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Brazilin and *Caesalpinia sappan* L. extract protect epidermal keratinocytes from oxidative stress by inducing the expression of GPX7

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[ABSTRACT] *Caesalpinia sappan* L., belonging to the family Leguminosae, is a medicinal plant that is distributed in Southeast Asia. The dried heartwood of this plant is used as a traditional ingredient of food, red dyes, and folk medicines in the treatment of diarrhea, dysentery, tuberculosis, skin infections, and inflammation. Brazilin is the major active compound, which has exhibited various pharmacological effects, including anti-platelet activity, anti-hepatotoxicity, induction of immunological tolerance, and anti-inflammatory and antioxidant activities. The present study aimed to evaluate the antioxidant activity and expression of antioxidant enzymes of *C. sappan* L. extract and its major compound, brazilin, in human epidermal keratinocytes exposed to UVA irradiation. Our results indicated that *C. sappan* L. extract reduced UVA-induced H_2O_2 production *via* GPX7 activation. Moreover, brazilin exhibited antioxidant effects that were similar to those of *C. sappan* L. *via* glutathione peroxidase 7 (GPX7), suggesting that *C. sappan* L. extract and its natural compound represent potential treatments for oxidative stress-induced photoaging of skin.

[KEY WORDS] *Caesalpinia sappan* L.; Brazilin; Ultraviolet; GPX7; Antioxidative

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Introduction

In human skin, epidermal keratinocytes play an important role in the formation of primary defensive skin barrier against environmental damage. Epidermal keratinocytes in the basal layer of epidermis move upward, and ultimately differentiate into cornified cells in the epidermal stratum corneum, thus forming the epidermal permeability barrier [1-2]. Skin aging can be divided into intrinsic and extrinsic aging. Intrinsic aging is the process of senescence that affects all body organs, and extrinsic aging occurs as a consequence of exposure to environmental factors. Solar ultraviolet (UV) irradiation is a strong extrinsic factor that damages the structure and function of skin, and it also causes premature skin aging (photoaging), which is clinically characterized by laxity, roughness, coarse wrinkles, thickness, irregular pigmentation, and dryness [3-5]. UV light is conventionally classified into UVA (320–400 nm), UVB (280–320 nm), and UVC (200–280 nm). UVA and UVB penetrate the atmosphere and cause most skin disorders [3, 6], and both can induce the apoptotic cell death of skin. The mechanisms of UV irradiation-induced apoptosis involve the synergistic contributions of the following three independent pathways: formation of reactive oxygen species (ROS), death receptor activation, and DNA damage [7–10]. The direct or indirect production of ROS affects signaling pathways related to cell death and mediates inflammation, carcinogenic processes, and photosensitivity [11–14].

Glutathione peroxidases (GPXs) reside in different subcellular compartments where they catalyze the reduction of H_2O_2 to H_2O [15-16]. GPX7, a GPX isoform, exhibits a novel structure that contains a cysteine instead of selenocysteine in the catalytic center of mouse embryonic fibroblasts [17]. GPX7 knockout mice show dramatic changes such as a shortened lifespan as compared to the control mice, and oxidative DNA damage and apoptosis are predominantly detected in the kidneys. Furthermore, multiple organ dysfunctions are diagnosed, including splenomegaly, cardiomegaly, glomerulonephritis, fatty liver, and carcinogenesis [18-19]. Carcinogenesis and premature death are thought to reflect systemic oxidative stress.

Caesalpinia sappan L. (CSL), belonging to the Legumi-

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nosae family, is a medicinal plant that is distributed in Southeast Asia. The dried heartwood of this plant is used as a traditional ingredient of food, red dyes, and folk medicines that treat diarrhea, dysentery, tuberculosis, skin infections, and inflammation [20–25]. Chemical analyses of CSL extracts have resulted in the isolation of phenolic components with various structural types, including campesterol, coumarin, xanthone, chalcones, homoisoflavonoids, flavones, and brazilin [26]. Brazilin (7, 11b-dihydrobenz[b]indeno[1, 2-d]pyran-3, 6a, 9, 10(6H)-tetrol) is a major compound found in CSL, and recent studies have shown that brazilin exhibits various pharmacological effects, including anti-platelet activity, anti-hepatotoxicity, induction of immunological tolerance, anti-inflammatory activity, and antioxidative activity [27–31].

In the present study, numerous pharmacological properties of CSL (especially its antioxidant effects) led to evaluation of the effects of CSL on antioxidant activity and antioxidant enzyme expression in human epidermal keratinocytes (NHEKs) that were exposed to UVA irradiation.

Materials and Methods

Preparation of plant extract

CSL plants were purchased from the Kyungdong conventional market (Seoul, Korea), and the samples were dried under forced-air circulation. The dried samples were ground and then boiled (100 °C) for 2 h to obtain the extract. The crude extract was concentrated in a rotary evaporator (Eyela) under reduced pressure and subsequently lyophilized.

Cell culture

Normal human epidermal keratinocytes (NHEKs; Lonza, Sweden) were cultured in KBM medium with KGM2 growth supplements containing human epidermal growth factor, insulin, bovine pituitary extract, epinephrine, hydrocortisone, transferrin, and gentamicin/amphotericin B (Lonza). NHEKs were serially passaged at 70%–80% confluence and were used within three passages. NHEKs were starved for 24 h in KBM medium without transferrin and cortisone and subsequently treated with CSL extracts or brazilin for 24 h.

Cell viability assay

The viabilities of NHEKs treated with CSL extracts and brazilin or control were determined using a cell counting kit-8 (CCK-8; Dojindo, USA. The NHEKs were plated in 96-well plates at a density of 3×10^3 cells/well, and the proliferation capacity was measured using the CCK-8 assay. The cells were treated with 10 μL of the CCK-8 solution in 90 μL of DMEM (phenol red-free; Welgene, Korea), and were then incubated at 37 °C for 1.5 h. The absorbance was measured at 450 nm with an Epoch microplate reader (BioTek, USA).

Ultraviolet A irradiation

Prior to the application of UVA irradiation, the NHEKs were washed twice with PBS and protected from drying with the addition of DMEM (phenol red-free). The NHEKs were irradiated with 5 $J \cdot cm^{-2}$ of UVA using a BioSun irradiator (Vilber Lourmat, Germany) [41-42]. After irradiation, the normal DMEM was replaced with the DMEM containing either CSL extract (20 $\mu g \cdot mL^{-1}$) or brazilin (1 $\mu g \cdot mL^{-1}$) and incubated for 24 h.

RNA isolation and quantitative real-time RT-PCR

Total RNA was extracted from the cells using TRIzol® Reagent (Invitrogen, USA), and the RNA concentration was assessed with an Epoch Take3 micro-Volume spectrophotometer (BioTek). RNA (2 μg) was reverse-transcribed into cDNA using a ReverTra Ace Kit (Toyobo, USA), and reverse transcription was stopped by adding Tris-EDTA buffer (pH 8.0) to 200 μL of the cDNA solution. Quantitative real-time RT-PCR was performed using a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific Inc., USA), according to the manufacturer's instructions. Briefly, 20 μL of PCR mixture contained 10 μL of 2X TaqMan Universal PCR Master Mix, 50 ng of cDNA, and 1 μL of 20X TaqMan Gene Expression assay solution (Applied Biosystems). The gene identification numbers for the TaqMan Gene Expression assay used in the real-time RT-PCR analysis are presented in Table S**1**. Human *GAPDH* (43333764F, Applied Biosystems, USA) was used to normal control.

H_2O_2 production measurement

H_2O_2 released from UVA-irradiated NHEKs was measured using an Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen), according to the manufacturer's instructions. The NHEKs were treated with CSL extracts or brazilin for 24 h prior to UVA-irradiation. The medium (50 μL) of each condition after UVA irradiation was harvested and incubated for 10 min with 100 μL of the reaction solution, which contained 100 $\mu mol \cdot L^{-1}$ of Amplex Red reagent and 0.2 $U \cdot mL^{-1}$ of horseradish peroxidase. The fluorescence of each condition was measured at a 590 nm emission following excitation at 560 nm using an Epoch microplate reader (Biotek).

Immunoblot analysis

The cells were lysed on ice for 1 h with RIPA lysis buffer (Millipore, Germany) with a protease inhibitor cocktail. After incubation, cell lysates were centrifuged at 12 000 $r \cdot min^{-1}$ for 15 min at 4 °C, and the supernatants were collected into fresh tubes. For immunoblot analyses, 30 μg of total protein was separated on 4%–12% gradient Bis-Tris gels before transfer to PVDF membranes (Thermo Fisher Scientific Inc., USA). The membranes were blocked by incubation in Tris-buffered saline Tween-20 (TBST) buffer containing 1% BSA for 45 min at room temperature (RT). The membranes were then incubated overnight at 4 °C with the *β*-actin primary antibody (1 : 1 000; Santa Cruz Biotechnology, USA) or the GPX7 primary antibody (1 : 1 000; Abcam, USA) in TBST containing 1% BSA. The blots were washed thirce with TBST and incubated for 1.5 h at RT with HRP-conjugated secondary antibodies (1 : 3 000; Bio-Rad, USA). The blots were developed using the Clarity Western ECL blotting substrate (Bio-Rad), according to the manufacturer's instructions.

HPLC

Caesalpinia sappan L. (CSL) and brazilin (ChemFaces, Purity: 98%) samples were dissolved in a standard solution of

methanol. Samples were subjected to an X-bridge C18 column (250 mm × 4.6 mm; Waters) after dilution with DMSO (Sigma). Samples were chromatographed on a column eluted with 25 : 75 (*V*/*V*) of methanol and 0.3% acetic acid at a flow rate of 1.0 mL·min^{-1}, and were monitored at 280 nm using HPLC 20A (Shimadzu).

Statistical analysis

Statistical analyses of data were performed using one-way analysis of variance (ANOVA). The results were expressed as means ± standard deviation (SD) of at least three independent experiments, and $P < 0.05$ was considered statistically significant.

Results and Discussion

In order to make a photoaged epidermal model, we irradiated NHEKs with various doses of UVA. Before investigating the mRNA expression of moisturizing-associated proteins in NHEKs in response to UVA irradiation, we first determined the non-cytotoxic UVA-irradiation dose using a CCK-8 assay. The cell death of NHEKs induced by UVA irradiation gradually increased as the irradiation dose increased. The cell viability after UVA irradiation (7 J·cm^{-2}) decreased by 28.3%, compared to that of the control (Figs. 1A and 1B). To determine age effects of UVA on NHEKs, we used quantitative real-time RT-PCR to measure the mRNA levels of genes associated with the moisturizing process. We observed a decrease in *AQP3* and *HAS2* gene transcription in response to UVA irradiation (5 J·cm^{-2}). Therefore 5 J·cm^{-2} of irradiation was selected as the photoaged condition for NHEKs in the present study.

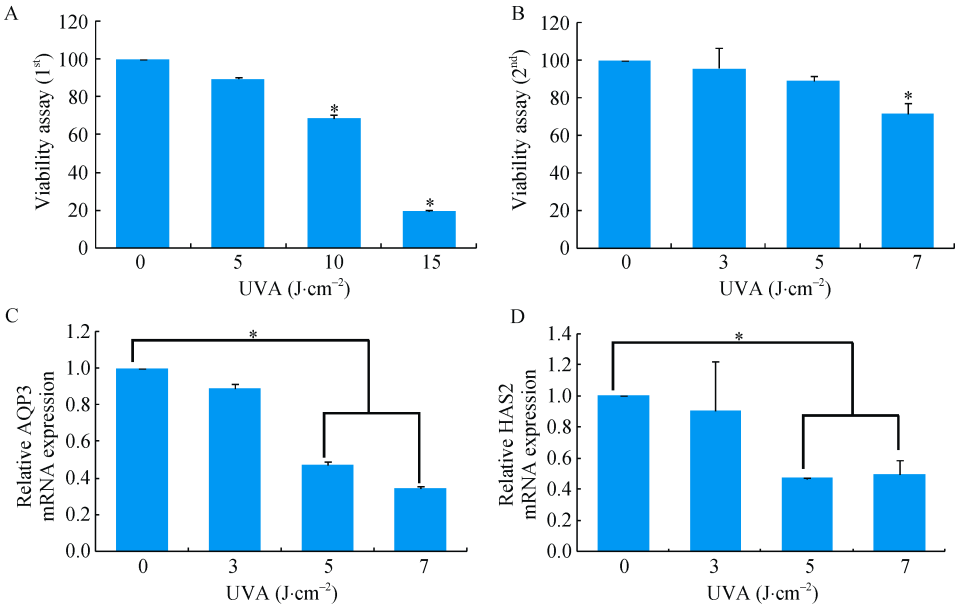

Fig. 1 Viability and characterization of UVA irradiation in normal human epidermal keratinocytes (NHEKs). NHEKs (3 × 10^3 cells) were seeded in 96-well plates, and were treated with UVA irradiation (A, B). The cell viability results, based on a CCK-8 assay, are presented as means ± SD of the percentage of the control optical density (OD). The results of real-time RT-PCR analyses of the epidermal keratinocyte markers *AQP3* (C) and *HAS2* (D) are shown as means ± SD of three independent experiments ($^*P < 0.05$)

Previous studies show that CSL exhibits antioxidant activity [32-33], so we conducted a DPPH assay to confirm the antioxidant effects of CSL extracts. The results indicated that the CSL extract exhibited a DPPH free radical scavenging effect in a dose-dependent manner. Compared to L-ascorbic acid, which was used as a positive control, the CSL extract showed similar antioxidant activity. We next used the non-cytotoxic concentrations of CSL extracts in NHEKs based on the results from CCK-8 assay toexamin whether the CSL extract could suppress ROS generation in UVA-irradiated NHEKs. The results indicated that UVA-irradiated NHEKs produced ROS levels approximately six times higher than normal epidermal keratinocytes and the CSL extract treatment significantly reduced H_2O_2 generation (Fig. 2).

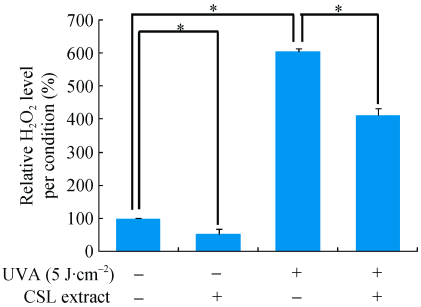

Fig. 2 *C. sappan* L. (CSL) extract inhibits H_2O_2 generation in UVA-irradiated NHEKs. Basal H_2O_2 levels in UVA-irradiated NHEKs following treatment with the indicated medium containing CSL extract are shown. Graphs depict means ± SD of three independent experiments ($^*P < 0.01$)

We next investigated whether the CSL extract could restore the mRNA expression of specific antioxidant enzymes. The accumulation of ROS induced by UV-irradiation could cause direct deleterious chemical modifications of cellular components (e.g., proteins, lipids, and DNA). Moreover, it could also overwhelm the elaborate antioxidant defense system associated with antioxidant enzymes containing superoxide dismutases (SODs), catalase (CAT), peroxiredoxins (PRDXs), and glutathione peroxidases (GPXs), thereby leading to a state of oxidative stress [3, 34-35]. In the present study, UVA irradiation caused the downregulation of antioxidant enzymes such as *SOD2* and *CAT*, whereas CSL extract could not recover the mRNA expression of *SOD2* and *CAT* (Fig. 3). Choung *et al.* [36] have observed that *SOD1* and *SOD2* are active at the 5 kJ·cm^{-2} UVA exposure level, while there is little response of *SOD3* expression at this lower UVA irradiation level. In our experiments, the detection of *SOD3* mRNA expression was not in accordance with the results of previous studies (data not shown). Interestingly, the mRNA expression level of *GPX7* was significantly increased in response to the treatment with CSL extract as compared to that of UVA-irradiation treatment alone (Fig. 3D). We also investigated the mRNA expression of other antioxidant enzymes (*GPXs* and *PRDXs*), but no changes were found after the treatment with the CSL extract (data not shown).

To elucidate the protein expression of *GPX7* in UVA-irradiated NHEKs treated with CSL extract, *GPX7* protein levels were measured by Western blotting. The results indicated that UVA irradiation downregulated the expression of *GPX7*, and CSL extract significantly increased *GPX7* protein expression. These results were consistent with the mRNA expression analyses.

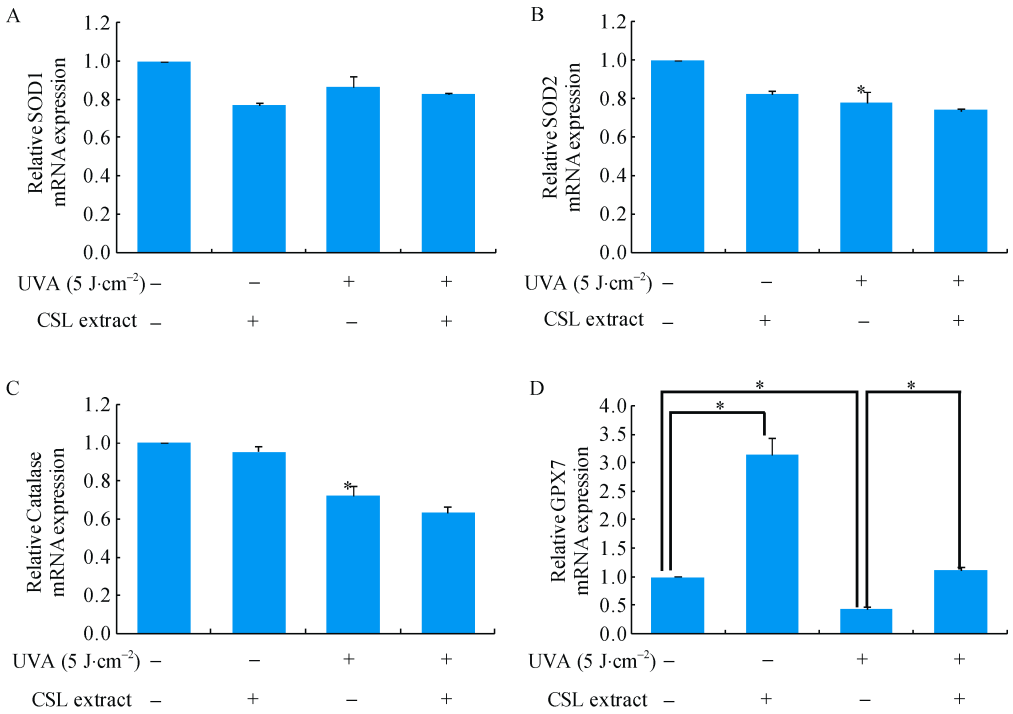

Fig. 3 Effects of CSL extract on the gene expression of antioxidant enzymes in NHEKs. The results of real-time RT-PCR analyses of the antioxidant enzyme markers, *SOD1* (A), *SOD2* (B), *CAT* (C), and *GPX7* (D) are shown. The data represent the means ± SD of three independent experiments ($^{*}P < 0.05$)

Previous studies show that brazilin is the major compound found in CSL [37-38], and an HPLC analysis was performed to confirm that brazilin was the major compound in the CSL extract obtained using boiled water. The HPLC method was coupled with ultraviolet detection for the quantitative determination of brazilin in CSL extract. The typical chromatograms of standard and CSL extracts are shown in Fig. 4S, and the retention time of brazilin was 6.4 min. Therefore, the results indicated that brazilin was the major compound of CSL extract.

A previous study found that the brazilin content was 8%–22% *W/W* of the CSL extract [37], and the results of the current study indicated that 20 μg·mL^{-1} of the CSL extract contained 1.74–4.4 μg·mL^{-1} of brazilin. Furthermore, based on the results of the non-cytotoxic CCK-8 assay of brazilin in NHEKs, we chose 1 μg·mL^{-1} of brazilin for further investigations of antioxidative activity (data not shown). Brzailin showed a DPPH free radical scavenging effect in a dose-dependent manner. Compared to L-ascorbic acid, which was used as a positive control, CSL extracts showed similar radical scavenging activity. Furthermore, we examined whether brazilin could suppress ROS generation in UVA-irradiated NHEKs. UVA-irradiated NHEKs produced higher ROS levels than normal NHEKs. The brazilin treatment significantly reduced H_2O_2 generation.

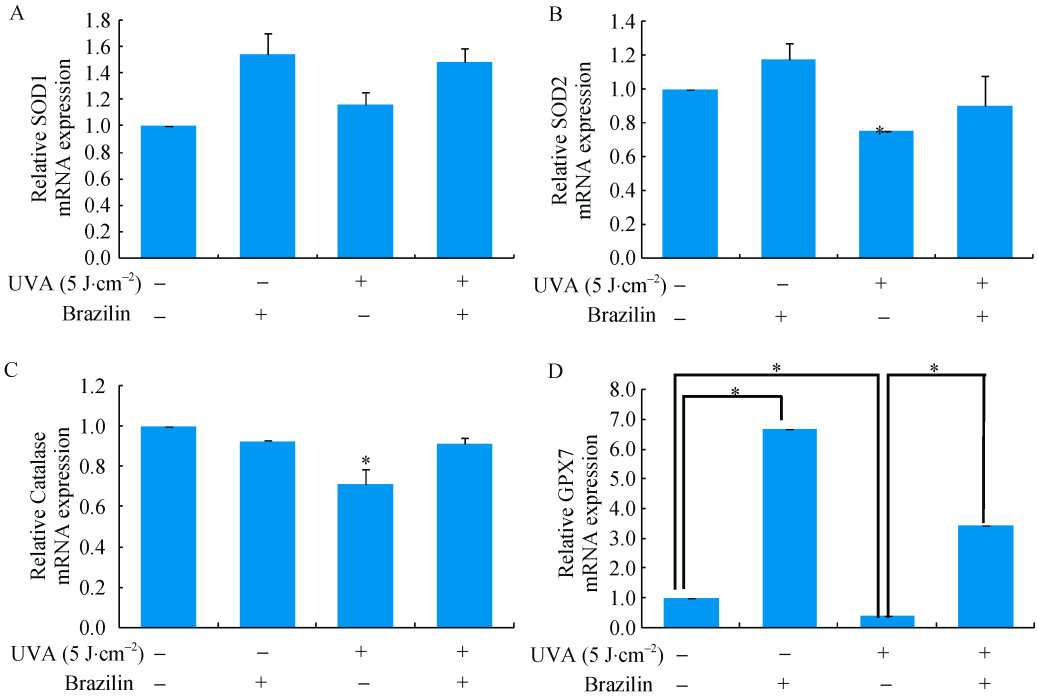

Fig. 4 Effects of brazilin on the gene expression of antioxidant enzymes in NHEKs. Results of real-time RT-PCR analyses of the antioxidant enzyme markers, *SOD1* (A), *SOD2* (B), *CAT* (C), and *GPX7* (D) are shown. The data represent means ± SD of three independent experiments ($^*P < 0.05$)

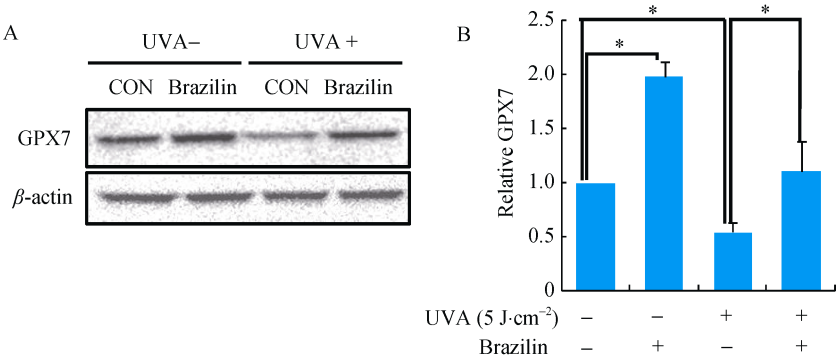

Fig. 5 Immunoblot analysis of GPX7. The results indicate that the expression of the GPX7 protein was significantly increased compared to the UVA-irradiated group (A). Densitometrically quantified relative intensity values for GPX7 are shown (B). The results are expressed as means ± SD of three independent experiments ($^*P < 0.05$)

To evaluate the antioxidant effects of brazilin on NHEKs, we used quantitative real-Time RT-PCR to measure the mRNA levels of genes associated with antioxidant enzymes. The *GPX7* mRNA expression level was significantly increased following brazilin treatment as compared to the UVA-irradiated treatment alone (Fig. 4). When UVA-irradiated NHEKs were treated with brazilin, *GPX7* transcription significantly increased as compared to the CSL extract treatment (Figs. 3D and 4D).

To elucidate the protein expression of GPX7 in in UVA-irradiated NHEKs treated with brazilin, GPX7 protein levels were measured. The results indicated that UVA irradiation downregulated the expression of GPX7 in NHEKs, and brazilin significantly increased GPX7 protein expression (Fig. 5). These results wre consistent with the results of the mRNA expression analyses.

The use of an *in vitro* model is relevant to the field of cosmetic research, which is banned from using animal tests for a number of end-points, including the efficacy evaluation of cosmetic ingredients. Several researchers have attempted to develop better *in vitro* analytical tools to replace animal tests, especially in light of regulations such as the 7th Amendment to the Cosmetics Directive [39] and REACh [40]. These regulations restrict the use of animal tests to determine the utility of CSL extract or brazilin as cosmetic ingredients. Although the antioxidant activities CSL extract and brazilin have been re-

ported [41–42], the results of the present study provided the first evidence supporting that the induction of *GPX7* expression by these two treatments may in part provide protection against UVA-induced photoaging. Our results demonstrated that CSL and its major compound, brazilin, scavenged UVA-induced secretions of H_2O_2 and enhanced antioxidant enzyme expression (especially that of GPX7). Moreover, CSL extract and brazilin exhibited protective effects against oxidative stress, so this natural compound isolated from CSL represented a potential treatment for oxidative stress-induced skin photoaging. Additional studies will be conducted to elucidate the role of brazilin in various skin cell types (e.g., melanocytes and dermal fibroblasts) to illuminate the mechanisms associated with the protective effects of the compound and to determine the full skin-protective abilities of brazilin and CSL extract.

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