Antioxidant, Anti-photoaging, Anti-inflammatory, and Skin-barrier-protective Effects of *Gleichenia japonica* Extract

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Ferns native to Korea, such as *Davallia mariesii, Dicranopteris pedata*, and *Gleichenia japonica*, possess antioxidant and antibacterial properties. However, their inhibitory effects on skin photoaging have not been demonstrated. Measurement and comparison of the antioxidant activity of three types of ferns revealed that the extract from *G. japonica* had the best effect. This study evaluates the potential of *G. japonica* extract as a new functional material for preventing skin damage caused by ultraviolet radiation. *G. japonica* extracts showed protective effects against ultraviolet B (UVB) radiation in human epidermal keratinocyte cells; the extracts inhibited intracellular reactive oxygen species production. In addition, collagen biosynthesis increased, and matrix metalloproteinase-1 activity and protein expression level decreased in human primary dermal fibroblast irradiated with UVB. The main peak (compound 1) of the extract was separated through high-performance liquid chromatography analysis and preparative liquid chromatography. Compound 1 is strongly inferred to be the main active ingredient because it showed better antioxidant activity and UVB protection effect than *G. japonica* extract. These results demonstrate the physiological effects of *G. japonica* extract and suggest its applicability as a new functional substance for preventing skin damage caused by ultraviolet radiation.

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Keywords: *Gleichenia japonica*; Ferns; Antioxidant; Anti-photoaging; Anti-inflammatory; Skin-barrier

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INTRODUCTION

The skin is continually exposed to the external environment, either directly or indirectly. The main cause of extrinsic aging is ultraviolet radiation, and the resulting aging is called photoaging (Kwon *et al.* 2009).

Ultraviolet radiation is classified into three types according to the wavelength: ultraviolet A (UVA, 320 to 400 nm), ultraviolet B (UVB, 280 to 320 nm), and ultraviolet C (UVC, 200 to 280 nm). Ultraviolet A and UVB radiation can easily pass through the epidermal and dermal layers of the skin, respectively. Ultraviolet B radiation directly acts on the deoxyribonucleic acid (DNA) in cells, causing skin damage such as pigmentation, dermatitis, and cancer (Lee *et al.* 2013; Kim *et al.* 2019). In addition, continuous exposure to UV radiation induces the production of active oxygen, which can damage DNA, proteins, and lipids in the cells, negatively affecting the skin (Kwak and Yang 2016). Collagen is a major extracellular protein present in the dermal layer that plays an important role in maintaining skin elasticity and hydration.
role in skin elasticity. Type I procollagen makes up 80 to 90% of collagen and is essential for preventing skin damage caused by UV rays. It also helps maintain elasticity and moisture balance and suppresses wrinkles (Yoo et al. 2012; Panich et al. 2016). Collagen degrades when matrix metalloproteinase-1 (MMP-1) activity increases. Therefore, when the activity of MMP-1 is reduced, collagen degradation is inhibited, and skin aging and wrinkle formation are prevented. When reactive oxygen species (ROS) are excessively generated, they can induce oxidative stress in human epidermal keratinocyte (HaCaT) cells and fibroblasts, leading to increased apoptosis and synthesis of MMPs that degrade collagen and elastin in the dermal layer of the skin (Davinelli et al. 2018).

Hyaluronic acid (HA) is an important component of the dermal and epidermal layers. The reduction of HA content by UVB radiation reduces the elasticity and moisture content of the skin, which causes the deterioration of skin barrier function (Chen et al. 2021).

Various studies have been conducted to develop safe natural antioxidants, confirm the physiological activities of natural plants, and identify functional materials (Kim et al. 2012; Choi et al. 2010). In addition, because the use of natural raw materials increases the suppression of oxidative stress caused by active oxygen, studies have investigated safe and effective natural raw materials that can help with skin aging (Sim et al. 2015). Ferns have been widely used in traditional medicine because they are generally effective against skin wounds, fever, and cough and have antibacterial, antiviral, and anticancer properties (Kwon et al. 2009; Oh et al. 2020). There are more than 30 types of ferns; however, research is lacking because of the limited understanding of their functions. *Gleichenia japonica* has been identified as a fern with excellent antioxidant and antibacterial activities, as well as high productivity (Zakaria et al. 2011; Mizutani and Masaki 2014). Based on these prior studies, it was hypothesized that *G. japonica* has various activities that can contribute to the prevention of skin aging; however, the effect of *G. japonica* extract on the physiological activities of the skin had not yet been investigated.

**EXPERIMENTAL**

**Chemicals and reagents**

For cell culture, Dulbecco’s Modified Eagle’s Medium (DMEM) and phosphate buffered saline (PBS) were purchased from Lonza (Walkersville, MD, USA). Fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Gibco (Thermo Fisher Scientific, Rockford, IL, USA). Fibroblast medium (FM), fibroblast growth supplement (FGS), and penicillin/streptomycin were purchased from ScienCell (Carlsbad, CA, USA) for primary cell culture. The compounds 1,1-diphenyl-2-picrylhydrazyl (DPPH), 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) used in the antioxidant activity test were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The Quantikine Human Pro-MMP-1 ELISA Kit was purchased from R & D Systems (Minneapolis, MN, USA). The procollagen Type I C-peptide (PIP) EIA Kit was purchased from Takara Bio, Inc. (Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), used for protein expression analysis, was purchased from Enogen Biotechnology (New York, USA), and MMP-1 antibody was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The experiments were performed using a UV/Vis spectrophotometer (Optizen 2120 UV, Mecasys, Daejeon, Korea), microplate spectrophotometer (Thermo Fisher Scientific, Multiskan Sky, Boseong, Korea), centrifuge
Preparation of G. japonica extract

*D. mariesii*, *D. pedata*, and *G. japonica* were purchased from the Korea Plant Extract Bank (KPEB., Daejeon, Korea). *D. mariesii* was collected in 2003 from Namwon-eup, Seogwipo-si, Jeju, special self-governing province, whereas *D. pedata* and *G. japonica* were collected in 2016 from Goheung, Jeollanam-do. Sixty-one g of dried, powdered sample was extracted using 1 L methyl alcohol and then concentrated. The concentrated sample was dissolved in DMSO at a concentration of 20 mg/mL and stored at -20 °C before use.

Cell culture

Human epidermal keratinocyte cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin under 37 °C and 5% carbon dioxide (CO₂) conditions. Human primary dermal fibroblasts (HDFs) were purchased from ScienCell (Carlsbad, CA, USA) and cultured in FM containing 2% FBS, 1% penicillin/streptomycin, and 1% FGS in a humidified atmosphere with 5% CO₂ at 37 °C.

Total phenolic content (TPC)

Total phenolic content was determined using a modified Folin-Ciocalteu's reagent (FCR) method (Folin and Denis 1912). The sample was diluted in distilled water to a concentration of 1,000 µg/mL; next, 500 µL of diluted sample was mixed with 500 µL of FCR and reacted at room temperature for 3 min. Thereafter, 500 µL of 10% sodium carbonate was added and mixed, light was blocked, and the absorbance was measured at 760 nm using a UV-visible spectrometer after standing at room temperature for 1 h. A calibration curve was prepared using gallic acid (10 to 60 µg/mL) as a standard (R² = 0.999).

Total flavonoid content (TFC)

Total flavonoid content was measured using an aluminum chloride (AlCl₃) colorimetric assay (Jia et al. 1999). Samples were diluted to a concentration of 1,000 µg/mL in distilled water. Next, 800 µL of 80% ethanol and 60 µL of 5% sodium nitrate were added to 200 µL of the sample, mixed, and allowed to stand for 5 min. Then, 60 µL of 10% AlCl₃ was added, mixed, and allowed to stand at room temperature for 5 min. Subsequently, 400 µL of 1 M sodium hydroxide solution was added and mixed, and the absorbance was measured at 510 nm using a UV-visible spectrometer. A calibration curve was prepared using catechin (50 to 600 µg/mL) as a standard (R² = 0.999).

DPPH radical scavenging activity

The radical scavenging activity of DPPH was measured using a modified Blois method (Blois 1958). After diluting the sample in methanol (MeOH), 100 µL of the sample and 100 µL of 0.2 mM DPPH reagent were dispensed into a 96-well plate and mixed. After diluting the sample in MeOH, 100 µL of the sample and 100 µL of 0.2 m mM DPPH reagent were dispensed into a 96-well plate and mixed. After mixing, the mixture was allowed to stand at 25 °C for 30 min, and the absorbance was measured at 515 nm using a microplate spectrophotometer. L-ascorbic acid was used as a positive control, and the DPPH radical
scavenging rate was calculated according to the following formula, where A stands for absorbance.

\[
\text{DPPH radical scavenging (\%) } = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100
\]

**Measurement of reactive oxygen species (ROS) scavenging activity**

Human epidermal keratinocyte cells were dispensed into a 24-well plate at 5.5 × 10⁴ cells/well and cultured for 24 h in a 37 °C, 5% CO₂ incubator. Ultraviolet B radiation (15 mJ/cm²) was irradiated, and the samples were treated at each concentration and cultured for 24 h. Afterwards, CellROX™ Orange Reagent was used to measure the amount of ROS generation. The concentration of CellROX was measured using a fluorescence microscope EVOS™ M5000 cell imaging system (Thermo Fisher Scientific, MA, USA) after reacting at 37 °C for 30 min after treatment with 5 μM. Each digitized value was calculated as an average by counting the CellROX-stained cells in the three images.

**Cell viability assay**

Cell viability was measured using the MTT method (Mosmann 1983). Human epidermal keratinocyte cells and HDF were dispensed into a 96-well plate and cultured for 24 h in a 37 °C, 5% CO₂ incubator. Thereafter, the extract was treated with 10 μL at each concentration and cultured for 72 h. Subsequently, the culture medium was removed, treated with MTT reagent prepared at a concentration of 0.5 mg/mL and cultured for 3 h. The treated MTT reagent was removed, DMSO was added to each well, incubated for 15 min at room temperature, and the absorbance was measured at 570 nm using a microplate spectrophotometer.

**UVB irradiation**

The evaluation of the cytotoxic protective effect of UVB in HaCaT cells was measured by modifying Wang’s method (Cao et al. 2013). Human epidermal keratinocyte cells were dispensed into a 24-well plate at 5.5 × 10⁴ cells/well and cultured for 24 h in a 37 °C, 5% CO₂ incubator. Then, UVB (15 mJ/cm²) was irradiated, and the extract was treated for each concentration and cultured for 24 h. After removing the medium, it was treated with MTT reagent prepared at a concentration of 0.5 mg/mL and cultured for 3 h. After removing the MTT reagent, 500 μL of DMSO was added to each well, reacted at room temperature for 15 min, and then absorbance was measured at 570 nm using a microplate spectrophotometer.

**Measurement of type I pro-collagen synthesis**

Type I pro-collagen biosynthesis was measured using a pro-collagen Type I C-peptide (PIP) EIA Kit (No. MK101), according to the manufacturer’s protocol as follows. Human primary dermal fibroblast was dispensed into a 6-well plate at 1.5 × 10⁵ cells/well and cultured for 24 h in a 37 °C, 5% CO₂ incubator. Then, UVB (10 mJ/cm²) was irradiated, and the extract was treated and cultured for 72 h. After culturing, 1 mL of the medium was taken and centrifuged at 4 °C at 12,000 rpm for 10 min. After diluting the supernatant, 100 μL of the antibody-POD conjugate solution and 20 μL of the diluted supernatant was added to each well of the microplate, mixed well, and left in an incubator at 37 °C for 3 h. After leaving the solution, the solution was removed, washed with 1X PBS, and substrate solution was added at 100 μL each, and left for 15 min. Finally, 100 μL of the stop solution
was added, mixed well to stop the reaction, and the absorbance was measured at 450 nm using a microplate spectrophotometer.

**Measurement of MMP-1 inhibitory activity**
Matrix metalloproteinase-1 inhibitory activity was measured using the Human Pro-MMP-1 Quantikine ELISA Kit (No. PDMP100) according to the manufacturer’s instructions. Human primary dermal fibroblast was dispensed into a 6-well plate at 1.5 × 10⁶ cells/well and cultured for 24 h in a 37 °C, 5% CO₂ incubator. Thereafter, UVB (10 mJ/cm²) was irradiated, and the extract was treated and cultured for 72 h, and then 1 mL of the medium was taken and centrifuged at 4 °C at 12,000 rpm for 15 min. After diluting an appropriate amount of the supernatant in diluent buffer, 100 μL of diluent RD1-52 and 100 μL of the sample were dispensed into wells of a microplate, covered, and allowed to react at room temperature for 2 h. After removing the contents of each well and washing four times with wash buffer, anti-human pro-MMP-1 conjugate was added, covered, and allowed to react for 2 h. Thereafter, the contents were removed and washed four times with wash buffer, and the substrate solution was added and allowed to react for 20 min in the dark at room temperature. Finally, the stop solution was added to each well and mixed well, and the absorbance was measured at 450 and 540 nm using a microplate spectrophotometer. The final absorbance was calculated by subtracting the 540 nm absorbance value from the 450 nm absorbance value.

**Measurement of hyaluronic acid production**
Hyaluronic acid production was measured using a Hyaluronan Immunoassay Kit (R&D Systems, USA). Human epidermal keratinocyte cells were dispensed into a 6-well plate at 5.5 × 10⁴ cells/well, cultured for 24 h, irradiated with UVB (15 mJ/cm²), and samples were treated by concentration and cultured for 24 h. After culturing, 1 mL of the medium was taken and centrifuged at 4 °C at 12,000 rpm for 15 min. After diluting the supernatant, 50 μL of Diluent RD1-14 and 50 μL of sample were dispensed into the wells of the plate, covered, and reacted for 2 h in a shaker (500 ± 50 rpm) at room temperature. After removing the contents of each well, washing five times with 1X wash buffer, 100 μL of hyaluronan conjugate was added, covered, and allowed to react for 2 h. Thereafter, the contents were removed and washed five times with wash buffer, and the substrate solution was added and allowed to react for 30 min in the dark at room temperature. Finally, stop solution was added to each well, mixed well, and the absorbance was measured at 450 nm and 540 nm using a microplate spectrophotometer. The final absorbance was calculated by subtracting the absorbance at 540 nm from that at 450 nm.

**Western blot analysis**
Human primary dermal fibroblasts were seeded at a density of 1.5 × 10⁵ cells/well (6-well plate), cultured for 24 h, irradiated with UVB (10 mJ/cm²), incubated with the extract, and cultured for 72 h. The cells were lysed in Pro-Prep lysis solution for 15 min on ice. Cell lysates were centrifuged at 13,000 rpm, 4 °C for 5 min. Proteins in the lysed supernatant were measured using the Bradford assay. Protein (20 μg) was subjected to 10% sodium do-decyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane. After blocking the transferred membrane with 5% skim milk for 1 h, the primary antibody was diluted in 5% skim milk and reacted at room temperature for 1 h. The secondary antibody was also reacted for 1 h at room temperature in the same manner. Membrane washing was performed four times with tris-
buffered saline and Tween 20 for 10 min each, and protein expression levels were measured using an enhanced chemiluminescence western blotting reagent (Advansta, USA).

**Measurement of nitric oxide (NO) production**
To measure the inhibitory effect on NO production at RAW 264.7 cells activated with lipopolysaccharide (LPS), RAW 264.7 cells were dispensed into a 96-well plate at 3 × 10^4 cells/well and cultured for 24 h. Then, LPS was treated at a concentration of 50 ng/mL and cultured for 24 h. After recovering the cultured supernatant, it was centrifuged at 4 °C and 12,000 rpm for 10 min, mixed 50 µL of the supernatant and 50 µL of 1X Griess reagent in a 1:1 ratio in a new 96-well plate, and incubated in a 25 °C incubator. After recovering the cultured supernatant, it was centrifuged at 12,000 rpm, 4 °C for 10 min, mixed with 50 µL of 1X Griess reagent in a 1:1 ratio in a new 96-well plate, and incubated for 15 min at 25 °C. Subsequently, absorbance was measured at 540 nm using an ELISA reader.

**High-performance liquid chromatography (HPLC) analysis**
The sample was placed into 25 mL of 1% DMSO in MeOH and subjected to 1 h of sonication. Subsequently, the sample was filtered using a 0.45 µm nylon filter after ultrasonic extraction. Each standard was diluted in 1% DMSO in MeOH and used as the reference solution for the assay. Dimethyl sulfoxide, neo-chlorogenic acid, chlorogenic acid, rutin hydrate, ferulic acid, quercetin, quercitrin, and myricetin A were purchased from Sigma-Aldrich (USA). Acetonitrile (100%) and glacial acetic acid were purchased from J.T. Baker (USA). Component analysis of the *G. japonica* extract was performed using an Agilent 1260 HPLC (Agilent Technologies, Santa Clara, CA, USA) instrument. The column used was the YMC-Pack ODS-AM (250 × 4.6 mm, 5 µm, YMC CO., Kyoto, Japan). Distilled water containing 1% acetic acid (solvent A) and 100% acetonitrile (solvent B) was used as the mobile phase, and all solvents were filtered through a filter before use. The absorbance was measured at 254 and 350 nm. The elution of the gradient and analytical conditions is described in Table 1.

**Table 1. Analytical Conditions of HPLC for *G. japonica* Extract Analysis**

<table>
<thead>
<tr>
<th>Gradient Condition</th>
<th>YMC-Pack ODS-AM (250 × 4.6 mm, 5 µm), 40 °C</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>250, 350 nm, 0.8 mL/min, 5 µL</td>
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<tr>
<td></td>
<td>A: 1% acetic acid, B: acetonitrile</td>
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<tr>
<td>Time (min)</td>
<td>A (%)</td>
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<td>0</td>
<td>95</td>
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<td>40</td>
<td>60</td>
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<td>43</td>
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<td>55</td>
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<td>63</td>
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**Preparative-liquid chromatography (Prep-LC) analysis**
The extract was diluted to a concentration of 20 mg/mL using 1% DMSO in MeOH and then subjected to ultrasonic extraction for 60 min. The extract was passed through a 0.45 µm nylon filter, and 1 mL was injected into the instrument. Preparative liquid chromatography was performed using an Agilent 1260 infinity II prep binary pump with a
1260 infinity II variable wavelength detector and a 1290 infinity II open-bed sampler/collector, with a column of infinity lab pursuit XRs C18, 21.2 × 250 mm, 5 μm (Agilent Technologies, Palo Alto, CA, USA). Distilled water containing 0.1% formic acid (solvent A) and 100% acetonitrile (solvent B) were used as the mobile phase, and all solvents were filtered through a filter before use. The absorbance was measured at 350 nm. The elution of the gradient and analytical conditions is described in Table 2.

Table 2. Analytical Conditions of Prep-HPLC for G. japonica Extract Analysis

<table>
<thead>
<tr>
<th>Gradient Condition</th>
<th>Column</th>
<th>Detector</th>
<th>Flow Rate</th>
<th>Injection Volume</th>
<th>Mobile Phase</th>
<th>Time (min)</th>
<th>A (%)</th>
<th>B (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Infinity lab Pursuit XRs C18 (250 × 21.2 mm, 5 μm), 25 °C</td>
<td>350 nm</td>
<td>1 mL</td>
<td>15 mL/min</td>
<td>A:1% acetic acid, B: acetonitrile</td>
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<td>17</td>
<td>95</td>
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Statistical analyses

Tests of all experimental conditions were repeated three times and expressed as mean ± standard deviation (SD). The normality test was performed using the Shapiro-Wilk test of the SPSS program. Statistical significance was determined using Student’s t-test and ANOVA. All statistical analyses were performed using SPSS statistical software 27.0. The values *p < 0.05, **p < 0.01 and ***p < 0.001 were considered to indicate significant differences.

RESULTS AND DISCUSSION

Total Phenolic and Flavonoid Content of G. japonica Extract

Table 3 shows the results quantifying the total polyphenol and flavonoid compounds contained in the D. mariesii, D. pedata, and G. japonica extracts. Total phenolic content and TFC were measured by converting the amounts of gallic acid and catechin, respectively. Table 3 demonstrates that the G. japonica extract exhibited a high TPC of 93.2 ± 0.16 μg GAE/g and TFC of 95.3 ± 0.23 μg CE/g.

Table 3. Total Flavonoids and Flavonoid Contents of D. mariesii, D. pedata, and G. japonica Extract

<table>
<thead>
<tr>
<th>Sample</th>
<th>TPC (μg GAE/g)</th>
<th>TFC (μg CE/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. mariesii</td>
<td>72.5 ± 0.1a</td>
<td>60.9 ± 0.14a</td>
</tr>
<tr>
<td>D. pedata</td>
<td>87.2 ± 0.25b</td>
<td>76.4 ± 1.2b</td>
</tr>
<tr>
<td>G. japonica</td>
<td>93.2 ± 0.16c</td>
<td>95.3 ± 0.23c</td>
</tr>
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</table>

* Results are expressed as mean values ± standard deviation (n = 3).
* Means with different superscript letters (a–c) indicate statistically significant differences (p < 0.05).
DPPH radical scavenging activity of the G. japonica extract

Radical scavenging activity of DPPH was measured, and the antioxidant properties of the extracts of *D. mariesii*, *D. pedata*, and *G. japonica* were compared. Each extract was treated at concentrations from 10 to 200 μg/mL, and the DPPH radical scavenging activity was measured and compared. *G. japonica* extract showed the highest activity among the extracts with an EC_{50} value of 49.8 μg/mL for DPPH radical scavenging activity (Table 4).

**Table 4. DPPH Radical Scavenging Activity**

<table>
<thead>
<tr>
<th>Sample</th>
<th>EC_{50} for DPPH (μg/mL)</th>
</tr>
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<tbody>
<tr>
<td><em>D. mariesii</em></td>
<td>51.2 ± 0.9^a</td>
</tr>
<tr>
<td><em>D. pedata</em></td>
<td>52.9 ± 3.17^b</td>
</tr>
<tr>
<td><em>G. japonica</em></td>
<td>49.8 ± 1.47^c</td>
</tr>
<tr>
<td>L-ascorbic acid</td>
<td>12.2 ± 0.05^d</td>
</tr>
</tbody>
</table>

**Fig. 1.** Effects of *G. japonica* extract on intracellular ROS production (Nor: normal-control, Con: control, NAC: N-acetylcysteine). (a) The levels of intracellular ROS in *G. japonica* extract treated cells were evaluated using CellROX. (b) Quantity of production of intracellular ROS by (a). The cells were irradiated with UVB 15 mJ/cm², and then treated with the extract and cultured for 24 h. ###p < 0.001 compared with normal-control, ***p < 0.001 compared with the control.
Cell viability of the G. japonica extract

To investigate the effect of G. japonica extract on cytotoxicity in HDF, cell viability was measured by MTT assay. The extract was treated at concentrations of 10, 25, 50, 100, and 200 μg/mL, and cell viability was confirmed after 72 h. As a result, it was observed that the extract showed no cytotoxicity up to a concentration of 50 μg/mL and exhibited a cell viability of about 90% or more. However, at concentrations of 100 and 200 μg/mL, the cell viability was slightly decreased (Fig. 2). Therefore, subsequent experiments were conducted using HDF using three concentrations (10, 25, and 50 μg/mL).

Effect of G. japonica on UVB-induced damage in HaCaT Cells

Human-derived normal skin keratinocytes have been used in many experiments related to skin regeneration and aging. To confirm whether G. japonica extract protects cells from cell damage caused by UVB, the MTT assay was performed on UVB-irradiated HaCaT cells and evaluated.
When HaCaT cells were irradiated with UVB (15 mJ/cm²), cell viability decreased to approximately 80% of that in the untreated control group. However, when the *G. japonica* extract was treated at concentrations of 10, 25, 50, and 100 μg/mL, the cell viability improved in a concentration-dependent manner, demonstrating a cytoprotective effect (Fig. 3).

**Effect on type I pro-collagen biosynthesis**

To evaluate the effect of *G. japonica* extract on the biosynthesis of Type I pro-collagen secreted into cells in UVB-irradiated HDF, HDF was irradiated with UVB at 10 mJ/cm², and then the extract was treated at concentrations 10, 25, and 50 μg/mL. *G. japonica* extract increased the content of pro-collagen, which was reduced by UVB irradiation in a concentration-dependent manner, and at a concentration of 50 μg/mL, it increased by about 54% or more (Fig. 4). These results suggest that *G. japonica* extract can effectively increase the pro-collagen biosynthesis reduced by UVB irradiation.

**Fig. 4.** Effect of *G. japonica* extract on type I procollagen synthesis in HDF (Nor: normal-control, Con: control). Cells were treated with 10 to 50 μg/mL *G. japonica* extract for 72 h. Data are expressed as mean ± SD of three independent experiments. #**p < 0.001 compared to unirradiated normal-control, *****p < 0.001 compared to UVB irradiated control group.
**Effect on MMP-1 inhibitory activity**

The effect of *G. japonica* extract on the production of extracellular MMP-1 after UVB irradiation was investigated. Matrix metalloproteinase-1 activity increased in HDF following UVB (10 mJ/cm²) irradiation. The activity was inhibited by approximately 55% or more compared to that of the control (Fig. 5). *G. japonica* extract can fundamentally resolve wrinkles caused by skin aging by effectively inhibiting extracellular matrix (ECM) decomposition and enhancing collagen synthesis.

**Measurement of hyaluronic acid (HA) production**

To verify the moisturizing effect of *G. japonica* extract on skin-barrier protection, the amount of HA produced was measured in HaCaT cells. Human epidermal keratinocyte cells irradiated with UVB (15 mJ/cm²) were treated with *G. japonica* extract at concentrations of 10, 25, 50, and 100 μg/mL for 24 h, and the amount of HA produced was measured using an ELISA kit. Ultraviolet B irradiation can reduce HA production; here, HA production was increased in a concentration-dependent manner, and at a concentration of 100 μg/mL, it increased by about 60% or more compared to the control (Fig. 6). These results indicate that *G. japonica* extract can be effective in moisturizing the skin.
**Fig. 6.** Effect of *G. japonica* extract in the production of hyaluronic acid in HaCaT cells (Nor: normal-control, Con: control, L-AS: L-Ascorbic acid). Cells were treated with 10 to 100 μg/mL *G. japonica* extract for 24 h. Data are expressed as mean ± SD of three independent experiments. ###*p* < 0.001 compared to unirradiated normal-control, ***p* < 0.001 compared to UVB irradiated control group.

**Effect on MMP-1 protein expression**

Western blot analysis was performed to confirm the effect of *G. japonica* extract on the extract on the expression of MMP-1, an enzyme that degrades collagen. Upon irradiation of HDF with UVB (10 mJ/cm²) and subsequent treatment with *G. japonica* extract at concentrations of 10, 25, and 50 μg/mL, a concentration-dependent inhibition of MMP-1 expression induced by UVB irradiation was observed, suggesting a potential protective effect of the *G. japonica* extract against photoaging. At a concentration of 50 μg/mL, MMP-1 expression level was reduced by approximately 80% or more (Fig. 7). These results indicate that *G. japonica* extract can prevent skin photoaging by downregulating MMP-1 expression in HDF.
Fig. 7. (a) Effect of *G. japonica* extract on MMP-1 protein expression in HDF (Nor: normal-control, Con: control). Cells were treated with 10 to 50 μg/mL *G. japonica* extract for 24 h. Data are expressed as mean ± SD of three independent experiments. GAPDH was used as a loading control and MMP-1 protein levels were normalized by GAPDH. (b) Band intensity compared to the control (GAPDH) was determined using TotalLab 1D software. Data are expressed as mean ± SD of three independent experiments. ### p < 0.001 compared to unirradiated normal-control, ***p < 0.001 compared to UVB irradiated control group.

NO production measurement

Lipopolysaccharide is a gram-negative bacterial cell wall component that stimulates macrophages to secrete various pro-inflammatory mediators such as NO, interleukin 6, and tumor necrosis factor α to trigger an immune response in the body (Pillai *et al.* 2005). To confirm the potential anti-inflammatory effect of *G. japonica* extract, changes in activated NO production were measured in LPS-induced RAW 264.7 cells. In 100 μg/mL, it was reduced by more than 49% compared to the control (Fig. 8). These results indicated that *G. japonica* extract may have anti-inflammatory activity against LPS-induced excessive NO production.
Fig. 8. Effect of *G. japonica* extract on NO production in RAW 264.7 cells (Nor: normal-control, Con: control, LPS: lipopolysaccharide). Cells were treated with 10 to 100 μg/mL *G. japonica* extract for 24 h. Data are expressed as mean ± SD of three independent experiments. ###*p* < 0.001 compared to unirradiated normal-control, ***p* < 0.001 compared to LPS treated control group.

**HPLC and Prep-LC analysis of *G. japonica* extract**

Rutin and quercetin are the well-known components of ferns (Kim et al. 2012). To confirm the major components of *G. japonica* extract, rutin and quercitrin, neochlorogenic acid, chlorogenic acid, ferulic acid, quercetin, myricetin were used as standards and analyzed by HPLC to confirm the major components of *G. japonica* extract. After examining seven standards, results indicated that neochlorogenic acid, chlorogenic acid, quercetin, and myricetin were not present. The retention times were similar to rutin, ferulic acid, and quercitrin. However, the UV spectrum comparison of ferulic acid revealed a variance in the sample and standard. Therefore, it was not considered the same substance (Fig. 9).

To ensure accuracy, a standard was added to the sample and the chromatogram was verified. As a result, two distinct peaks were identified around rutin and quercitrin. It was thus established that they were not the same substance. Therefore, the main peak detected at 11 minutes was then isolated and the composition was confirmed (Fig. 10). After changing the assay for separation, the peak (compound 1) was separated using Prep-LC, and the UV spectrum was confirmed (Fig. 11).

As a result of comparing the DPPH radical scavenging ability and UVB protection effect, compound 1 showed a better effect than *G. japonica* extract, so it is strongly inferred to be the active ingredient (Table 5, Fig. 12). It is thought that quantitative analysis by LC-MS/MS analysis method is necessary to clearly verify the active ingredients of *G. japonica* extract in the future.
Fig. 9. High-performance liquid chromatography (HPLC) chromatogram and UV spectrum of *G. japonica* extract and standards. (A) Standards, (B) *G. japonica* extract 350 nm, and (C) *G. japonica* extract 253 nm chromatogram. Compared the UV spectrum of the sample to a standard detected at the same retention time. (D) Rutin hydrate, (E) Ferulic acid, (F) Quercitrin.

Fig. 10. High-performance liquid chromatography (HPLC) chromatogram and UV spectrum with standards added to the *G. japonica* extract. (A) Standards chromatogram (B) Added a standard to the *G. japonica* extract chromatogram.
Fig. 11. High-performance liquid chromatography (HPLC) chromatogram and UV spectrum with standards added to the *G. japonica* extract. (A) Standards chromatogram (B) Added a standard to the *G. japonica* extract chromatogram

**Table 5.** Effect of Compound 1 on DPPH Radical Scavenging Activity

<table>
<thead>
<tr>
<th>Sample</th>
<th>EC$_{50}$ for DPPH (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound 1</td>
<td>19.8 ± 0.4</td>
</tr>
<tr>
<td>L-ascorbic acid</td>
<td>12.2 ± 0.05</td>
</tr>
</tbody>
</table>

Note: Results are expressed as mean values ± standard deviation (n = 3).

Fig. 12. Effect of *G. japonica* and compound 1 on cell viability in HaCaT cell exposed to UVB irradiation (Nor: normal-control, Con: control). Cells were treated with 10 to 100 μg/mL *G. japonica* and compound 1 for 48 h. Data are expressed as mean ± SD of three independent experiments. ###*p < 0.001 compared to unirradiated normal-control, ***p < 0.001 compared to UVB irradiated control group.
CONCLUSIONS

1. The *Gleichenia japonica* extract exhibited antioxidant, anti-photoaging, skin-barrier-promoting, and anti-inflammatory effects in HaCaT, HDF, and RAW 264.7 cells.

2. These results indicated that *G. japonica* extract can be used as an active ingredient to prevent and treat skin damage caused by ultraviolet radiation by maintaining skin homeostasis.

3. However, since the active ingredient analysis and mechanisms of action for the anti-photoaging and anti-inflammatory effects of *G. japonica* extract have not yet been fully clarified, more detailed research is needed in the future.

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