) and incubated with various concentrations (0−10000 mg/L) of pomegranate polyphenolic extracts for 3 h. Cells were then washed with PBS and preloaded with DCF diacetate (DCFH-DA) substrate by incubating with 10 μM DCFH-DA for 30 min at 37 °C. Fluorescence was determined after 30 min of incubation with polyphenolics using a BMG Labtech FLUOstar fluorescent microplate reader (485 nm excitation and 538 nm emission, BMG Labtech Inc., Durham, NC).

Antioxidant Capacity. SKU-1064 human skin fibroblast cells were prepared and treated as described for the generation of ROS assay. The antioxidant capacity of the cell medium was assessed by the oxygen radical absorbance capacity (ORAC) assay (*27*), adapted to be performed in a BMG Labtech FLUOstar fluorescent microplate reader (485 nm excitation and 538 nm emission, BMG Labtech Inc., Durham, NC). Results were quantified in μmol Trolox equivalents/mL.

Semiquantitative RT-PCR. Cells (6×10^6) were seeded into 12 well plates, pretreated with various concentrations of PE for 2 h, washed twice with PBS, and exposed to UV irradiation (60 mJ) for 1 min with 0.4 mL of PBS/well. PBS was removed following UV exposure and replaced with DMEM media containing various concentrations of PE. Cells were incubated with the extract for 2 h at 37 °C and 5% CO_2, followed by incubation for 24 h in fresh DMEM media. Total RNA was extracted using RNeasy Mini kit (Qiagen, Valencia, CA), and 470 ng of RNA was used to synthesize cDNA using Reverse Transcription System (Promega, Madison, WI). Real-time polymerase chain reactions (PCRs) were performed using 2 μL of cDNA as previously described (*28*). Primers were obtained from Integrated DNA Technologies. Optimal semiquantitative conditions were set to fall in the linear PCR product range (data not shown). As an internal control, TBP (TATA-box binding protein) was amplified in each RNA sample. The sequences of the primers used were as follows. MMP-1: F, 5′-TTTGATGGACCTGGAGGAAAT-3′; R, 5′-TGAGCATCCCCTCCAATACC-3′. MMP-13: F, 5′-ATTAAGGAGCATGGCGACTTC-3′; R, 5′-CCCAGGAAAAGCATGAG-3′. SIRT-1: F, 5′-GAACAGGTTGCGGGAATCC-3′; R, 5′-CAGGCAAGATGCTGTTGCA-3′. Caspase 3: F, 5′-CTGGACTGTGGCATTGAGACA-3′; R, 5′-CGGCCTCCACTGGTATTTTATG-3′. TBP: F, 5′-TGCACAGGAGCCAAGAGTGAA-3′; R, 5′-CACATCACAGCTCCCCACCA-3′.

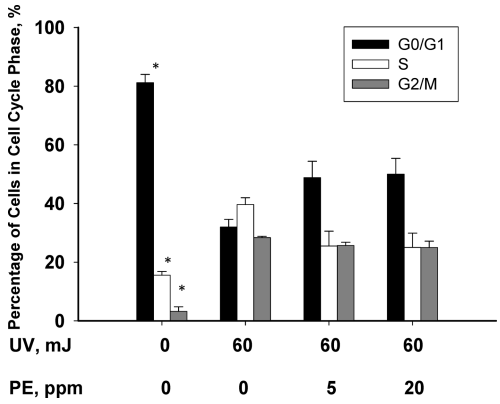

Figure 4. Flow cytometry of SKU-1064 cells following UVB irradiation and treatment with PE (mg/L) for 2 h. Values are means ± standard errors ($n = 3$). Asterisks (*) indicate differences at the $P < 0.05$ significance level.

Cell Cycle Kinetics. Cells were treated as described for the proliferation assay. Cells were trypsinized, collected by centrifugation, resuspended in staining solution [50 μg/mL propidium iodide, 30 units/mL RNase, 4 mmol/L sodium citrate, and Triton X-100 (pH 7.8)], and incubated at 37 °C for 10 min. Sodium chloride solution was added to a final concentration of 0.15 mol/L. Stained cells were analyzed on a FACS Calibur Flow Cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) using Cell Quest acquisition software (Becton Dickinson Immunocytometry Systems, San Jose, CA) as previously described (*28*).

Immunofluorescence Analysis by Phase Contrast Microscopy. Cells seeded on coverslips were exposed to UV radiation and treated with pomegranate polyphenolics as described for the proliferation assay. Monolayers were washed twice with PBS, fixated with 3.8% paraformaldehyde, and permeabilized with 0.5% Triton X-100. Blocking was performed for 2 h in PBS containing 5% BSA. A primary antibody (NF-κB, antirabbit p105/p50, Abcam, Cambridge, MA) (1:250 dilution) was applied for 2 h in blocking solution while a secondary antibody (Alexa Fluor^{G}488 goat antirabbit IgG, Invitrogen, Carlsbad, CA) (1:1000 dilution) was similarly applied for 45 min. After rinsing with PBS, DAPI (4′,6-diamidino-2-phenylindole solution (Invitrogen, Molecular Probes, Carlsbad, CA) (5 μg/mL) was applied for 5 min. Mounting of the coverslip was performed with Prolong Gold Antifade reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Microscopic images were taken with a Zeiss Axioplan 2 microscope (Carl Zeiss, Thornwood, NY) fitted with an Axiocam high152 resolution digital camera and Axiovision 4.1 software.

Statistical Analysis. Data were analyzed using SPSS version 15.0 (SPSS Inc., Chicago, IL). One-way analysis of variance (ANOVA) followed by pairwise comparisons was performed with posthoc Tukey−Kramer HSD ($p < 0.05$).

RESULTS AND DISCUSSION

Cell Proliferation. Protective effects of pomegranate polyphenolics against UVA and UVB were evaluated by measuring the extent of inhibition of UV-induced reduction in cell numbers using SKU-1064 human skin fibroblasts. Total cell numbers were indicative of the proliferative activity of SKU-1064 cells, and reductions were associated with UVA- or UVB-induced cell death. Both UVA and UVB radiation induced significant ($p < 0.01$) decreases in total cell numbers, while treatment with PE inhibited UV-induced cell death in a dose-dependent fashion (**Figure 1**). PEs inhibited the UVB-induced cell death at concentrations as low as 20 mg/L ($p < 0.05$), whereas the inhibition of cell death induced by UVA was achieved at higher doses (60 mg/L). These results suggest PEs may exert a higher protection against UVB-induced cell death, which has been identified as the most carcinogenic solar light component, accounting for the majority of adverse effects that result in a variety of skin disorders (*29*). These observations are in agreement with previous reports indicating the potential of pomegranate fractions in facilitating skin repair, dermis, and epidermis regeneration (*30*). Furthermore, the protective effect of PE against cell death may be related to its potential for regulation of different signal transduction pathways associated with skin aging (*4, 31*).

Generation of ROS. Intracellular generation of ROS following UV exposure was evaluated by the DCF assay. Both UVA and UVB radiation induced major increases (up to 70%) in ROS generation rates, while treatment with PEs at concentrations above 100 mg/L significantly reduced such effects ($p < 0.05$) (**Figure 2**). Polyphenolic concentrations greater than 2000 mg/L completely inhibited UVA- and UVB-induced generation of ROS. Even when the skin possesses an elaborate internal antioxidant defense system to deal with UV-induced oxidative stress, excessive exposure to UV and other environmental factors can overwhelm this intrinsic antioxidant defense, resulting in oxidative damage and skin cancer, immune-suppression, and premature skin aging (*32, 33*). The high production of intracellular ROS has been involved with the initiation process in UVB light-induced skin carcinogenesis mediated by DNA damage, which, if not repaired, can be replicated to generate UVB light-induced mutations (*4*). Therefore, treatment of the skin with antioxidant polyphenolics from pomegranate may be a useful preventive strategy against the injurious UV-induced production of ROS.

Antioxidant Capacity. Antioxidant capacity levels were not significantly ($p < 0.05$) altered following UVA or UVB exposure; however, treatment with pomegranate polyphenolics at concentrations greater than 50 mg/L significantly increased the antioxidant capacity levels in a dose-dependent manner (**Figure 3**). Conversely, UV exposure did not affect the antioxidant capacity. Intracellular antioxidant enzymes, responsible for neutralizing reactive species, may have contributed to maintaining the antioxidant capacity after treatment with UV (*9*). Higher doses of pomegranate may have acted through counteracting UV-induced generation of ROS and/or increasing the antioxidant activity of cells. Both mechanisms may confer extra protection to cells when longer UV exposure time is used.

DNA Flow Cytometry Analysis. UV treatment of skin fibroblast cells resulted in cell cycle alterations, specifically in the suppression of G0/G1 phase progression and induction of S and G2/M phase arrest. Treatment of UVB-exposed cells with pomegranate polyphenolics decreased the UVB-induced cell cycle arrest in S phase and partially restored the suppressed G0/G1 progression (**Figure 4**); however, these effects were not significant. Cell cycle perturbations in UVA-exposed skin fibroblast cells remained unchanged ($p < 0.05$) following treatment with pomegranate polyphenolics (data not shown).

Analysis of Gene Expression. Results obtained from the irradiation with UVA were not significant (data not shown). Exposure of skin cells to UVB radiation resulted in the alteration of important signal pathways associated with skin aging. UVB exposure significantly increased gene expression of pro-apoptotic caspase 3, MMP1, and MMP13 and decreased SIRT1 expression in fibroblast cells (**Figure 5**). Matrix MMPs are a family of endoproteases with the ability to degrade extracellular matrix proteins. The activity of MMPs can be regulated at three levels: synthesis (gene

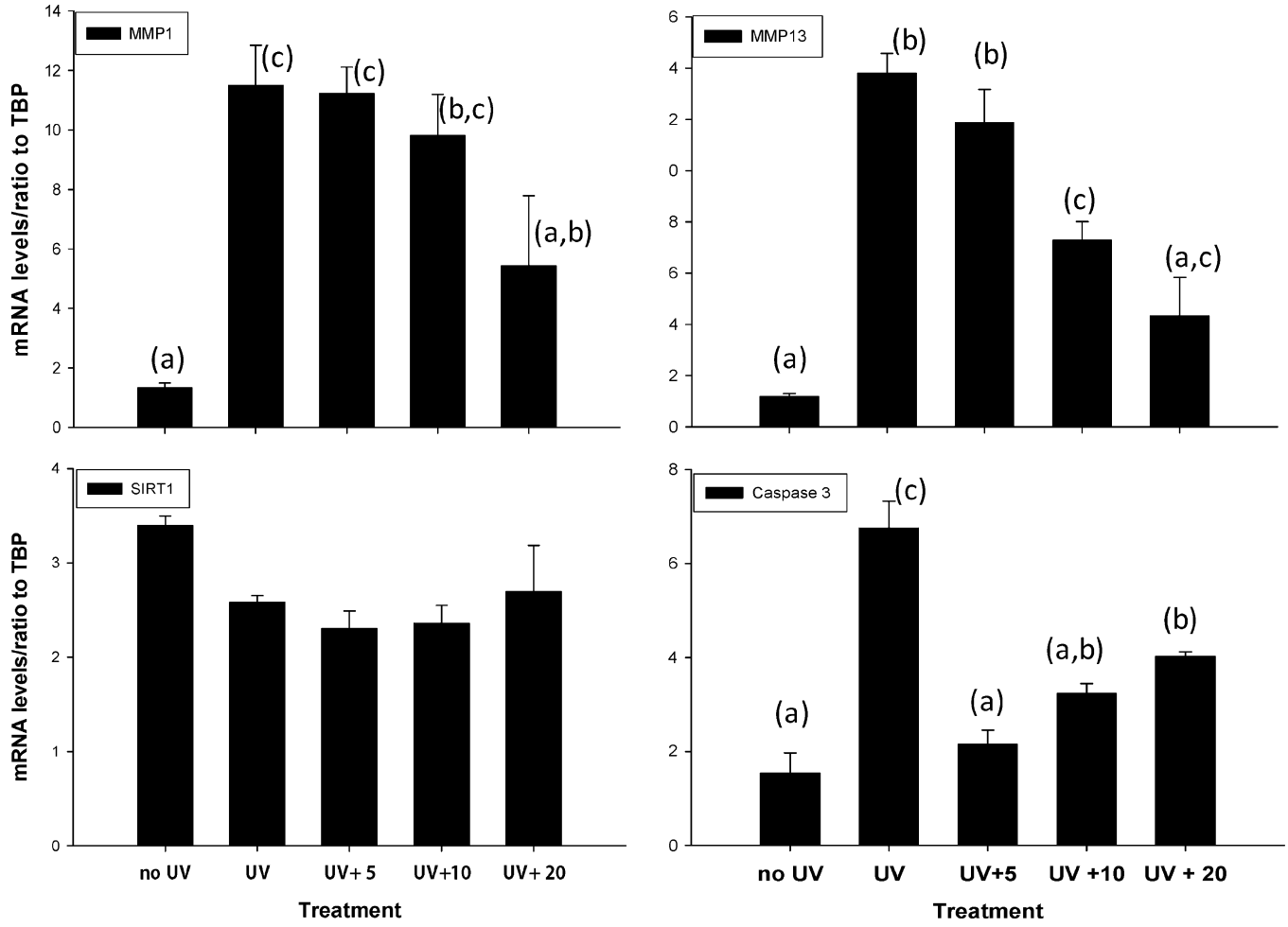

Figure 5. mRNA expression of SKU-1064 cells following UVB irradiation and treatment with PE (mg/L) using real time PCR, expressed as a ratio to TATA-binding protein (TBP) mRNA. Values are means ± standard errors ($n = 3$). Statistical significance of the differences between treatments was determined using ANOVA followed by paired-group comparisons. Different letters indicate significance at $P < 0.05$.

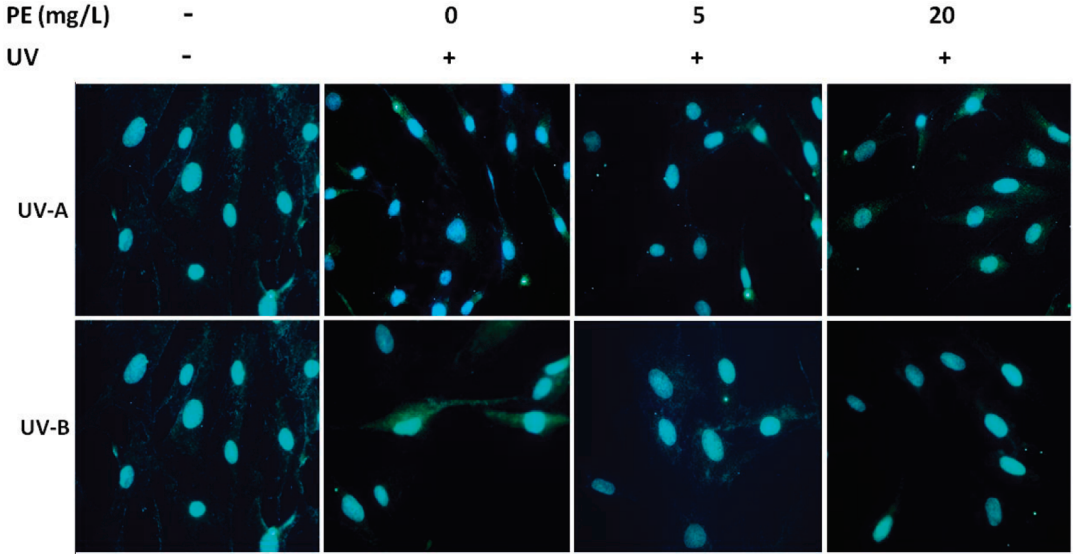

Figure 6. Effects of pomegranate polyphenolics on the NF-κB (p50) activation and nuclear localization induced by UV irradiation in SKU-1064 cells. The nucleus is stained blue with DAPI that binds to DNA, and p50 is tagged with green fluorescent secondary antibody. The images were captured sequentially using an Axiocam high152 resolution digital camera and then overlaid to give a complete image. The green brightness provides an estimated level of p50 protein in nucleus. These experiments were repeatedly performed for three times, obtaining analogous observations.

expression), activation, and inhibition of proteolytic activity (*34*). Expression of MMP genes following UV radiation has been involved in degradation of fibrillar skin collagen (*35, 36*), leading to skin wrinkling and sagging (*37*). Treatment of

UVB-exposed cells with PE concentrations higher than 10 mg/L decreased ($p < 0.05$) the expression of MMP1 and MMP13 induced by UVB. Moreover, treatment of UVB-exposed cells with 20 mg/L of PE reduced MMP levels to those of nonexposed cells.

Expression of SIRT1 in human skin cells has been related to visible improvement of the aged skin (*17*), while a decline in SIRT1 expression has been associated with accelerated aging of mice (*38*). Exposure of skin fibroblasts to UVB radiation significantly decreased SIRT1 expression. Treatment with PEs appeared to increase SIRT1 expression in UVB-exposed cells in a dose-dependent manner; however, effects were not significant (**Figure 5**).

Cell exposure to UV radiation can also trigger excessive programmed cell death (apoptosis) in skin cells through the activation of caspases. Caspase 3 is an effector caspase that results in cellular disassembly when activated (*39*). Exposure of fibroblast skin cells to UVB up-regulated the expression of caspase 3, while treatment with pomegranate (5 mg/L) reversed this effect to basal levels ($p < 0.05$). However, increased PE concentrations (>5 mg/L) induced caspase 3 expression to some extent (**Figure 5**).

Immunofluorescence Analysis by Phase Contrast Microscopy. Immunofluorescence analyses were conducted to visualize the p50 active subunit of NF-κB when translocated to the nucleus. By blocking the fixed cells with a primary antibody for the p50 subunit and by the subsequent use of secondary antibody with a fluorescent dye that recognized the primary antibody, we were able to detect the extent of p50 translocation in the DAPI stained nucleus as an indicator of NF-κB activation. NF-κB activates genes responsible for inflammatory and immunosuppressive responses in skin (*13, 14*) and genes responsible for production of matrix MMPs involved in collagen degradation (*15*).

PE seemed to inhibit the UV-induced activation and nuclear translocation of p50, the functionally active subunit of NF-κB, up to near basal levels (**Figure 6**). These findings are consistent with previously observed inhibition of mRNA MMP levels by pomegranate polyphenolics in UV-exposed cells. Similar results have also been reported when testing the UV-protective effects of pomegranate polyphenolics in other in vitro systems (*40*).

In summary, results from this study provide evidence supporting the protective and chemopreventive properties of standardized PEs in human skin fibroblasts against UVA- and UVB-induced damage. Protective effects of pomegranate polyphenolics against UVA- and UVB-induced cell death of human skin fibroblasts may be attributed to reduced generation of intracellular ROS and increased intracellular antioxidant capacity. Molecular mechanisms involved in the protective effects of PEs against photodamage in skin fibroblast cells seem to be related to the regulation of NF-κB inflammatory cascade, gene expression, and transcription of proteins that play key roles in cell integrity. Overall, these findings provide evidence supporting the use of pomegranate polyphenolics in skin repair and as chemopreventive agents in topical applications.

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