Supplementary material:

Title: Unraveling the time-dependent mechanism of quercetin glycosides from natural green silk in ameliorating UV-induced photoaging

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Experimental Section

Construction of the hydroxyproline standard curves

A hydroxyproline standard curve was prepared by serially diluting a 0.5 mg/mL stock solution with distilled water to create a series of concentrations at 15, 7.5, 3.75, 1.875, 0.938, 0.469, 0.234, and 0.117 μg/mL. The assay produced a standard curve with excellent linearity, represented by the regression equation $y = 0.0632x + 0.0031$, where y is the absorbance and x is the hydroxyproline concentration (μg/mL). The correlation coefficient (R^2) was 0.9992.

HPLC analysis conditions

HPLC analysis was performed on a Shimadzu system equipped with a photodiode array detector. Separation was achieved on a Nova-Pak C18 column (150 × 3.9 mm i.d., 4 μm, Waters) maintained at 40 °C. The mobile phase consisted of solvent A: water containing 0.2% formic acid (v/v), and solvent B: acetonitrile containing 0.2% formic acid (v/v). The gradient program was as follows: 0–20 min, linear gradient from 7% to 40% B; 20–30 min, gradient to 100% B; followed by re-equilibration. The flow rate was 1.0 mL/min, and the injection volume was 10 μL. Detection wavelength was set at 365 nm for flavonoid glycosides. The identification of quercetin glycosides was based on comparison of retention times and UV–Vis spectra with authentic standards [1, 2].

Folin-Ciocalteu method

The adaptation was performed as follows: 20 μL of sample solution was mixed with 100 μL of 0.3 M Folin-Ciocalteu reagent and allowed to react for 5 min at room temperature. Then, 80 μL of 10% Na_2CO_3 solution was added, and the mixture was incubated in the dark for 60 min. Absorbance was measured at 760 nm using a microplate reader. To validate the method for molar concentration determination, a calibration curve was constructed using quercetin-3-O-glucoside as the reference standard at concentrations ranging from 5 to 200 μM. The linear regression equation was $y = 0.00165x + 0.028$, with a coefficient of determination (R^2) of 0.9995, indicating excellent linearity over the tested concentration range.

qPCR experimental methodology

1. RNA extraction and cDNA synthesis

Total RNA was extracted from treated cells using the PureLink™ RNA Mini Kit (#12183016; Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The RNA concentration and purity were determined spectrophotometrically. Subsequently, 1 μg of total RNA from each sample was reverse-transcribed into first-strand cDNA using the PrimeScript RT Master Mix (#RR036A; Takara, Japan) in a 20 μL reaction volume.

2. Quantitative PCR (qPCR)

qPCR amplification was performed using the SYBR Premix Ex Taq II (#RR820A; Takara, Japan) on a real-time PCR detection system. Each 20 μL reaction mixture contained 10 μL of SYBR Premix, 2 μL of cDNA template, 0.8 μL of each gene-specific forward and reverse primer (10 μM), and 6.4 μL of RNase-free water. The thermal cycling protocol was as follows: initial denaturation at 95°C for 30 seconds, followed by 40 cycles of denaturation at 95°C for 5 seconds and annealing/extension at 60°C for 30 seconds. A melt curve analysis was performed at the end of each run to confirm the specificity of the amplification.

3. Data analysis

The cycle threshold (Ct) values were determined using the instrument's built-in software. The relative mRNA expression levels of the target genes (COL1A1, MMP1, TGFB1) were normalized to the endogenous control ACTB and calculated using the comparative 2^(–ΔΔCt) method. Statistical analyses were conducted with GraphPad Prism 9.0 software. Data from at least three independent experiments are presented as the mean ± standard deviation (SD). Differences between groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test, with p-value < 0.05 considered statistically significant.

Immunofluorescence staining protocol

1. Fixation:

The culture medium in the chambers was aspirated and discarded. Cells were gently rinsed once with pre-cooled phosphate-buffered saline (PBS). Subsequently, 4% paraformaldehyde was added to cover the cells and fixed at room temperature for 15-20 minutes. After fixation, the cells were washed three times with PBS, 5 minutes per wash.

2. Permeabilization:

After aspirating the PBS, 0.3% Triton X-100, freshly prepared in PBS, was added to permeabilize the cells for 10-15 minutes at room temperature. Following permeabilization, the cells were washed three times with PBS, 5 minutes per wash.

3. Blocking:

The PBS was aspirated, and the cells were blocked with 100 μL of ready-to-use goat serum at room temperature for 60 minutes to minimize non-specific binding.

4. Primary antibody incubation:

After blocking, the blocking solution was carefully removed. The primary antibody, Phospho-Histone H2A.X, was diluted in PBS containing 1% BSA. Then, 70-100 μL of the diluted primary antibody solution was added to each well. The cells were incubated with the primary antibody in a humidified chamber at 4°C overnight. Following incubation, unbound primary antibody was removed by washing the cells three times with PBS, 5 minutes per wash.

5. Secondary antibody incubation:

After aspirating the PBS, 80 μL of the fluorescently-labeled secondary antibody, Goat Anti-Rabbit IgG

Fc, diluted in an antibody dilution buffer, was added to each well. The cells were incubated with the secondary antibody in a humidified, light-protected chamber at room temperature for 1 hour. Post-incubation, unbound secondary antibody was removed by washing the cells three times with PBS in the dark, 5 minutes per wash. This was followed by two additional 1-minute rinses with ddH_2O.

6. Mounting:

The coverslips were mounted using an Antifade Mounting Medium containing DAPI. The mounted samples were stored in a light-protected, humidified chamber at 4°C.

7. Observation:

For fluorescence visualization, DAPI staining (blue fluorescence) was detected using an excitation wavelength of approximately 330-380 nm and an emission wavelength of approximately 420 nm. FITC-labeled γ-H2AX foci (green fluorescence) were detected using an excitation wavelength of approximately 465-495 nm and an emission wavelength of approximately 515-555 nm. Images were acquired using the appropriate fluorescence filter channels.

Supporting Figures

Fluorescent properties of natural green silk and its quercetin glycosides under UV irradiation

Natural green silk exhibits intense fluorescence under UV light due to its unique quercetin glucoside content. This characteristic was further confirmed by UV imaging system analysis, which demonstrated consistent fluorescence in both the raw silk and the purified quercetin glycosides extracted from it [3]. The ^{1}H and ^{13}C NMR spectral data for quercetin-5,4'-O-diglucoside were recorded in DMSO-d_6:

^{1}H NMR (600 MHz, DMSO-d6): δ 4.77 (1H, d, J=7.2 Hz, H-1 in 5-O-glc), 4.91 (1H, d, J=7.2 Hz, H-1 in 4′-O-glc), 6.64 (1H, d, J=1.8 Hz, H-8), 6.80 (1H, d, J=1.8 Hz, H-6), 7.33 (1H, d, J=8.4 Hz, H-5′), 7.75 (1H, dd, J=1.8, 8.4, H-6′), 7.92 (1H, d, J=1.8 Hz, H-2′). ^{13}C NMR (150 MHz, DMSO-d6): δ 61.1(4'-O-Glc-C-6), 62.4(5-O-Glc-C-6), 69.2(5-O-Glc-C-4), 70.6(4'-O-Glc-C-4), 73.4(5-O-Glc-C-2), 74.2(4'-O-Glc-C-2), 76.2d(5-O-Glc-C-3), 76.8d(4'-O-Glc-C-3), 77.6(4'-O-Glc-C-5), 78.4f(5-O-Glc-C-5), 97.1(C-8), 101.0(4'-O-Glc-C-1), 103.2(5-O-Glc-C-1), 104.3(C-6), 106.4(C-10), 115.1(C-2'), 116.5(C-5'), 120.1(C-6'), 125.8(C-1'), 138.2(C-3), 143.7(C-2), 146.9c(C-3'), 147.6c(C-4'), 157.0(C-9), 158.8(C-5), 164.2(C-7), 172.9(C-4).

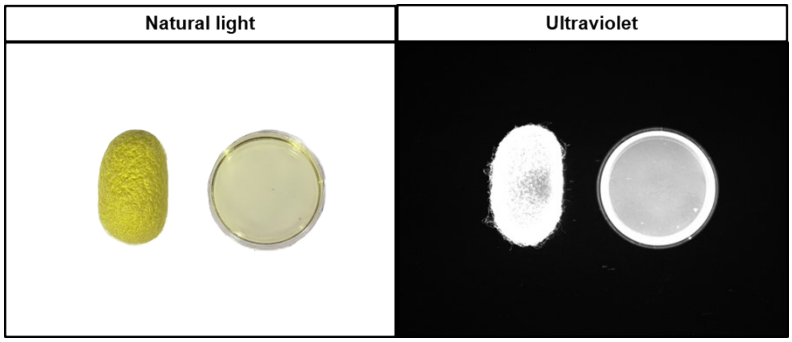

Fig. S1. Comparative visualization of fluorescence in green silk and purified quercetin glycosides

Concentration-gradient screening of quercetin glycosides using the CCK-8 assay

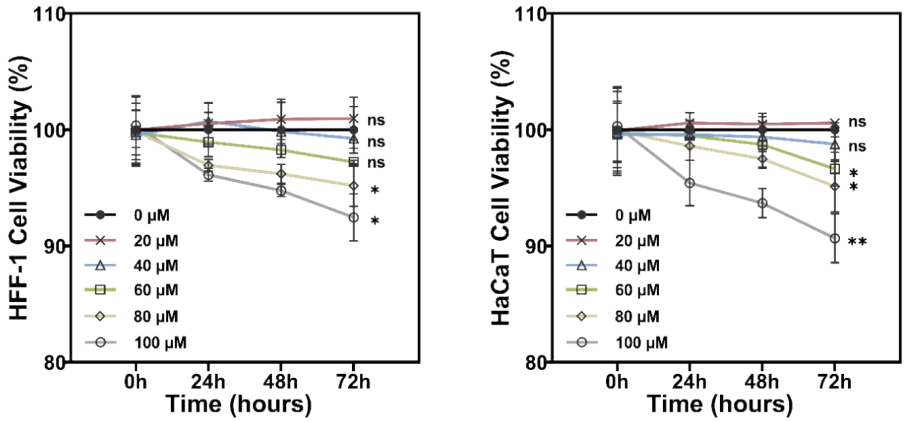

Fig. S2. Cytotoxicity assessment over a 72-hour period

Effects of 24–96 h QGT post-treatment on the viability of UV-irradiated HFF-1 and HaCaT cells

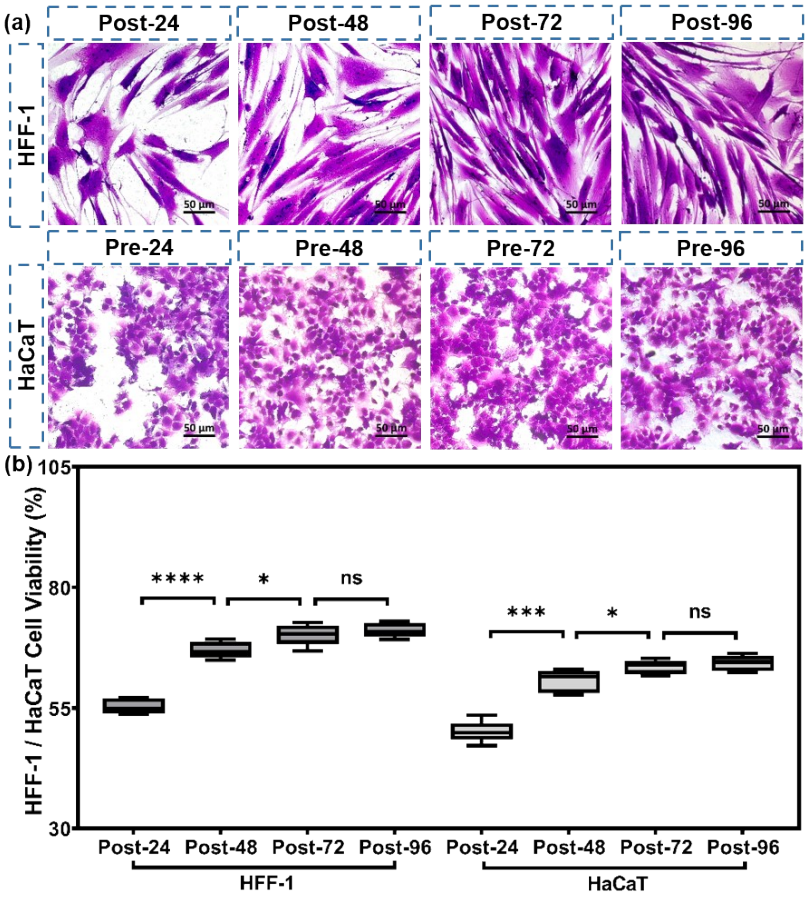

Fig. S3. Cell morphology and viability of HFF-1 and HaCaT cells assessed by crystal violet staining and trypan blue exclusion assay (24-96 h) (a) Crystal violet staining of HFF-1 / HaCaT cells (b) Viability of HFF-1 / HaCaT cells determined by trypan blue exclusion

References

1. C. Hirayama, H. Ono, Y. Tamura, K. Konno and M. Nakamura, Phytochemistry, 2008, 69, 1141-1149.
2. Y. Tamura, K. Nakajima, K. Nagayasu and C. Takabayashi, Phytochemistry, 2002, 59, 275-278.
3. T. Daimon, C. Hirayama, M. Kanai, Y. Ruike, Y. Meng, E. Kosegawa, M. Nakamura, G. Tsujimoto, S. Katsuma and T. Shimada, Proceedings of the National Academy of Sciences of the United States of America, 2010, 107, 11471-11476.