Electronic Supplementary Material (ESI) for Analyst.

Supplementary Information for:

Evaluation of UV radiation-induced toxicity and biophysical changes in various skin cells with photo-shielding molecules

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SECTION S1

Experimental section

Materials

HEKa (derived from the foreskin of a man (13 age) belonging to the yellow color skin, HEMa (derived from the foreskin of a man (18 age) belonging to the yellow color skin, and HDFa (derived from the foreskin of a man (12 age) belonging to the yellow color skin cells and their respective growth media and supplements were purchased from CEFO Ltd (Seoul, South Korea). Fetal bovine serum (FBS), trypsin–EDTA, and penicillin and streptomycin were purchased from Gibco Laboratories (Grand Island, NY). Cell culture flasks were purchased from SPL life Sciences Co., Ltd, Pocheon-Si, South Korea). Melanin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Alexa Fluor 488, 4,6-diamidino-2-phenylindole dihydrochloride (DAPI), keratin (KRT1), rhodamine phalloidin (PHDR1), bFGF, phosphate buffered saline (pH 7.4) and trypan blue were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Live–Dead Cell Staining Kit was purchased from BioVision (Milpitas Boulevard, Milpitas, CA, USA). All other reagents were of pharmaceutical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA). Milli-Q water (18.2 MΩ) was used for all the experiments.

Instrumentation

Lamp for producing UVA (365 nm) and UVC (254 nm) was purchased from Vilber Lourmat (Cedex, France) and the irradiance of 0.350 mW/cm^2 were used for all experiments. Biophysical characteristics were studied using the ECIS system, software, electrode arrays, and lock-in amplifier obtained from Applied Biophysics (Troy, NY, USA). Morphological characteristics were studied using a conventional microscope. Topography and biomechanical

changes were studied using a bio-atomic force microscope (NanoWizard® II; JPK Instruments, Berlin, Germany) in liquid contact mode. After exposure of cells to UVR, culture media of the cells were replaced with fresh media at each time point and the cells were used for bio-AFM studies.

Culture of human skin cells

HEKa, HEMa, and HDFa cells were cultured in T-75 cell culture flasks containing specific growth media (phenol red free) supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL of streptomycin and were incubated at 37 °C in an atmosphere of 95% air and 5% CO_2. The cells were subcultured every 3 days before starting the experiments. For all the experiments, monodispersed cell suspensions containing 2- to 4-times passaged cells were used according to the standard tissue culture protocol. Cell viability was measured using trypan blue dye exclusion method.

Impedance-based cell assays and UVR exposure: A biophysical approach

Before starting the experiment, a UV light setup was built inside a digitally controlled incubator for real-time monitoring. A detailed description of the UV light setup along with the ECIS system is presented in Figure 1. The UV light setup was built just above the ECIS cell culture well such that all culture wells received the same light through a hole in a screen (Figure 1). The eight holes in a screen are ensured that all eight cultured wells received the same lighting. For control experiment, a single hole is covered with black cap to prevent light exposure, and was used to the darkness control. The UV lamp was exposed to cool air from an electrical cooling fan to prevent an increase in temperature during the investigation period. Temperature in the well station was controlled using typical input sensors, including a resistive thermal device with thermocouple (K type; Omega). Temperature of the cultured media was maintained at 37 °C to prevent any unwanted effects. All the devices were

controlled externally. The UV lamp produced UVR of wavelengths 254 (UVC) and 365 nm (UVA) was used with irradiance of 0.350 mW/cm^2.

All equipment was sterilized using 70 % ethanol for 20 min, and was irradiated with ultraviolet radiation for 15 min prior to the start of experiment. For all experiment, L-cysteine (10 mM) treated 8-well 10 electrode arrays (ECIS-8W10E arrays) were used. Each well contained 10 circular micro-patterned tiny gold electrodes (250 μm) that could measure the activity of approximately 500–1000 cells. These electrodes were connected to a counter electrode. All the electrodes were connected to the edge of the ECIS chip and were plugged into an ECIS well station containing the lock-in amplifier. The electrodes were initially incubated in the cell culture medium specific to each cell type at 37 °C for 30 min to record background impedance signals (Z_0). The impedance signals were recorded by applying alternating potentials to 2 electrodes through a 1-MΩ resistor.

Real-time measurement of UVR-induced damage in different skin cells

Monodispersed cell suspensions containing desired densities of HEKa, HEMa, and HDFa cells were prepared from 48-h subconfluent cultures. Before the experiment, each cell type (final cell density, 5×10^5 cells per well) was seeded in 400 μL of the respective growth medium and was cultured to confluence at 37 °C in a humidified incubator with an atmosphere of 5% CO_2. The cell lines were then cultured on the 8W10E culture chip. Of the 8 wells, 2 wells were seeded with HEKa cells, 2 were seeded with HEMa cells, and 2 were seeded with HDFa cells. The remaining 2 wells were used for cell-free experiments. A control was maintained for each cell type under the same conditions without exposure to UVR. A screen without any hole was used to prevent the exposure of control cells to UVR. Resistance in all the wells was monitored every second at different frequencies (4, 8, 12, 16, 24, 32, 48, and 64 kHz). We selected an appropriate frequency for each cell type and

monitored changes in the behavior of the cells at these selected frequencies. The incubated cells adhered to the surface of the electrodes after an increase in impedance. After reaching a steady state, the cells were exposed to intermittent doses of UVR at different time points to determine the response of these cells in real time. Changes in cellular behavior in response to irradiation with intermittent doses of UVR were recorded as impedance signals. Analysis of UVR-induced toxicity in real time was performed continuously from 1 min to 2.5 h by using the ECIS system. Data collection and processing were performed using the ECIS software. The resultant average biophysical data from all the cells was normalized as Z_x/Z_0 to negate the background impedance and to obtain the exact UVR-induced toxicity responses.

Immunofluorescence analysis of UVR-induced cell death and changes in cytoskeleton ultrastructure

Simultaneously, UVR-induced cell activities were imaged using Live–Dead Cell Staining Kit, according to the manufacturer's protocol. After the exposure of the ECIS cell culture chip to UVR at each time point (5, 20, 40, 80, and 150 min), the medium was replaced with a previously prepared Live–Dead Cell Staining solution. The chip was then incubated in the dark for 15 min, and the stained samples were photographed using a fluorescence microscope. Control samples were also photographed similarly and were compared with experimental samples. Live cells were stained green by a cell-permeable fluorescent dye (calcein-AM) while dead cells stained red with a cell nonpermeable fluorescent dye (propidium iodide). The cell index of the cultures was calculated, in order to quantify the Live/Dead of cells on the different intermittent UVR exposed samples. The total number of live (green) dead (red) cells in representative each image was counted. The percentage of dead cells was calculated as the number of dead cells divided by the total number of cell in

each image. Results of percentage of cells are expressed, and the mean and standard error of these values were calculated.

UVR-induced changes in cell morphology and cytoskeleton ultrastructure were investigated in-vitro by using a fluorescence microscope. The medium was replaced after exposure to UVR at each time point (5, 15, 60, and 150 min). The samples were treated with previously prepared Alexa Fluor 488, PHDR1, and DAPI staining solutions and were incubated in the dark for 50, 30 and 10 min, respectively, to stain the microtubules (green, Alexa Fluor 488), nuclei (blue, DAPI), and F-actin (red, PHDR1), respectively. The stained samples were photographed using a fluorescence microscope. Control samples were also photographed similarly and were compared with experimental samples. The spatial distribution of cell spreading area and fluorescence intensities of actin microfilament distribution were quantified using the ImageJ software (ImageJ, National Institutes of Health (NIH), Bethesda, MD). The image processing step, the contrast of each fluorescence-stained cell image was enhanced using the color display option to identification of cell distribution expression around the cell center. Then adjust to threshold for identification of cell coverage area. Three independent cell of interest were selected from each image using a tracing tool, and analyzed the cell spreading area. The actin microfilament distribution expression in cell has been quantitatively measured by selecting the red color region of cells which was highly surrounded the nuclear region. The actin microfilament distributions per cell have been measured, and mean and standard error of these values were calculated.

UVR-induced changes in cell viability

Qualitative and quantitative changes in the viability of skin cells after exposure to UVR at each time point were measured by performing the MTT assay, according to a standard protocol. Briefly, 150-µL suspensions containing 5×10^4 HEKa, HEMa, and HDFa cells per

milliliter in their respective growth media were seeded in a 96-well culture plate and were grown to confluency by incubating at 37 °C in 5% CO_2 in a humidified incubator. The cells were then exposed to UVR for 5, 20, 60, 80, 120, and 150 min, and cell viability was measured by performing the MTT assay. The data were compared with those obtained for control cells.

UVR-induced changes in cell morphology and topography

Images of UVR-induced changes in cell morphology and topography were obtained using a high-resolution bio-atomic force microscope. After UVR exposure at each time point (5, 15, 60, and 150 min), the medium in the ECIS 8-well cell culture chip was removed, and the cells were washed with DI water. After washing, a thin liquid film was left on the cells to prevent dehydration. The top chambers of the 8-well chip were then removed by taking off the top well assembly by pressing the base. The electrode array was then mounted on an appropriate stable holder such as a live cell culture vessel of the atomic force microscope or Petri dish containing respective fresh culture media maintained at 37 °C and was used for bio-AFM studies. Data were acquired after the exposure of cells to UVR at 5, 15, 60, and 150 min. All images were acquired in the physiological liquid environment by using NanoWizard® II atomic force microscope mounted on an inverted optical microscope (Nikon Instruments Eclipse Ti; Amsterdam, Netherlands). The inverted optical microscope precisely positioned the tip of the atomic force microscope over the region of interest and allowed to establish a direct correlation between optical images and structural and nanomechanical changes in UVR-induced cells. Effects of UVC and UVA radiations on the different pigmented skin cells were determined by performing bio-AFM, which can examine cells in an aqueous environment.

Biomechanical analysis of UVR-induced cells by performing bio-AFM

Bio-AFM studies to determine the nanomechanical changes in UVR-induced skin cells were performed in liquid contact mode under physiological conditions. Biomechanical changes in cells exposed to UVR for 5, 15, 60, and 150 min were measured using nanoindentation method with conical silicon nitride cantilevers (Si3N4, Au surface) having a nominal spring constant of 0.2 N/m for imaging and force spectroscopic analysis. Initially, the cells samples were photographed in the liquid contact mode to locate the cells. Scans were performed at a rate of 0.2–0.4 Hz, with a high-resolution image quality, and a scan size of 100 × 100 μm. Biomechanical changes in each cell type were analyzed by scanning different positions in the central region of the cell surface, which were selected from the contact mode image. After selecting the desired area, the cantilever was approached onto the cell surface at a speed of 1 μm/s and a contact force of 1 nN. After the induction of force onto the cell surface, the cantilever was lifted and cantilever deflection was recorded. Tip–Cell deflection curve was plotted to evaluate the relative stiffness (Young's modulus; also called as elastic modulus 'E') of the cells. Young's modulus was calculated using Hertz's contact mechanics model with the JPK data processing software. To quantify the stiffness of the cells, Poisson's ratio and opening half-angle (α) of the conical cantilever tip was taken as 0.5 and 35°, respectively. Cantilever deflection was decreased in the range of 500 nm to obtain a gentle indentation, which prevented cell membrane defects and Hertz model limitation.

Analysis of surface roughness

Morphological and topographical differences between cells exposed to UVR at different time points and control cells were determined by obtaining 2D and 3D bio-AFM images. These differences were quantified by analyzing cell surface roughness. Surface roughness was analyzed using the bio-AFM system with the JPK offline data processing software v3.3.25. Data received from the height scale (x–y scan range, 100 × 100 μm) images obtained using

the atomic force microscope were used to estimate the surface roughness of control and UVR-induced cells. For analyzing surface roughness, the 20 μm scan area was selected from different center regions of the cells. Surface roughness was calculated by applying a mean filter to raw or original data. Root-mean-square value of surface roughness of the selected area of cells exposed to UVR for 5, 15, 60, and 150 min was obtained using the JPK software.

UVR-shielding effects of different bioactive compounds

Real-time drug screening test

Before examining the shielding effects of bioactive compounds melanin, KRT1, and bFGF, these compounds were screened for their cytotoxic effects. To achieve efficient shielding, we selected the safest concentration, i.e., non-toxic maximum tolerant concentration, of these compounds for screening their cytotoxic effects. HDFa, HEKa, and HEMa cells cultured for 16 h were used to screen the safest non-toxic concentrations of these bioactive compounds. The cells were exposed to 0.05, 0.50, and 1 mM of melanin, KRT1, and bFGF, which were prepared using a fresh medium and filtered using a 0.22μm membrane. Cytotoxicity was measured continuously for 24 h and was compared with that in control cells. Toxic concentrations of the bioactive compounds may decrease the impedance value. Higher concentrations of these compounds may decrease cellular activity while lower concentration may not decrease cellular activity compared with control cells. The results of cytotoxicity screening of the bioactive compounds are described in the Results and Discussion. Based on these results, we selected the maximum non-toxic concentration of 1 mM for all the bioactive compounds, which was non-toxic and was associated with maximum cellular activity.

Real-time measurement of the UVR-shielding effects of the bioactive compounds

To analyze the UVR-shielding effects of melanin, KRT1, and bFGF, HEKa, HEMa, and HDFa cells were pretreated with 1 mM concentrations of these compounds for 24 h. Briefly, HEKa, HEMa and HDFa cells were cultured in their respective growth media supplemented with 10% FBS, 1% antibiotics, and 1 mM of the abovementioned bioactive compounds and were incubated at 37 °C in 95% air and 5% CO_2. After 24 h, the cells were harvested and were used for examining the UVR-shielding effects of these compounds in real time. Monodispersed cell suspensions containing the desired densities of HEKa, HEMa, and HDFa cells were prepared from 24-h pretreated cell cultures. Approximately 5×10^5 cells per well were cultured to confluency in 300 μL of respective growth media at 37 °C in a humidified incubator with an atmosphere of 5% CO_2. Confluent cells adhered to the surface of the electrode after increasing the impedance. After reaching a steady state, the cells were exposed to intermittent doses of UVR from 1 min to 2.5 h. After exposure, the culture media were replaced with fresh media. Changes in cellular responses were detected as impedance signals. Changes in impedance signals occurred because of exposure to intermittent doses of UVR and because of the replacement of old media with fresh media. UVR-shielding effects of the bioactive compounds were determined continuously in real time. In addition, the resultant effects on the ECIS data were evaluated and compared among the 3 compounds.

Statistical analysis

The obtained data were analyzed using Student's *t*-test with Microsoft Office Excel 2010 and were expressed as mean ± standard deviation. All the experiments were performed in triplicate, and the results were compared with the corresponding control experiments. Responses of UVR-induced cell were compared with those of control cells, with the level of significance set at ***$P < 0.0005$, **$P < 0.005$, and *$P < 0.05$. Statistically significant

differences in the shielding effects of the bioactive compounds were determined by setting the level of significance set at ***P < 0.0005, **P < 0.005, and *P < 0.05.

SECTION S2

Real-time toxicity screening

Before determining the shielding effects of melanin, KRT1, and bFGF, these compounds were screened for their cytotoxicity to determine a safe concentration that promoted maximum cellular activity. To determine the safest concentration of melanin, KRT1, and bFGF, we treated the skin cells with 3 different concentrations (0.05, 0.50, and 1.00 mM; Figure S7) of these compounds for 24 h. Real-time measurements of cellular activity were compared with those of control cells. Results of screening showed that the selected concentrations of the bioactive compounds were non-toxic against the treated cells. Moreover, these concentrations did not induce any significant changes compared with control cells. If the selected concentrations had toxic effects on the treated cells, they would decrease the impedance values of these cells, indicating a decrease in cellular activity. And if the selected concentrations had no toxic effects on the ells, they would not decrease the impedance values of these cells, it's all depends on concentration effects.[1] In this study, 0.05-, 0.5-, and 1-mM concentrations of melanin, KRT1, and bFGF did not exert toxic effects on the treated cells. Therefore, we selected the highest non-toxic and well-tolerated concentration of 1 mM of all the compounds for performing UVR-shielding studies. This measurement approach allows high-throughput screening because it shows early-onset changes in cells that can be used to develop new drugs.

Bioactive compounds	**HDFa**		**HEKa**		**HEMa**	
	254 nm	365 nm	254 nm	365 nm	254 nm	365 nm
bFGF	*	***	***	*	-	-
Melanin	***	*	**	***	***	***
KRT1	-	-	*	**	*	**

Table S1. **Survival/Recovery of irradiated cells because of the shielding effects of bioactive compounds**. ***High survival rate and increase in impedance, **moderate survival rate and minimum increase in impedance, and *low survival rate and steady state or no change in impedance. -No survival and decrease in impedance.

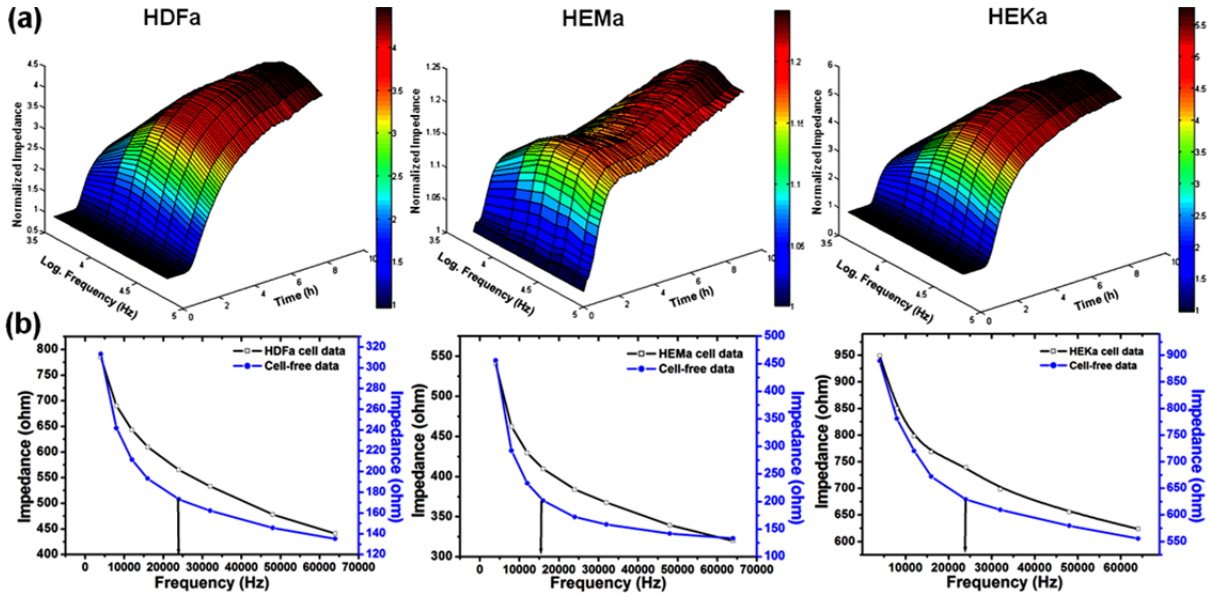

Figure S1. **Characteristic responses of skin cells to different frequencies to determine the best frequency for studying their behavior**. (a) 3D images of changes in impedance as a function of frequency and time during the growth of different pigmented skin cells on the ECIS electrodes. The images show a normalized impedance scale with log frequencies of 4, 8, 12, 16, 24, 32, 48, and 64 kHz for cell behavior. (b) 2D representations of frequency-dependent electrical impedance from data obtained for the cell-covered and cell-free electrodes. Frequency-dependent impedance: Data obtained from the cell-covered and cell-free electrodes were re-plotted from the 3D data to obtain the best signal output for HDFa, HEKa, and HEMa cells.

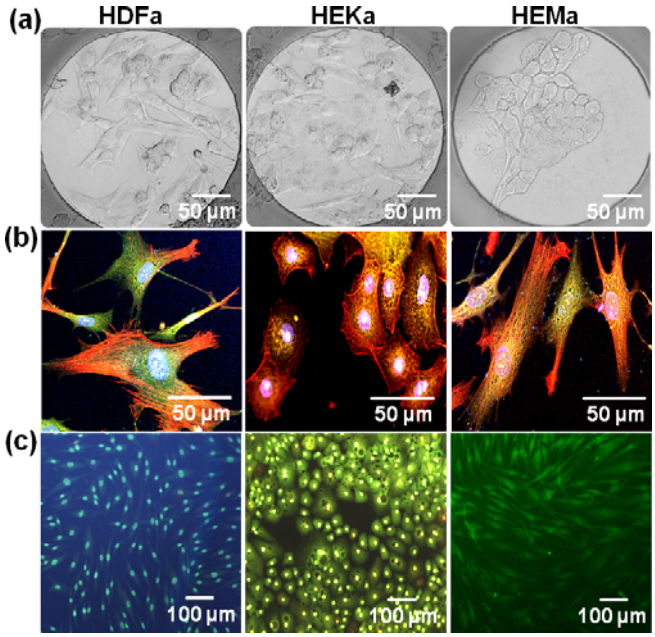

***Figure S2*. HDFa, HEKa, and HEMa cells showing different structural morphology with the control cells after the second passage**. (a) Fluorescence images of cells stained for microtubules (green, Alexa Fluor 488), nuclei (blue, DAPI), and F-actin (red, PHDR1). (b) Live–Dead Cell Staining of control cells. (c) Microscopic images of cells covering the electrode.

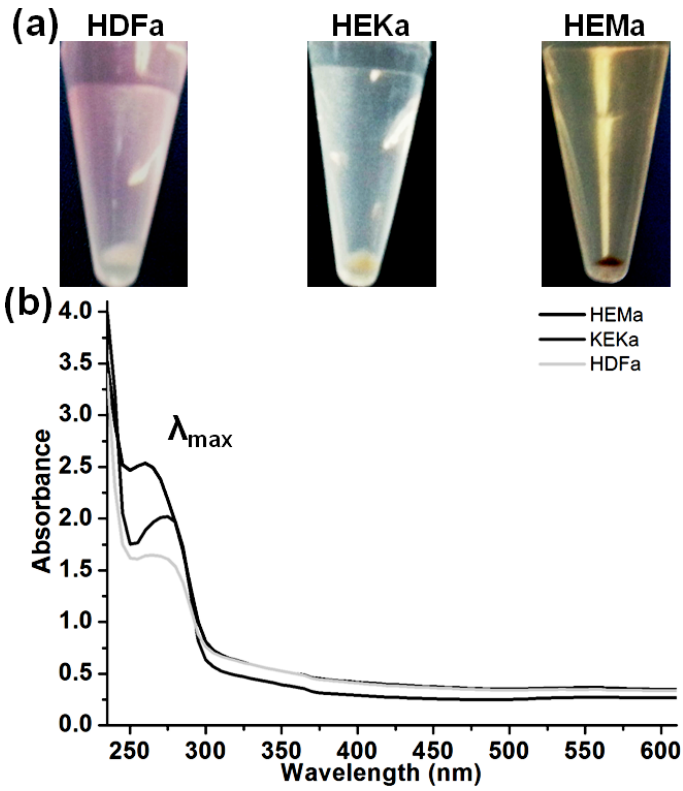

Figure S3. **Spectral absorption by chromophores (photosensitizers) extracted from different skin cells**. (a) Variations in pigmentation were visible in all the 3 cell lines. (b) Absorption spectra of corresponding cells; total concentration of chromophores (photosensitizers) in each cell line.

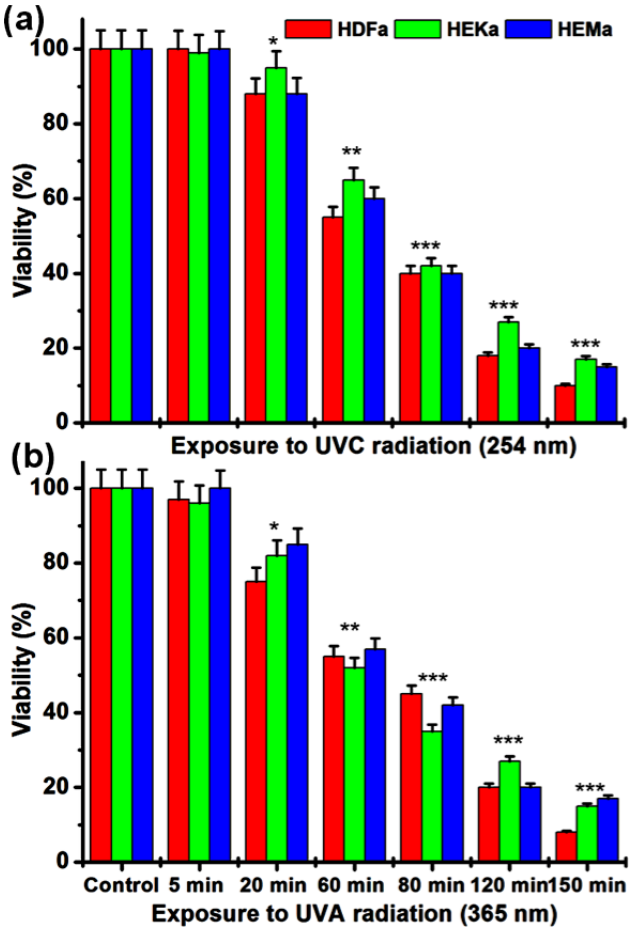

Figure S4. **Time-dependent changes in the viability of HDFa, HEKa, and HEMa cells exposed to UVR**. (a) Effects of exposure to UVC radiation (254 nm). (b) Effects of exposure to UVA radiation (365 nm).

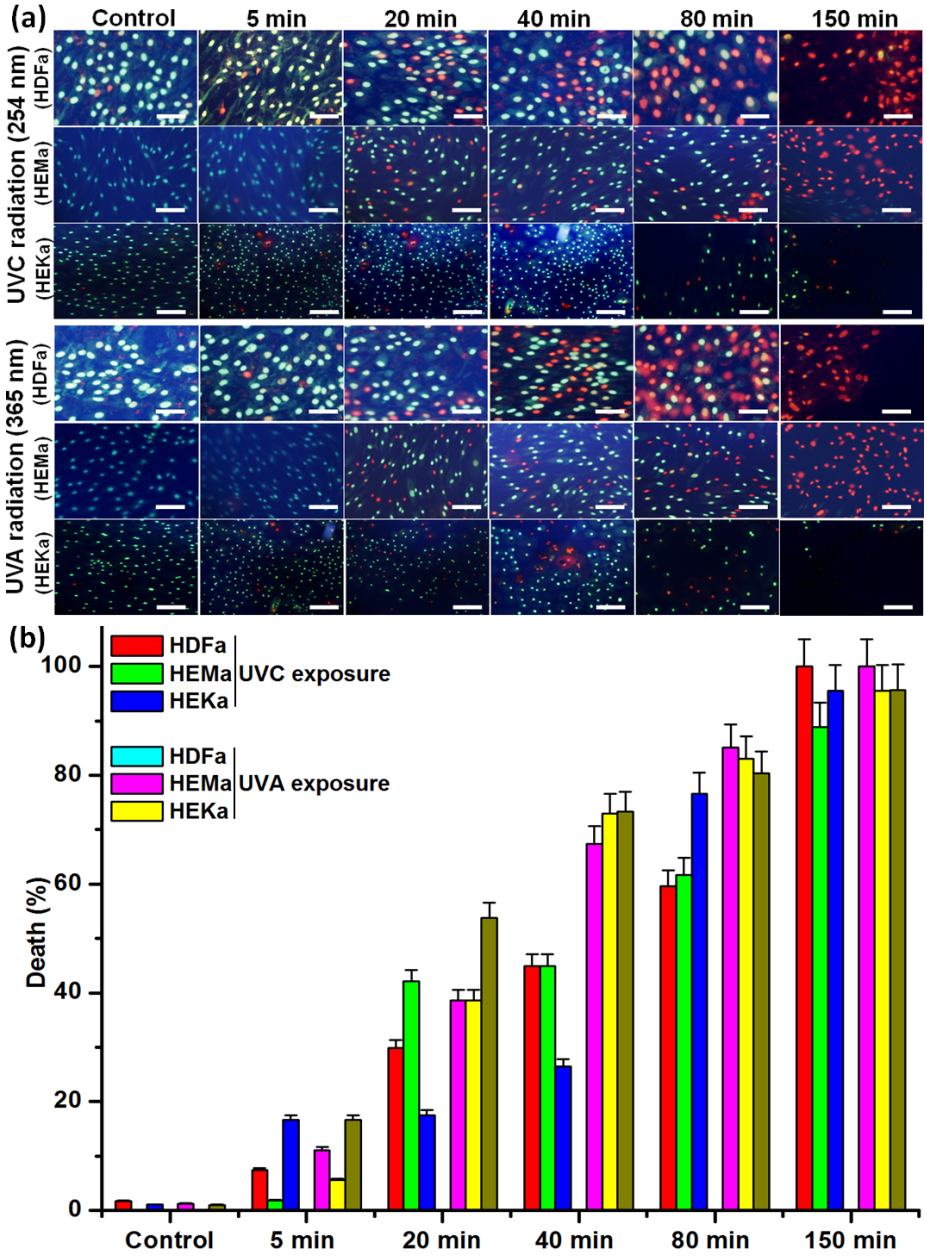

***Figure S5*. Fluorescence microscopy images of the Live–Dead Cell Staining assay of HDFa, HEKa, and HEMa cells after exposure to different UVRs.** (a) Upper part of the image: cells exposed to UVC [254 nm]; lower part of the image: cells exposed to UVA [365 nm]). The images were taken at different time points. Red spots indicate dead cells, and green spots indicate live cells. Scale bars = 50 µm. (b) Effect of different UVRs radiation on percentage of dead cells.

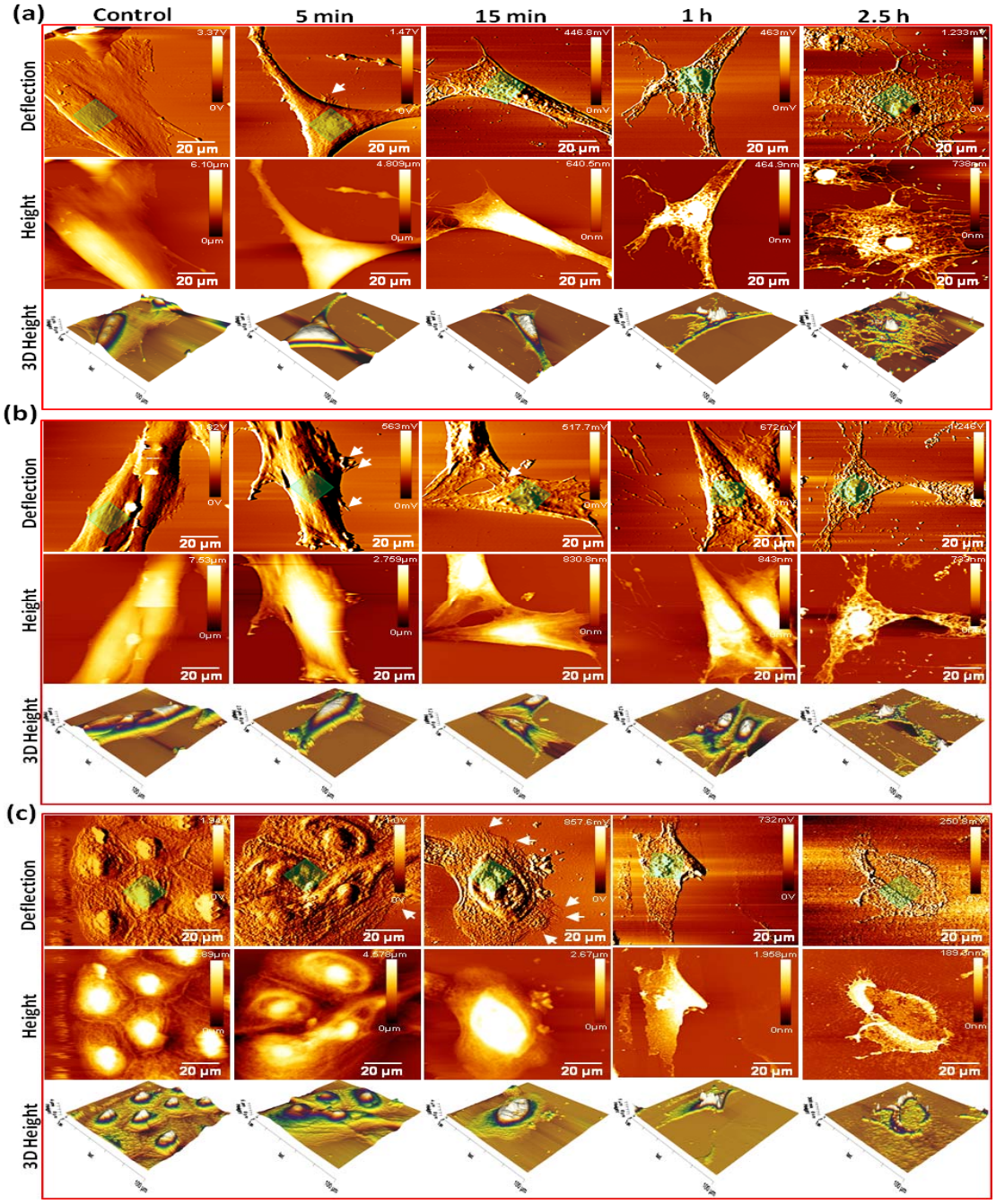

***Figure S6*. Bio-AFM images showing morphological changes (vertical deflection, height, and 3D height) in living cells exposed to UVA radiation (365 nm) at different time points**. (a) Bio-AFM images of HDFa cells, (b) bio-AFM images of HEKa cells, and (c) bio-AFM images of HEMa cells. Control cells show relatively smooth surface. Cells irradiated with UVA show continuous changes such as projections, surface roughness, membrane damage, rupture, and death. Scan size = 100 × 100 µm for all images.

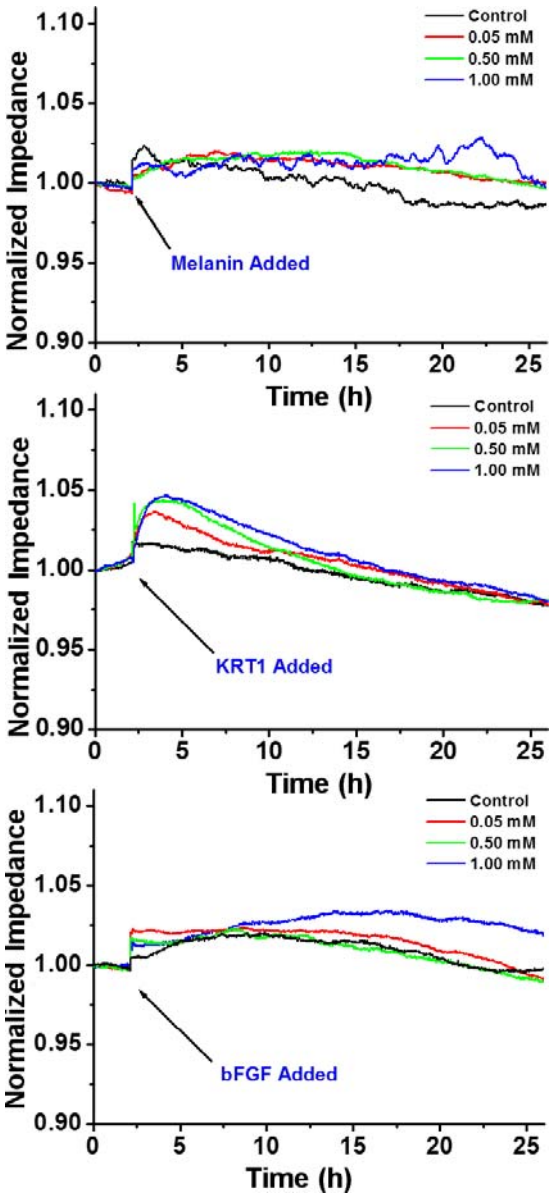

***Figure S7*. Cytotoxic effects of (a) melanin, (b) KRT1, and (c) bFGF on fibroblasts**. Data show normalized impedance profiles of human dermal fibroblasts exposed to different concentrations of these bioactive compounds for 24 h.

Reference

1. D. Bennet, S. Kim, *Anal. Chem.* 2013, **85**, 4902-4911.