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Identification of the Phytochemical Compounds and Their Type I Procollagen Induction in *Astragalus membranaceus* Sprouts Grown under Different Light Conditions

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ABSTRACT

Astragalus membranaceus, a medicinal herb, has been used for diuretic, tonic, anti-viral and antioxidant effects. Its root is widely used in Chinese and Korean traditional medicines, but in this study, the sprout grown optimally in our indoor system was evaluated for cosmetic efficacies. Interestingly, *A. membranaceus* sprout extract activated the production of type I procollagen by human dermal fibroblast cells in a dose-dependent manner, without showing cell toxicity. A phytochemical study was conducted to identify the active constituents in the extract by solvent fractionation and column purifications. As a result, three compounds: tryptophan (**1**), linoleic acid (**2**) and adenine (**3**) were identified by NMR spectroscopy. Isolated tryptophan was verified to induce the synthesis of the type I procollagen in a dose-dependent manner. These results demonstrate that *A. membranaceus* sprouts containing tryptophan can be developed as skin care ingredient with the optimization of indoor cultivation system.

INTRODUCTION

Astragalus membranaceus (Fisch.) Bge is an annual or perennial herb in the Leguminosae family and called milk-vetch root (English), huangqi (Chinese), ogi (Japanese), or hwanggi (Korean) (Ma *et al.*, 2000). It is an important medicinal plant in Korea and China. Hwanggi root has been traditionally used for draining pus, reducing pain, treating leprosy, boosting immunity, recovering from fatigue and alleviating skin diseases (Tzu *et al.*, 2014). It has been known to contain saponins, cycloastragenols, and polysaccharides. A group of cycloastragenol (CAG) such as an aglycone of acetylastragaloside I, isoastragalosides and astragalosides, has been reported (Luo *et al.*, 2016) and more than 40 saponins have been reported from hwanggi root (Ren *et al.*, 2013). More recently, new saponins from hwanggi root were reported: astrolano-saponin A1, A2, B, C, D, and E (Wang *et al.*, 2016). Astragalosides are known as active compounds having various pharmacological activities (Kwon *et al.*, 2013). A number of studies reported cardiotonic (Zhang *et al.*, 2006), anti-aging (Lei *et al.*, 2003), anti-cancer (Cho *et al.*, 2007), anti-allergy (Kim *et al.*, 2013), and anti-inflammatory effects (Zhang *et al.*, 2003). Moreover, glabridin, one of hwanggi peptides has been shown to inhibit expression of MMPs in human dermal fibroblasts, suggesting anti-wrinkle efficacy (Sun *et al.*, 2014).

Anti-aging effects are critical for cosmetic ingredients. Aging is perceived by increased wrinkles and decreased elasticity of the skin. Wrinkle and skin elasticity depend upon the amount of collagen and elastin production in the extracellular matrix (ECM). Collagen accounts for 70-80% of the human dermis and collagen synthesis are vital to maintain youthful looking skin (Sher *et al.*, 1997). Type I and III collagens are the major components of human dermis. Amount of Type I in normal skin is 80%, while type III is 15%. Hence, type 1 collagen is the major component of ECM and it is directly related to skin aging (Lovell *et al.* 1987). Collagen is degraded naturally by MMPs (matrix metalloproteinases), which are responsible for the physiological degradation of ECM proteins. Repeated

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exposure to sunlight causes skin damage by activating MMPs that breakdown collagens (Fisher *et al*., 2002) and stimulates tumor necrosis factor-α, -γ, nitric oxide and interleukin, all of which inhibit the synthesis of collagen in the skin and increase the expression of collagen-degrading enzymes. These changes lead to photo-aging, skin erythema, roughness, wrinkles and blackening (Sher *et al*., 1997).

We have developed novel cosmetic agents derived from plants, especially sprouts cultivated in controllable conditions, such as safflower sprout extract (Chang *et al*., 2014) and peanut sprout extract (Mi, 2016). In this study, we cultivated hwanggi sprout by using previously reported cultivation system (Chang *et al*., 2014) and examined the collagen synthesis activation properties. In addition, the active constituents were identified by phytochemical investigations.

MATERIALS AND METHODS

Plant materials and growth conditions

Hwanggi seeds were purchased from Aramseed Co. (Seoul, Republic of Korea) in 2016. The seeds were kept at 5°C prior to use. Before germination, they were sterilized with 70% (v/v) ethanol for 3 min followed by 2% NaOCl for 5 min. After three washes, seeds were germinated in a room maintained at 20 ± 2°C with steady temperature and humidity controls. In two days, the germinated seeds were collected and exposed to different LED lights; red (660 nm), blue (450 nm), white (5000 k) or dark (no light) at 30~40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetic photon flux density (PPFD) and the daily photoperiod of 24 hours conditions constantly. They were watered 3~4 times daily, until harvest. The collected sprouts were dried in a 60°C oven for 24 hours. The dried sprouts were extracted with 1:20 extraction ratio (w/w) using 40% 1, 3-propanediol (DuPont Tate & Lyle) at 60°C for 24 hours. The extract was filtered and used for *in vitro* tests.

Chromatographic apparatus

The high performance liquid chromatographic (HPLC) instrument (Agilent model 1200 series, Hewlett Packard, Palo Alto, CA, USA) equipped with a Kromasil C18 column (5 μm × 4.6 mm × 250 mm), an autosampler, a photodiode array detector, and a vacuum degasser was used to analyze compounds of the extracts. The flow rate was at 1.0 ml/min, and the detector was set at 320 nm. For the HPLC analysis, a linear gradient of mobile phase (solvent A, 0.1% acetic acid in HPLC grade water; solvent B, HPLC grade acetonitrile) was used and the elution solvent was applied as follows: 90% A/10% B at 0 min, 90% A/10% B at 15 min, 60% A/40% B at 30 min, 30% A/70% B at 42 min, 5% A/95% B at 44 min, and 5% A/95% B at 45 min.

Isolation of pharmacologically active components

Hwanggi sprouts were harvested from the cultivation system (Chang *et al*., 2014), cleaned and dried at 60°C for 24 hours. The total extract was prepared from the dried sprouts (385 g) extracted in 50% (v/v) ethanol for 24 hours at room temperatu re, followed by filtration. It was concentrated in a vacuum rotary evaporator (N-1100, Eyela Co., Tokyo, Japan). We obtained 81.6 g of ethanol extract from 385 g powdered sprout (21%). To prepare the fractions, an aliquot of 81.6 g was re-suspended in 1 L of distilled water and serially fractionated with *n*-hexane, chloroform, ethyl acetate, and butanol. The hexane fraction (596 mg) was further purified on a silica gel column (2 × 43 cm) and eluted with $CHCl_3$–CH_3OH (10:1, v/v) to get compound 2 (128.5 mg). The ethyl acetate fraction (997.6 mg) was further separated on a silica gel column (2.5 × 44 cm) with $CHCl_3$–CH_3OH (3:1, v/v) to get compound 3 (13.5 mg). The butanol fraction (1.3 g) was subjected to silica gel column chromatography (2.5 × 45 cm) and eluted with $CHCl_3$–CH_3OH (4:1, v/v) to get 27 fractions. Fractions 21 to 27 were purified on a Sephadex LH-20 column (2 × 36) eluted with $CHCl_3$–CH_3OH (3:1, v/v) to get compound 1 (11 mg) (Fig. 1).

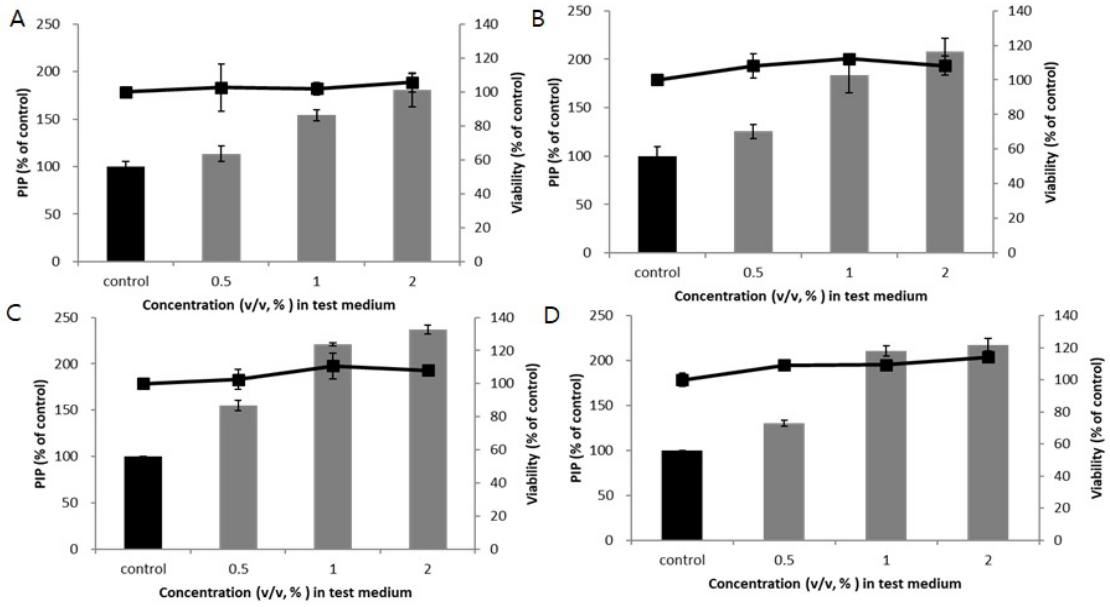


Fig. 1: Effect of the hwanggi sprouts extract on procollagen type I C-peptide (PIP) synthesis and cell toxicities. Black and grey bars indicate the levels of PIP and the lines indicate cell viability. A, dark; B, white; C, red; D, blue.

Compound 1 (Tryptophan)

Yellow powder. ^{1}H NMR (CD_3OD, 400 MHz) δ 7.19 (1H, S), δ 7.11 (1H, *dd*, *J* = 7.3), δ 7.05 (1H, *t*, *J* = 7.3), δ 7.70 (1H, *d*, *J* = 8.2), δ 7.36 (1H, *d*, *J* = 8.2), δ 3.86 (1H, *dd*, *J* =9.6, 3.6 Hz), δ 3.52 (1H, *dd*, *J* = 4.1, 15.1 Hz), δ 3.52 (1H, *dd*, *J* = 9.6, 15.1 Hz); 13C NMR (CD3OD, 400 MHz) δ 125.2 (C-2), 109.6 (C-3), 122.8 (C-4), 120.2 (C-5), 119.4 (C-6), 112.5 (C-7), 138.5 (C-8), 128.6 (C-9), 28.6 (C-10), 56.8 (C-11), 174.6 (C-12) (Table 1).

Table 1: NMR spectroscopic data for compound **1** (400 MHz CD_3OD).

No.	compound 1	
	δH (int, multi, J Hz)	δC (ppm)
2	7.19 (1H, *s*)	125.2
3		109.6
4	7.11 (1H, *dd*, 7.3)	122.8
5	7.05 (1H, *t*, 7.3)	120.2
6	7.70 (1H, *d*, 8.2)	119.4
7	7.36 (1H, *d*, 8.2)	112.5
8		138.5
9		128.6
10		28.6
11	3.86 (1H, *dd*, 9.6, 3.6)	56.8
12	3.52 (1H, *dd*, 4.1, 15.1) 3.52 (1H, *dd*, 9.6, 15.1)	174.6

Compound 2 (Linoleic acid)

Yellow powder. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.34 (2H, t, *J* = 7.5), δ 1.63 (2H, *m*), δ 1.30 (*m*), δ 2.04 (2H, *m*), δ 5.36 (1H, *m*), δ 5.36 (1H, *m*), δ 2.79 (2H, *m*), δ 5.36 (1H, *m*), δ 5.36 (1H, *m*), δ 1.30 (*m*), δ 0.87 (3H, *t,* *J* = 6.4); ^{13}C NMR ($CDCl_3$, 400 MHz) 179.5 (C-1), 34.3 (C-2), 24.9 (C-3), 29.2~29.8(C-5~C6), 27.3 (C-8), 130.1 (C-9), 128.2 (C-10), 25.7 (C-11), 128.0 (C-12), 130.3 (C-13), 27.3 (C-14), 29.3 (C-15), 32.0 (C-16), 22.8 (C-17), 14.2 (C-18) (Table 2).

Compound 3 (Adenine)

Yellow powder. ^{1}H NMR (CD_3OD, 400 MHz) δ 8.10 (1H, *s*), δ 7.09 (1H, *s*);^{13}C NMR (CD_3OD, 400 MHz) δ 155.8(C-1), 152.3 (C-2), 150.2 (C-3), 13.8.8 (C-4), 118.4 (C-5) (Table 3).

Total polyphenol content and antioxidant activity

Total polyphenol content was determined by the oxidation of phenolic compounds by a mix of phosphotungstic and phosphomolybdic acids in a base medium, producing blue acids of tungsten and molybdenum. Absorbance was then read at 765 nm. The total polyphenol content was expressed as mg gallic acid equivalents. Each assay was performed in triplicate (Singleton *et al.*, 1965). Total flavonoid content was determined by aluminum trichloride method using quercetin as a reference. 125 µl of the extract is mixed with 75 µl of a 5% $NaNO_2$ solution. The mixture was allowed to stand for 6 min, before 150 µl of aluminum trichloride (10%) was added and incubated for 5 min. Finally, 750 µl of 1 M NaOH was added and the final volume was adjusted to 2.5 ml with distilled water. After 15 min of incubation, the mixture turned to pink and the absorbance was measured at 510 nm. The total flavonoid content was expressed as mg quercetin equivalents. The antioxidant activity of the extract was measured according to the method described by Bracca *et al.* with slight alterations. The reaction mixture consisted of 0.1 ml extract with 0.2 ml 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution (0.15 mM in 80% methanol solution). The mixture was shaken strongly and left to stand for 30 min at room temperature in the dark. Ascorbic acid was taken as positive control. The absorbance was measured at 517 nm and the percent of inhibition was calculated.

DPPH Scavenging activity (%) = $[1 - \{(sample_{ABS} - blank_{ABS})/control_{ABS}\}] \times 100$.

Table 2: NMR spectroscopic data for compound **2** (400 MHz $CDCl_3$).

No.	compound 2	
	δH (int, multi, J Hz)	δC (ppm)
1		179.5
2	2.34 (2H, *t*, 7.5)	34.3
3	1.63 (2H, *m*)	24.9
4		
5	1.30 (*m*)	29.2~29.8
6		
7		
8	2.04 (2H, *m*)	27.3
9	5.36 (1H, *m*)	130.1
10	5.36 (1H, *m*)	128.2
11	2.79 (2H, *m*)	25.7
12	5.36 (1H, *m*)	128.0
13	5.36 (1H, *m*)	130.3
14		27.3
15	1.30 (*m*)	29.3
16		32.0
17		22.8
18	0.87 (3H, *t*, 6.4)	14.2

Table 3: NMR spectroscopic data for compound **3** (400 MHz CD_3OD).

No.	compound 3	
	δH (int, multi, J Hz)	δC (ppm)
1		155.8
2	8.10 (1H, *s*)	152.3
3		150.2
4	7.09 (1H, *s*)	138.8
5		118.4

Cell viability assay

Human dermal fibroblast (HDFn) cells were cultured in the Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal

bovine serum (FBS), penicillin (100 units/ml) and streptomycin sulfate (100 µg/ml) growth medium in a humidified atmosphere of 37°C, 5% CO_2. The cytotoxicity assay was based on the cleavage of the yellow tetrazolium salt to form an orange formazan dye by active cells (Mosmann *et al.*, 1983). Firstly, cells were cultured in 96-well plate (1×10^4 cells/well) and kept in CO_2 incubator (37°C with 5% CO_2) overnight for complete adhering to the plate. After incubation for 24 hours, MTT(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent was added and the media were removed in 4 hours. Pigments were dissolved in DMSO and the absorbance was measured at 450 nm.

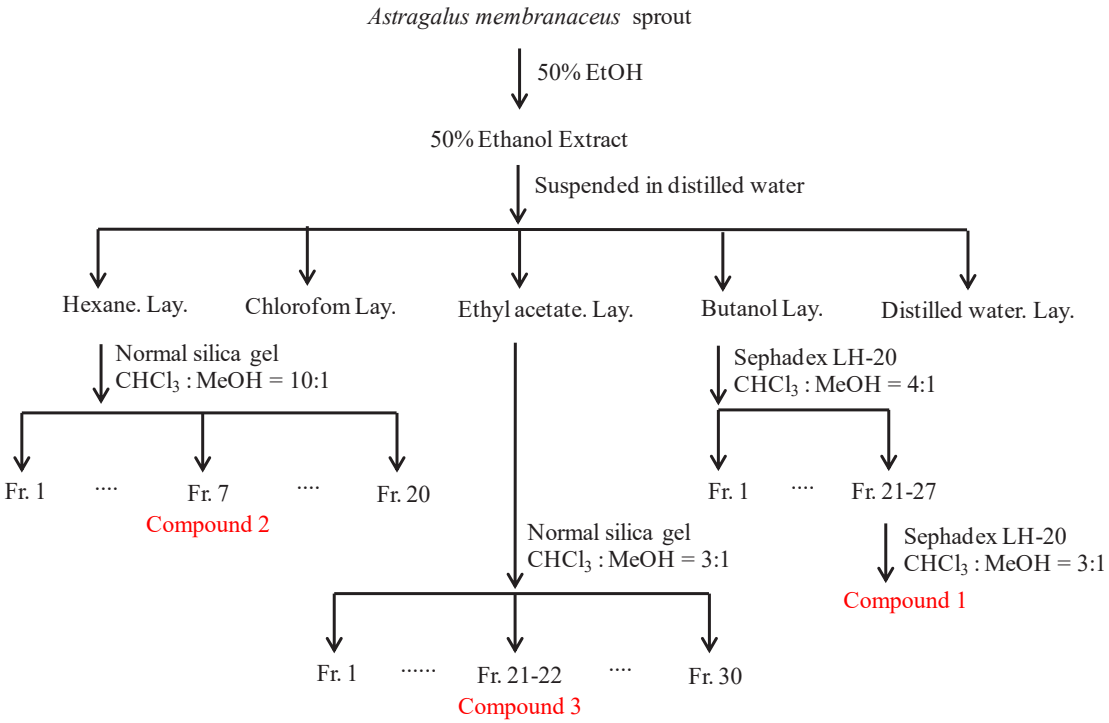
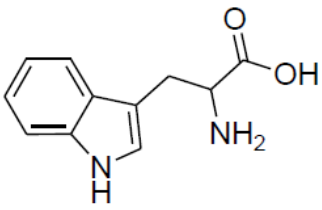
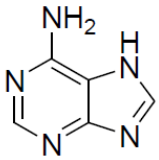


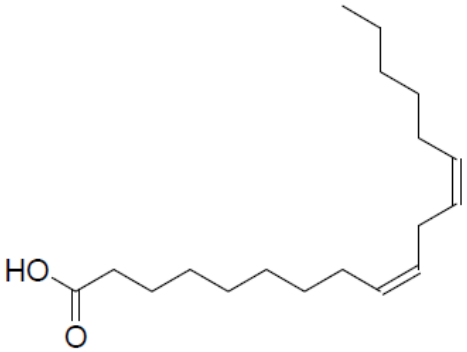
Fig. 2: Isolation scheme of the compounds **1**, **2** and **3** from the extract of hwanggi sprouts.



Compound 1. Tryptophan

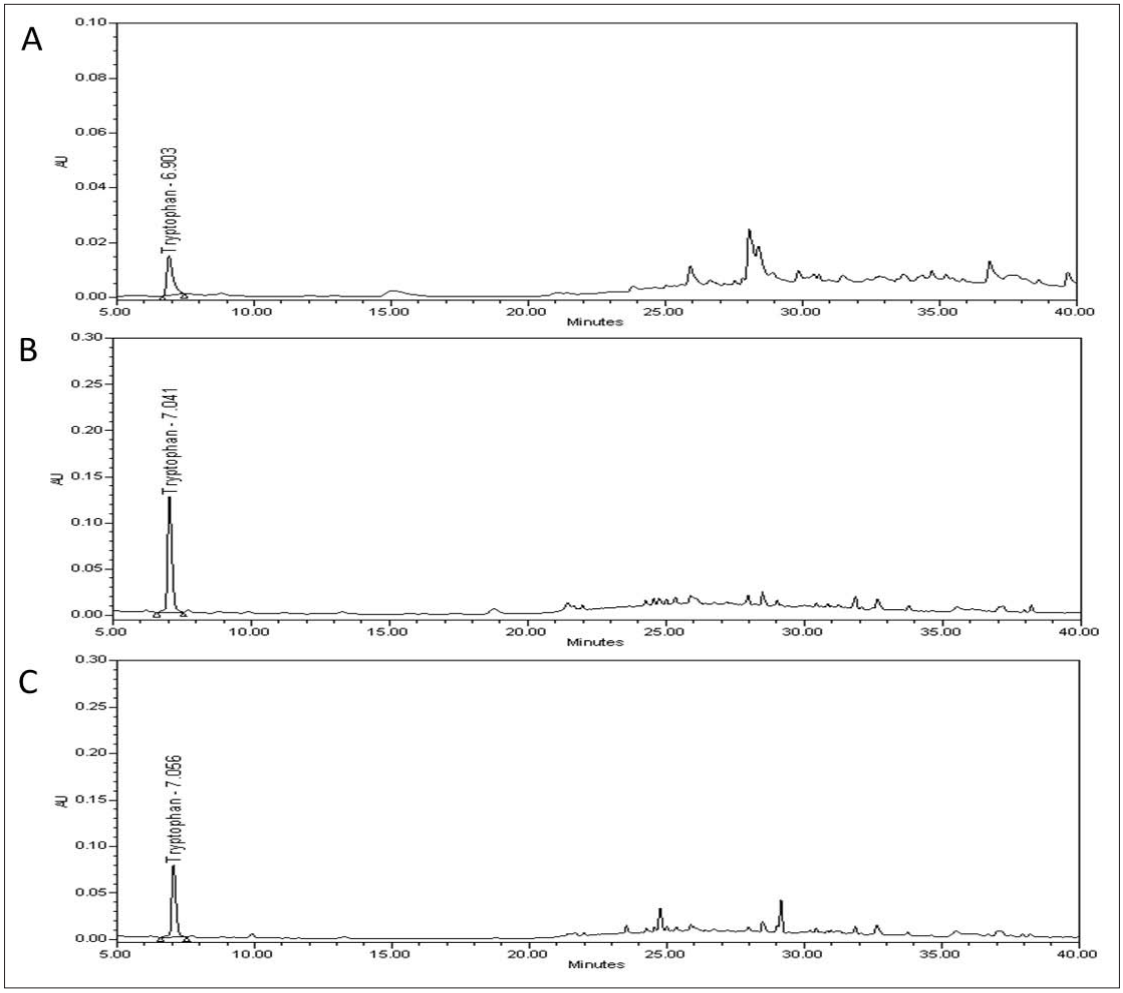


Compound 3. Adenine



Compound 2. Linoleic acid

Fig. 3: Chemical structures of compounds **1**, **2** and **3** isolated from hwanggi sprouts.



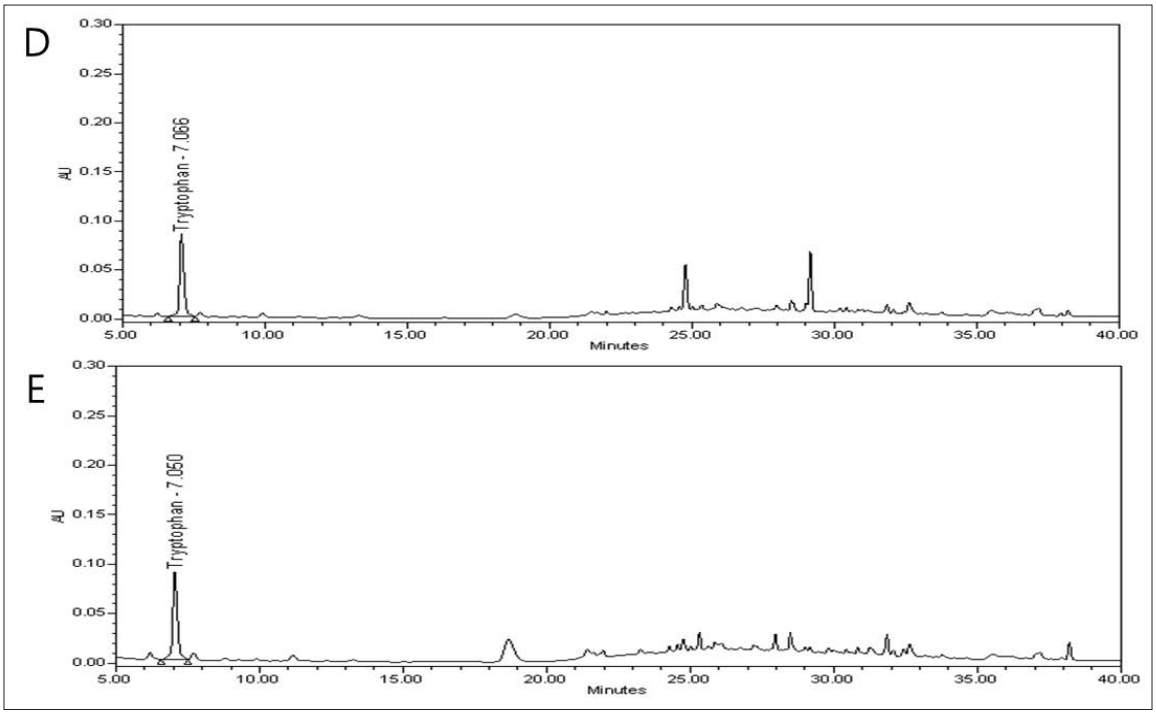


Fig. 4: HPLC profile of hwanggi sprouts extracts. A, isolated tryptophan; B, dark; C, white; D, red; E, blue.

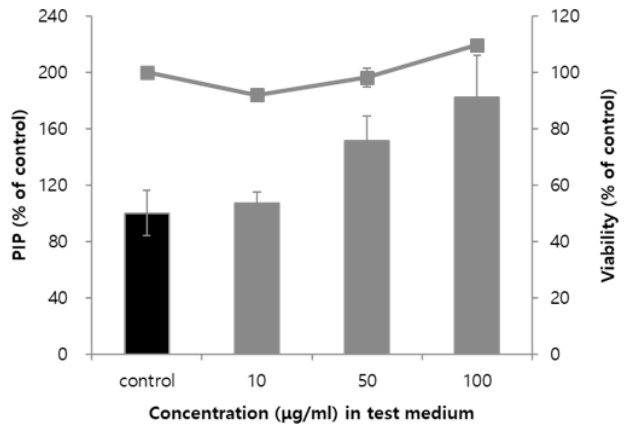


Fig. 5: Effect of the tryptophan on collagen synthesis and cell toxicities. Black and grey bars indicate the levels of PIP and the lines indicate cell viability.

Determination of the amount of procollagen type 1 C-peptide (PIP)

The cell suspension was transferred from the 10 cm^2 plate into a 24-well plate (5×10^4 cells/well) for type 1 procollagen assay and incubated in CO_2 incubator (37°C, 5% CO_2). After incubation, hwanggi sprout extract or each isolated compound was added to the plate in serum-free DMEM which kept in CO_2 incubator (37°C, 5% CO_2) for 24 hours. The supernatants were gathered after a centrifugation at 13,000 rpm in 4°C for 20 min. The amount of PIP was measured according to the supplier's instructions (MK101, Takara, Japan).

Statistical analysis

Experimental data from more than triplicate are shown as means ± standard deviations (SD).

RESULTS

To know the optimal growth conditions, total polyphenol content and antioxidant activity were assessed in the extracts of hwanggi sprouts grown under different light conditions, as shown in Table 4. The highest level of total polyphenols (0.34 ± 0.002 mg/ml) was found in the white light sample, which also showed best antioxidant activity in a dose-dependent manner with SC_{50} value of 18%. Then they were subjected to PIP assays, examining a cosmetic efficacy (Fig. 1). They showed enhanced collagen synthesis by HDFn cells at all conditions, while those grown under red and blue had slightly higher activity than the others.

A phytochemical isolation process was performed on hwanggi sprouts extracts to dissect the biologically active components (Fig. 2). In the series of fractions, pro-collagen synthesis activity was confirmed in the EtOAc, hexane and *n*-butanol fractions. From the *n*-butanol fraction, compound 1 (11 mg) was isolated and identified as tryptophan. Compound 2 (128.5 mg) isolated from the EtOAc layer was linoleic acid and Compound 3 (13.5 mg) isolated from the *n*-butanol layer was adenine. Compounds 1 and 3 were detected at 260-280 nm with the 8 to 9-day-old sprouts (Fig. 3 & 4), but not detected in the adult root extract (data not shown). These compounds may appear at a certain period after germination, but not be contained in adult roots as much. The amount of tryptophan in the extracts was affected by light conditions and was higher in the darkness than the others (Fig. 4, Table 5). Isolated linoleic acid and adenine did not increase pro-collagen synthesis (not shown). On the other hand, isolated tryptophan increased pro-collagen synthesis, confirming that tryptophan is one of the active components of the sprouts (Fig. 5).

Table 4: Total polyphenol content and antioxidant activity of hwanggi sprouts grown under different light conditions.

Light conditions	Total polyphenols (mg/mL)	Total flavonoids (mg/mL)	DPPH Scavenging activity (SC_{50}, %)
Dark	0.23 ± 0.004	0.04 ± 0.007	77 ± 0.01
White	0.34 ± 0.002	0.07 ± 0.003	18 ± 0.007
Red	0.22 ± 0.015	0.03 ± 0.013	51 ± 0.003
Blue	0.24 ± 0.002	0.01 ± 0.002	84 ± 0.007

Table 5: Quantification of tryptophan in hwanggi sprouts extracts.

Light conditions	Tryptophan (μ/mL)
Red	3,770
Blue	4,088
Dark	6,247
White	4,555

DISCUSSION

We here report that *A. membranaceus* sprouts grown under a controllable environment could be developed as a novel anti-aging agent for skin care products. As far as we know, this is the first report of research on *A. membranaceus* sprouts and the first identification of plant tryptophan as a collagen inducer, although *A. membranaceus* is an important and well-studied medicinal herb (Tzu *et al.*, 2014). The cosmetic application has been made by peptides hydrolyzed from its adult roots, inhibiting expressions of MMPs in human dermal fibroblasts (Sun *et al.*, 2014).

In this study, we demonstrated that tryptophan from hwanggi sprout extract can play a role in collagen synthesis of HDFn cells. Collagen does not contain a particularly high amount of tryptophan and there has been no report of the collagen-inducing activity of tryptophan. This result was unexpected but confirmed by the same activity with the commercially available tryptophan (Sigma Aldrich, data not shown). The detailed mechanism of the collagen induction by tryptophan which is not a major constituent of collagen is of high interest and needs more work to dissect from the sensing of external tryptophan and to the induction of Col1 genes by HDFn cells. But, tryptophan may not be a sole factor for these activities because the condition producing most of the tryptophan (dark) is not the same as the one for highest activity (red). It is more reasonable that multiple compounds in hwanggi sprouts act together to induce collagen synthesis of skin cells.

Conclusively, hwanggi sprouts grown under different light conditions showed distinct activities, and more specifically, one of its active compounds, tryptophan can play a role in the anti-wrinkle effect of cosmetic products. In addition, this is another example that indoor cultivation system for sprouts can optimize and standardize raw material production, which is a critical step in the natural product industry (Chang *et al.*, 2014).

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