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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 $J·cm^{-2}$ 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 $μg·mL^{-1}$) or brazilin (1 $μg·mL^{-1}$) 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 $μmol·L^{-1}$ of Amplex Red reagent and 0.2 $U·mL^{-1}$ 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 $r·min^{-1}$ 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 thrice 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

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