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Anti-Inflammatory Effects of Streamed *Platycodon grandiflorum* against UVB Radiation-Induced Oxidative Stress in Human Primary Dermal Fibroblast

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Abstract

Ultraviolet B (UVB) exposure is a risk factor for skin damage resulting in oxidative stress, inflammation, and cell death. The purpose of this study was to investigate the physicochemical properties of *Platycodon grandiflorum* (PG) to improve its biological activities using a three-step steaming process. We investigated the protective effects of PG and steamed PG extracts on human dermal fibroblasts (HDFs) against UVB radiation-induced oxidative stress and inflammation as well as the underlying mechanisms. The antioxidant potential of the PG extracts was evaluated by measuring the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) scavenging activity. ABTS and DPPH were shown by the 0, 30, and 70% ethanol extracts of 2S-PG and 3S-PG (IC_{50}, 28~45 and 27~30 μg/mL, respectively). Treatment of UVB-irradiated cells with steamed PG (25~400 μg/mL) did not affect their viability. The streamed PG extract suppressed UVB-induced generation of reactive oxygen species (ROS). In addition, streamed PG extract reduced cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) protein expression in UVB-irradiated HDF, regulating nuclear factor (NF)-κB expression. These findings suggest that steamed PG extract may be potentially effective against inflammation associated with UVB-induced oxidation stress.

Key words: streamed *Platycodon grandiflorum*, ultraviolet B, human primary dermal fibroblast, inflammation

Introduction

Ultraviolet (UV) exposure is a major etiologic factor for skin damage and has a number of biological effects such as epidermal pigmentation, melanin production, inflammation, and cancer (Akhalaya et al. 2014). There are three types of UV light and, in particularly, UVB with a wavelength range of 290~320 nm (Clydesdale et al. 2001) has the most cytotoxic skin-damaging effects (Lee et al. 2014). Oxidative damage by UVB is caused by singlet oxygen (O_2), hydrogen peroxide (H_2O_2), the superoxide anion (O^-), and hydroxyl radical (OH), which are reactive oxygen species (ROS) (Radhiga et al. 2016). UVB primarily generates ROS that play a key role in mediating most of the biology responses (Berneburg et al. 2000) that lead to inflammatory skin disorders and the direct interaction with DNA via induction of DNA damage (Pillai et al. 2005). The primary event in the generated of ROS by UVB is activation of the transcription factor, nuclear factor (NF)-κB (Kuo et al. 2017). Following its activation, NF-κB regulates the expression of almost 400 different genes including inducible nitric oxide (NO) synthase (iNOS) and cyclooxygenase-2 (COX-2) (Pillai et al. 2005). Therefore, UVB induces inflammation by stimulating the expression of iNOS (Ghosh et al. 2012), the enzyme that produces NO (Serasanambati & Chilakapati 2016). In addition, the expression of COX-2 in the human skin leads to increased production of proinflammatory mediators such as prostaglandins and throm- boxanes (Pillai et al. 2005). Consequently, understanding the effects of UVB on the human skin involves acquiring knowledge of the inflammatory

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effects mediated by ROS generation.

Platycodon grandiflorum (PG), which belongs to the Campanulaceae family, is a well-known herb cultivated in most parts of Korea and an ornamental plant (Cho 2011; Ryu 2014). Extracts of PG have been reported to have health benefits such as antioxidant, anticancer, anti-inflammatory, anti-allergic, and hepatoprotective in mice (Lee et al. 2001; Lee et al. 2004a; Jang et al. 2006; Oh et al. 2010; Lee et al. 2015). PG contains triterpenoid saponins, which are carbohydrates that protect hepatocytes against oxidative damage and HaCaT cells against the effects of UVA radiation (Tada et al. 1975; Lee et al. 2004b). Especially, the saponin contained in PG called platycoside has been found to increase with steaming, and it has various pharmacological effects (Lee et al. 2013; Lim et al. 2016).

PG has been studied for various bioactivities. However, the antioxidant and inflammatory activity of the steamed formulation on human dermal fibroblasts (HDFs) exposed to UVB radiation has not been investigated and, therefore, in this study, we sought to investigate this phenomenon.

Materials and Methods

1. Sample preparation

The PG was used in this study was collected from Icheon in Korea in 2016 purchased from the Korea Medicine Herbal Association. Non-steamed PG (NS-PG) and steamed PG (S-PG: 1S-PG, 2S-PG and 3S-PG) were prepared from samples of the same variety grown in 2016 and 5 kg of each sample was extracted. The S-PGs was prepared by steaming the plant material in a steamer at 90~95℃ for 4 h, drying it in a drying oven at 30 ℃ for 1 day, and the process was repeated one to three times. NS-PG and the S-PG were extracted with 0%, 30% and 70% ethanol (sample/solvent ratio, 1:10) by shaking overnight at room temperature (Kim et al. 2013). After sample extraction, the extracts were filtered, and vacuum evaporated. The extracts were dissolved in dimethyl sulfoxide (DMSO), and the extraction process is represented as Fig. 1.

2. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was determined using the method proposed by Brand-Williams (1995) with slight modifications (Karaś et al. 2015). Briefly, 25 µL of the sample was mixed with 225 µL DPPH solution while 25 µL each of DMSO and the sample in 225 µL ethanol were used as blank1 and 2. The mixtures were shaken vigorously and placed in a dark room for 30 min at room temperature and 25 µL DMSO with 225 µL DPPH was used as the control. The decrease in absorbance was measured at 517 nm using a multimode microplate reader. All experiments were performed in triplicate and the scavenging effect was calculated according to the following equation.

$$\text{Antioxidant activity of DPPH (\%)} = \left(\frac{A\,\text{sample} - A\,\text{blank2}}{A\,\text{control} - A\,\text{blank1}}\right) \times 100$$

The half-maximal inhibitory concentration (IC_{50}) was obtained by determining the free radical scavenging activity of the sample concentration that showed 50% inhibition.

3. 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) scavenging assay

The free radical scavenging activity was also determined using the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid ($ABTS^+$) radical cation as described by Re et al. (1999) with slight modifications (Karaś et al. 2015). The working ABTS solution was produced by reacting aqueous ABTS solution (7 mM) with potassium persulfate (2.45 mM), and the mixture was in the dark

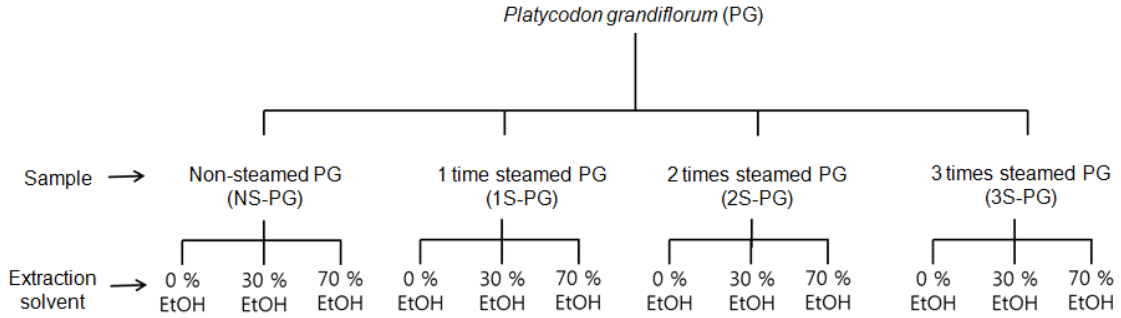

Fig. 1. Extraction scheme of *Platycodon grandiflorum*.

at room temperature for 24 h. The solution was diluted to obtain an absorbance value of approximately 0.7~0.8 at 734 nm. Then, 180 μL ABTS solution was mixed with 20 μL of each sample and the absorbance was measured at 734 nm using multimode microplate reader (Biotek Instrument, VT, USA). Water was used as the blank and the scavenging effect was calculated according to the following equation.

$$\text{Antioxidant activity of ABTS (\%)} = \left(\frac{A_{blank} - A_{sample}}{A_{blank}}\right) \times 100$$

The IC_{50} was determined by assessing the free radical scavenging activity of the sample concentration that showed 50% inhibition.

4. Cell culture and treatment

HDF cells (American Type Culture Collection, Manassas, VA, USA) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 1% penicillin and streptomycin (P/S) in a humidified 5% CO_2 atmosphere at 37℃. The cells were washed with Dulbecco's phosphate-buffered saline (DPBS), and then exposed to 100 mJ/cm^2 UVB irradiation using a UV-B lamp GL20SE (Sankyodenki, Japan) without the culture plate cover. Then, the UV-exposed cells were immediately placed in growth medium containing NS-PG and 1S-PG, 2S-PG and 3S-PG samples.

5. Cell viability assay

The potential effect of NS-PG and S-PG on the viability of UVB-irradiated HDFs was investigated using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay (Promega, Madison, WI, USA), based on the conversion of the yellow tetrazolium salt to a purple formazan product. HDF cells were seeded in 96-well culture plates at a density 1×10^5 cell/mL, cultured for 24 h, exposed to UVB radiation, and then they were treated with various concentrations of the test samples. Cells were incubated at 37 ℃ for 18 h, followed by the addition of MTS and measurement of the absorbance using the multimode microplate reader at 490 nm.

6. Quantitation of intracellular ROS

To measure the intracellular ROS content in UVB-irradiated HDF cells, they were seeded in 96-well plates at a density of 1×10^5 cell/mL and exposed to UVB-irradiation. The cells were then washed and resuspended in DPBS, followed by the addition of 2',7'-dichlorodihydrofluorescein diacetate DCF-DA (20 μM) and incubation at 37℃ for 30 min. ROS positive cells were measured using the multimode microplate reader at excitation and emission wavelengths of 485 and 528 nm, respectively.

7. Western blot assay

UVB-exposed cells were collected and lysed in ice cold radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling Technology Inc., Beverly, MA, USA). The protein levels were measured using the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Waltham, MA, USA). The extracted protein was separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto nitrocellulose membranes (GE, Healthcare, Chalfont St, Giles, UK). After blocking with Tris-buffered saline (TBS) containing 5% nonfat dry milk, the membranes were immunoblotted with specific antibodies. The protein bands were detected using a chemiluminescent imaging system (Davinch-K, Seoul, Korea).

8. Statistical analysis

Data are represented as means±standard deviation (S.D.) of three replicates. A student's *t*-test was also used to compare the group means and a $p<0.05$ was considered statistically significant.

Results and Discussion

1. Antioxidant effect of S-PG samples

As shown in Table 1, the antioxidant activity was determined using DPPH and ABTS radical scavenging activity assays. DPPH radical is scavenged by antioxidants compounds present in extracts that can capture radicals, whereas, in the ABTS assay, cationic $ABTS^+$ radicals are captured. The highest radical scavenging activities against ABTS and DPPH were shown by the 0, 30 and 70% ethanol extracts of 2S-PG and 3S-PG (IC_{50}, 28~45 and 27~30 μg/mL, respectively). NS-PG showed the weakest IC_{50} values of 148~160 and 640~1,604 μg/mL in the ABTS and DPPH assays, respectively. The results of our study show that the steaming process increased the antioxidant activity of 2S-PG and 3S-PG, leading to better results than those of the NS-PG. Moisture contents of NS-PG and 3S-PG were 78.07 and 7.7%. Polyphenol contents of 1S-PG and 3S-PG were 33 and 38 mg/

Table 1. $ABTS^+$ and DPPH radical scavenging activities of non-steamed and steamed *Platycodon grandiflorum* extract (*n*=3)

Sample		IC_{50} value(μg/mL)	
		ABTS	DPPH
NS-PG	0%	160±3.56	992±131.95
	30%	153±5.39	640±6.23
	70%	148±1.64	836±97.35
1S-PG	0%	118±5.11	126±23.43
	30%	65±9.47	50±19.59
	70%	104±5.55	109±11.04
2S-PG	0%	42±1.26	30±2.63
	30%	45±0.56	28±0.70
	70%	45±0.62	33±1.98
3S-PG	0%	30±0.47	27±2.94
	30%	27±1.48	27±1.70
	70%	29±0.73	29±1.32
Ascorbic acid		34±0.33	10±0.26

100 g. Crude saponin contents of NS-PG and S-PG were 57.0 and 107.92 mg%. When PG were steamed moisture, polyphenol and crude saponin contents were increased (Lee et al. 2013; Kim et al. 2015). Based on the results, we concluded that steaming released the saponins and polyphenols with antioxidant properties.

2. Effects of S-PG on cell viability of UVB radiation-damaged HDF cells

To examine the effect of NS-PG, 1S-PG, 2S-PG and 3S-PG on the viability of UVB-radiated HDF cells, we performed the MTS assay, which is commonly used for cell cytotoxicity analysis by measuring the activity of mitochondrial dehydrogenase. The various concentrations (0, 50, 100 and 200 μg/mL) of the different ethanol extracts of NS-PG, 1S-PG, 2S-PG and 3S-PG (0, 30 and 70%) did not affect the cell viability (Fig. 2). However, treatment with several different concentrations of NS-PG, 1S-PG, 2S-PG and 3S-PG, increased the cell viability in a concentration-dependent manner. We found no cytotoxicity with 200 μg/

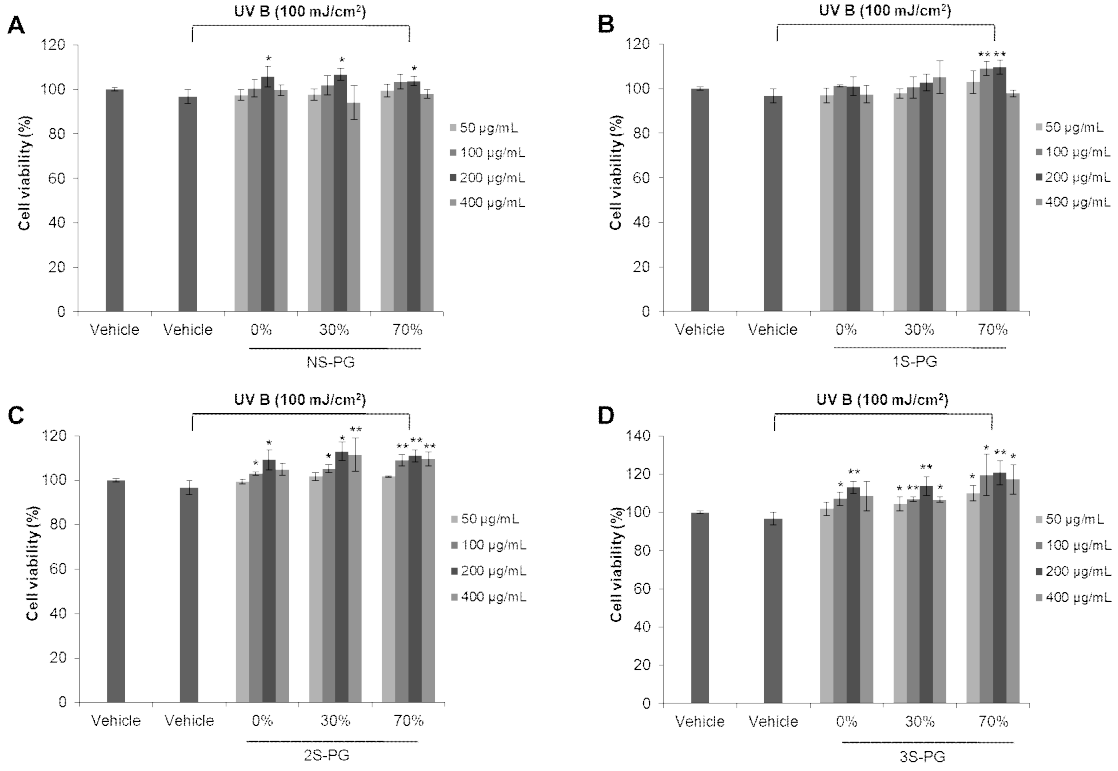

Fig. 2. Cell viability of *Platycodon grandiflorum* (PG) extract-treated ultraviolet B (UVB)- induced human dermal fibroblasts (HDFs). (A) Non-steamed PG (NS-PG), (B) Once-steamed PG (1S-PG), (C) twice-steamed PG (2S-PG) and (D) thrice-steamed PG (3S-PG). Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay. UVB-induced HDF cells were incubated with PG samples (0~400 μg/mL) for 0~12 h. Results are means±standard deviation (SD) of three determinations. Significance was determined using *t*-test; * $p<0.05$ and ** $p<0.01$ vs. UVB-damaged cells.

mL sample treatment in the MTS assay. Based on these results, 200 μg/mL was selected as the working sample concentration for the experiments.

3. Effect of S-PG on intracellular ROS generation in UVB radiation-damaged HDF cells

To elucidate whether the effects of NS-PG, 1S-PG, 2S-PG and 3S-PG (0, 30 and 70%) were linked to their antioxidant properties, ROS generation in UVB-exposed HDF cells was measured in UVB-damaged cells. We found that intracellular ROS generation in UVB-damaged cells was higher than that in undamaged cells. NS-PG, 1S-PG, 2S-PG and 3S-PG decreased intracellular ROS generation compare with that in untreated UVB-damaged cells (Fig. 3). The highest reduction of ROS generation was shown by the 30 and 70% ethanol extracts of 3S-PG (68.31±1.52 and 65.44±3.87), respectively. The various concentrations of the ethanol extracts of each PG sample significantly decreased the intracellular ROS generation compared to that of the untreated group. The 70% ethanol PG samples reduced the ROS generation the most (75.41±4.39, 81.52±2.33, 79.75±3.46 and 65.44±3.87 for NS-PG, 1S-PG, 2S-PG and 3S-PG, respectively). This finding confirms that effects of NS-PG and S-PG (1S-PG, 2S-PG and 3S-PG) on ROS generation can be correlated to their retained antioxidative activity.

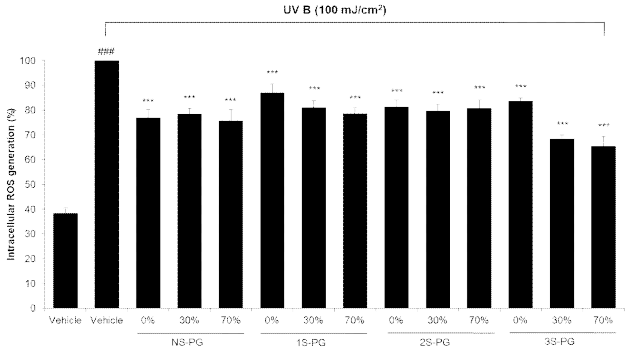

Fig. 3. Effect of *Platycodon grandiflorum* (PG) inhibitors on intracellular reactive oxygen species (ROS) production in ultraviolet B (UVB)-induced human dermal fibroblast (HDF) cells. Cells were induced without (vehicle) or with UVB at 100 mJ/cm^2 and then treated with non-steamed PG (NS-PG), and once-, twice-, or thrice-steamed PG (1S-PG, 2S-PG and 3S-PG, respectively). After 24 h, cells were stained with 10 mM 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) for 30 min, and ROS production was determined using a fluorescence microplate reader. Results are means±standard deviation (S.D.) of three replicates. Significance was determined using Student's *t*-test; ### $p<0.001$ vs. undamaged cells; *** $p<0.001$ vs. UVB-damaged cells.

4. Effect of PG samples on protein expression UVB radiation-damaged HDF cells

UVB radiation damages the skin by inducing the generation of ROS in HDF (Berneburg et al. 2000; Pillai et al. 2005). To elucidate whether the effects of NS-PG, 1S-PG, 2S-PG and 3S-PG 70% ethanol extracts were linked to their anti-inflammatory activity in UVB-induced oxidative stress, the expression levels of relevant proteins were measured. NF-κB activation by UVB is well known to induce the expression of inflammatory mediators such as COX-2 and iNOS (Pillai et al. 2005). Because ROS generation was decreased respectively at 70% extracts, we performed western blot analysis to investigate whether the PG extracts (N, 1S, 2S, 3S-PG 70% ethanol extracts) affected NF-κB activity (Fig. 4). PGs treatment at a concentration of 200 μg/mL reduced the levels of the inflammation-related COX-2, iNOS, p65 and phosphorylated-p65 (p-p65) proteins in UVB-induced HDF cells. Specifically, 2S-PG and 3S-PG reduced COX-2 and iNOS activities by 18 and 27% and 86 and 75%, respectively, while pp65/p65 was reduced by 3S-PG by 78% compared to the UVB damaged control. We confirmed that 2S-PG and 3S-PG attenuated lipopolysaccharide-induced inflammatory mediators by inhibiting NF-κB activation. Our results showed that 2S-PG and 3S-PG showed antioxidative effects and decreased intracellular ROS generation by UVB-induced oxidative stress in HDF cells. The extracts also sustained the expression of COX-2,

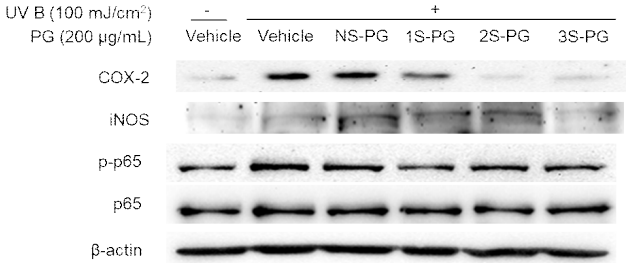

Fig. 4. Effect of *Platycodon grandiflorum* (PG) inhibitors on ultraviolet B (UVB)-induced inflammatory activation of human dermal fibroblast (HDF) cells. UVB-induced HDF cells were pretreated with 200 μg/mL once-, twice-, or thrice-steamed PG (1S-PG, 2S-PG and 3S-PG 70% extracts, respectively) for 24 h. Cell lysates were prepared and analyzed using western blotting with antibodies against inducible nitric oxide (NO) synthase (iNOS), cyclo-oxygenase 2 (COX-2), p65, and phosphorylated-p65 (p-p65) and actin.

iNOS and p65 protein in UVB-induced oxidative stress in HDF cells. This study confirmed the anti-inflammatory effects of 2S-PG and 3S-PG. These effects were mediated by the suppression of NF-κB activation in UVB-induced oxidative stress in HDF cells, which suppressed the release of COX-2, iNOS and ROS. Therefore, understanding the mechanism of S-PG samples were prepared by steaming more than two times may facilitate the development of more effective therapies for UVB-induced inflammatory skin damage.

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