2. Experimental details

2.1. Cell culture and treatment

HEK-293 cells were cultivated in DMEM supplemented with 10% FBS, 1% PSS and 1% MEM NEAA at 37 °C in 5% CO₂ and 95% saturated atmospheric humidity. Non-adherent cells were removed by changing the medium every 2-3 days until the cells attained confluence. HEK-293 cells were seeded in flat-bottom well plates and then incubated in a CO₂ incubator overnight, until all cells adhered to the wall. The cells were incubated with ginsenoside F2, C3G and their combination for 12 h and then were immediately exposed to hydrogen peroxide (H₂O₂) for 6 h in a humidified atmosphere of 5% CO₂.

2.2. Cell viability assay

Cell viability was determined by the MTS assay as previously mentioned with a few changes \(^1,^2\), which used the MTS Assay Kit according to the manufacturer’s instructions. MTS (20 μL) was added to the wells after treatment of four groups, followed by incubation at 37°C, 5% CO₂ for 1 hours. Then the plates were read in a multi-mode microplate reader (Bio Tek Instruments, USA) at 490 nm wavelength. The results were expressed as the mean optical density (OD) of each group and dose. All the experiments were repeated at least 3 times.

2.3. Cytotoxic and proliferation assessments of ginsenoside F2, C3G and their combination

The ginsenoside F2 was dissolved in DMSO and DMEM medium, and C3G was dissolved in DMEM medium at different concentrations (1.25, 5 and 20 μM). The
combination was made by mixing the ginsenoside F2 and the C3G with the volume proportion of 1:1, and the final concentrations were 1.25, 5 and 20 μM. HEK-263 Cells were seeded in a 96-well plate of 5 ×10^3 cells/well and incubated for 24 h. Then, the cells were treated with ginsenoside F2, C3G and their combination and were incubated for an additional 12 h at 37°C, 5% CO₂. Cell viability was determined using MTS method as described above.

2.4. Individual and combined protective effects of ginsenoside F2 and C3G against oxidative stress in HEK-293 cells

HEK-293 Cells were seeded in a 96-well plate of 5 ×10^3 cells/well and incubated for 24 h. The cells were pretreated with ginsenoside F2, C3G and their combination (1.25, 5 and 20 μM) for 12 h. Subsequently, 400 μM of H₂O₂ was added and performed for 6 h. Normal HEK-293 cells without ginsenoside F2, C3G and H₂O₂ treatment were used as a control. DMEM and H₂O₂ were added then to the treatment without the addition of ginsenoside F2, C3G as a damage group. Cell viability was determined using MTS method as described above.

2.5. Measurement of intracellular ROS

Production of intracellular ROS was assessed using DCFH-DA as described previously. HEK-293 Cells were seeded in a 24-wells plate of 5 ×10^4 cells/well and incubated for 24 h. After pretreatments of F2, C3G, the combination and 400 μM of H₂O₂, the medium was removed and the cells were incubated with DCFH-DA (10 μM) for 20 min at 37 °C in the dark. The green fluorescence images of DCF in HEK-293 cells were collected by Laser Scanning Confocal Microscopy (LSCM) (Olympus,
Japan). The fluorescence intensity was determined by a multi-mode microplate reader (BioTek Instruments, USA) at an excitation wavelength of 485 nm and an emission wavelength of 528 nm.

2.6. Measurement of antioxidant enzyme activities and MDA level

HEK-293 Cells were seeded in a 12-wells plate of $1 \times 10^5$ cells/well and incubated for 24 h. After pretreatments of F2, C3G, the combination and 400 μM of H$_2$O$_2$, the medium was removed and the cells then washed once with PBS, the cells were lysed in ice-cold radio immunoprecipitation assay (RIPA) lysis buffer containing 50mM Tris-HCl (pH 7.4), 150mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, sodium orthovanadate, sodium fluoride, EDTA, leupeptin and 1 mM phenylmethanesulfonyl fluoride (PMSF) for 10 min and then centrifuged at 12,000g, 4°C for 10 min. Activities of SOD, GSH-Px, CAT, MDA and BCA levels were measured according to the manufacturer’s protocol of corresponding assay kit.

2.7. Western blot analysis

Cultured cells were lysed in RIPA lysis buffer containing 1 mM PMSF on ice for 10 min and then centrifuged at 12,000g, 4°C for 10 min. The diluted suspensions were boiled in loading buffer containing DTT for 5 min. 30 μg of the proteins in supernatant were separated by 12% sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gels electrophoresis and the blots were transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were incubated in blocking buffer (5% nonfat dried milk in Tris-buffered saline with 0.05% (v/v) Tween 20, TBST) at room temperature for 1 h after washed in TBST. And then incubated with antibody against
Nrf2, Keap1 and GAPDH in TBST (1:400 dilution) overnight at 4 °C. The membranes were washed three times with TBST and incubated with horseradish peroxidase-conjugated secondary antibodies (1:4000 dilution) for 1 h at room temperature. The membranes were washed four times with TBST at room temperature and detected by DNR MiniBIS Pro Bio Imaging System (DNR, Israel) using ECL reagents. The images were collected and the bands corresponding to nrf2, keap1 and GAPDH protein were quantitated by densitometric analysis using the ImageJ software (http://rsb.info.nih.gov/ij/index.html). The data were obtained from at least three independent experiments and the data of Nrf2 and Keap1 were normalized on the basis of GAPDH level.

2.8. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

For the quantification of gene expression, quantitative real-time polymerase chain reaction (qRT-PCR) was conducted by using SYBR Green on a real-time system (CFX96; Bio-Rad) according to a previous publication with a few changes. Briefly, total RNA of cells was extracted with TRIzol reagent according to the manufacturer’s instructions. The concentration of total RNA was detected by BioTek Epoch microplate spectrophotometer (BioTek, USA), and cDNA was reversely transcribed from total RNA after erasing gDNA by using a reverse transcriptase kit according to the manufacturer’s instructions. PCR was performed in a real-time PCR system, and the reaction contained 1 μg of cDNA, primers, RNase-free water and SYBR premix. The sequences of the primer pairs used for the amplification of human Nrf2, Keap1 and GAPDH are as followed:
Nrf2 (forward primer: 5’-AACCCTTGTCACCATCTCAG-3’; reverse primer: 5’-
GCAGCCACTTTATTTCTTACC-3’),

Keap1 (forward primer: 5’-ACTCGTTGACGCCAAGCTT-3’; reverse primer: 5’-
GCAGGGCGACCACACTGATT-3’),

GAPDH (forward primer: 5’-ATCCCATCACCACCCTTCCTC-3’; reverse primer: 5’-
CCATCACGCCACAGTTT-3’).

Amplification conditions were set as followed: 95°C for 3 min, 40 cycles of 95°C for 15 sec, 51.6°C (nrf2) or 58°C (keap1) for 20 sec and 72°C for 30 sec. The relative index \(2^{-\Delta\Delta C_T}\) was calculated by comparing the average expression level to control samples which was defined as 1.00. Expression levels of nrf2 and keap1 genes were normalized by concurrent measurement of GAPDH levels.

2.9. Statistical Analysis

All of the assays were carried out in triplicate. Data analyses were performed using the SPSS 21.0 software. The results were expressed as the mean ± standard deviation in the text and figures. The statistical significance of differences between two groups was determined by the one-way ANOVA program with the LSD test unless specified. Probability values of less than 0.05 were considered significant.

Figure 1. Individual and combined protective effects of ginsenoside F2 and C3G on cell viability. Cells were treated with ginsenoside F2, C3G and their combination at the indicated concentrations (1.25, 2.5, 5, 10, and 20 μM) for 12 h and H₂O₂ (400 μM) for 6 h. The combination of 1.25 μM contains 0.625 μM F2 and 0.625 μM C3G, the combination of 2.5 μM contains 1.25 μM F2 and 1.25 μM C3G, the combination of 5 μM contains 2.5 μM F2 and 2.5 μM C3G, the combination of 10 μM contains 5 μM F2 and 5 μM C3G, the combination of 20 μM contains 10 μM F2 and 10 μM C3G. Cell viability was assessed by MTS assay. Vertical bars indicate mean values ± SD (n = 3).
Figure 2. Cytotoxic and proliferative effects of ginsenoside F2, C3G and their combination in HEK-293 cells. Cells were treated with ginsenoside F2, C3G and their combination at the indicated concentrations (1.25, 5 and 20 μM) for 12 h. The combination contains equal amounts of F2 and C3G. Cell viability was assessed by MTS assay. Vertical bars indicate mean values ± SD (n = 3).