Effect of Antioxidants and skin barrier function in of embryonic callus derived the domestic rose roots

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Abstract

Background: To maintain healthy skin cells, it is most important to prevent external invasion while strengthening the skin barrier. Roses that grow the most in the country have become used in many fields as a material that has a variety of benefits.

In this study, Sweet Yellow and 18R 120-11, which are fragrant and bred rose in Korea, was induced with callus to confirm their potential as an eco-friendly material for skin barrier improvement.

Methods: The embryogenic callus derived from the rose's root was induced, and the callus was extracted with water, and the ellagic acid was confirmed through HPLC fraction. Especially, DPPH assay was performed to determine the antioxidant of Sweet Yellow callus and 18R 120-11, and it was confirmed that free radical scavenging activity was activated. It was confirmed that the expression levels of the proteins filaggrin (FLG).

Results: These results lead that Sweet Yellow callus extract and others have been proven to be an antioxidant and have a significant effect in improving the skin barrier.

Conclusion: Therefore, it is expected that the embryonic callus extract derived from the domestic rose roots can be widely used in anti-aging cosmetic materials as the fragrant rose.

Keywords: *18R 120-11, Antioxidant, Cosmetic Ingredient, Embryogenic callus, skin barrier, Sweet Yellow callus extract*

Introduction.

The rose (Rosa hybrida) is an important flower all over the world. Domestication of roses has a long and complex history, and the rose species have been hybridized across vast geographic areas such as Europe, Asia, and the Middle East (Bendahmane et al., 2013). They are cultivated in Korea and are sold as garden plants, in pots, or as cut flowers, the latter accounting for approximately 20% of the market (MAFRA, 2015). However, the industry has been declining since 2005. It is necessary to develop the industry through area expansion. Roses are also used for many other purposes, as well as cut flowers for general ornamental purposes, differentiation, and gardening, as well as materials for food, medicine, and fragrance industries (Leus et al. 2018). Callus is defined as a group of cells derived from competent source tissue that is cultured under in vitro conditions to form an undifferentiated mass of cells (Kim, 2011). Moreover, a plant cell has an ability referred to as totipotency, which is the genetic potential of a plant cell to produce the entire mature plant. Various plant callus extracts have been reported as anti-inflammatory, antioxidant, and anti-obesity effects. In the rose plant, it is reproduced from the plant by two methods, one is organogenesis from the stem and somatic embryogenesis (SE) via the callus (Schum & Dohm, 2003). Plant regeneration via SE has been reported in rose since 1967. Somatic embryos can be induced from various rose explants, including leaves, stems, petioles, roots, petals, and immature seeds (Liu G et al.,2021; de Wit et al.,1990; Rout et al.,1991; Kim et al., 2009; Marchant et al.,1996; Murali et al.,1996). Rose-derived somatic embryonic callus has been used as a material for mass propagation or transformation, but recently it is also used as a cosmetic material, and the demand for sustainable rose callus material supply is increasing in the cosmetic industry (Lee et al., 2014).

A rose is a woody perennial flowering plant of the genus Rosa, in the family Rosaceae, or the flower it bears. Many species of roses have long been used in herbal and folk medicines to alleviate menstruation problems, treat blood circulation disorders, and control cancer growth. A recent study demonstrated that flowers of Rosa spp. contain a high level of phenolic compounds with a significant antioxidant capacity (Choi, 2015). Also, for various preparations of rose hip and seed, antioxidative and anti-inflammatory effects have been demonstrated (Chrubasik C. et. al., 2008).

The primary function of the skin is to act as a protective barrier between the host organism and its external environment, minimizing water loss from the body whilst, at the same time, preventing the entry of pathogens and allergens. Oxidative stress, caused by reactive oxygen species (ROS), is responsible for modulating several pathological conditions and skin barrier mutation. ROS directly causes skin aging by involving the oxidative damage of lipids, proteins, and DNA of the skin. Environmental challenges, such as UV irradiation, toxic chemicals, and mechanical wounding, result in increased reactive oxygen species (ROS), inflammation, skin aging, and cancer development (B. Ganguly et al., 2021). It is important to improving skin barrier and antioxidant as maintaining healthy skin. Recent human genetic studies strongly suggest that perturbation of skin barrier function as a result of reduction or complete loss of filaggrin expression leads to enhanced percutaneous transfer of allergens. Filaggrin is therefore in the frontline of defense, and pr1otects the body from the entry of foreign environmental substances that can otherwise trigger aberrant immune responses (A. Sandilands et al.,2009). Also, we need to develop a sustainable anti-aging active ingredient that does not damage nature.

The effects of rose callus extract as a natural cosmetic source are rarely reported. Therefore, in this study, Sweet Yellow and 18R 120-11 which are fragrant roses cultivate and breed in National Institute of Horticultural & Herbal Science (Lee et al., 2009). and we induced Sweet Yellow and 18R 120-11 callus from using plant tissue culture technology and established conditions. Each callus extract was analyzed using HPLC to confirm what kinds of substances or phytochemicals were contained. Also, we investigated the efficacy of the rose callus extracts derived from Sweet Yellow and 18R 120-11 roots using multiple assays from in vitro. These results lead to the prospect that callus extracts have significant antioxidant and skin barrier improvement effects and that the plant callus extracts can be widely used in anti- aging cosmetics as a sustainable, nature-friendly active material.

Materials and Methods.

2.1. Production of Sweet Yellow and 18R 120-11 callus

Sweet Yellow and 18R 120-11 roots were soaked in 70% ethanol for 30 s followed by washing with distilled water. Again, the roots were shaken in 0.5 % sodium hypochlorite (Waco, Osaka, Japan) for 5 min and washed with distilled water. The roots in the aseptic

condition were cut into small pieces (0.5 to 2 cm). We cultured the early stage of plant cells on SH culture medium containing 3 mg·L^{-1} 2,4-D, 300 mgL^{-1} proline with 3 mg•L^{-1} 2,4-D and 3 % sucrose in darkness at 25 ± 2 ℃. The induced callus was propagated in the petri dish in 3~6 weeks.

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Table 1. Embryogenic callus derived from in vitro root of rose used in this study

Cultivar or breeding line				Induction year
Name	Breeding year	Female and male parents	Flower color	
Sweet Yellow	2005	‘Saphir’(♀)×‘Diana'(♂)	Light yellow	2006
18R 120-11	2018	Whisper’(♀)×‘Whisper'(♂)	Pink	2021

2.2. Preparation of callus extraction.

We obtained Sweet Yellow and 18120-11 callus extract by hot water extraction with 2 g of Sweet Yellow and 18120-11 callus, 1 L of distilled water at 121 ℃ for 15 min. After heat extraction, the solids were removed by filtration through a 0.45 μm syringe filter (PTFE). Experiments were carried out at the concentrations described in paragraphs 2.3-2.7.

2.3. Analysis through HPLC

The chromatographic experiments were performed using HPLC system to confirm the active ingredients or phytochemicals contained in Sweet Yellow, 18R 120-11 callus extracts. Water and acetonitrile used in the mobile phase were purchased from Samchun Pure Chemicals (South Korea), and trifluoroacetic acid of Alfa Aesar (France) was added during the preparation of the mobile phase. HPLC was carried out under the conditions of Table 2.

Table 2. Analytical conditions of the callus extracts using HPLC

Instrument	HPLC System (Agilent 1260 Infinity II system (U.S.A.))		
Column	Shim-pack GIS C_{18} (4.6 × 250 mm, 5 μm, Shimadzu, Japan)		
Detector	Diode Array Detector (210, 255 nm)		
Mobile Phase	Mobile Phase A :	0.1% Trifluoroacetic acid in water	
	Mobile Phase B :	0.1% Trifluoroacetic acid in acetonitrile	
Flow	1 mL/min		
Injection volume	20 μL		
Mobile Phase Gradient	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0	100	0
	5	100	0
	75	30	70

2.4. Culture of Human Skin Cells.

HaCaT (Human kerartioncyte) cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) (Welgene, Gyeongsan-si, Korea) supplemented with 10 % Fetal Bovine Serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA) and penicillin- streptomycin (10,000 U/mL) (Thermo Fisher Scientific) at 37 °C with a 5 % CO_2 condition.

2.5. Assessment of Cell Viability by MTT Assay.

We conducted an MTT (3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide) assay to measure the effect of Sweet Yellow and 18R 120-11 callus extracts on the cell viability, proliferation, and cytotoxicity of the human keratinocyte cells (HaCaT). We treated HaCaT cells with three different concentrations (0.5, 1 and 5 %) of callus extracts. Distilled water was used as a control. We incubated HaCaT cells at a density of 5×10^4 cells per well in a 96-well plate for 24 h. Individual cells were treated with different concentrations of Sweet Yellow, 18R 120-11 callus extract and distilled water. After 24 h of treatment, the medium was removed, 4 μL of 5 mg/mL MTT (Sigma-Aldrich, St. Louis, MO, USA) was added, and they were incubated for 4 h. We again removed the medium, added 100 μL of dimethylsulfoxide (DMSO)(Sigma), and let it dissolve for 10 min. We measured the wavelength absorbance at 570 nm using a Termo Scientific Multiskan GO Microplate Spectrophotometer (Fisher Scientific Ltd., Vantaa, Finland). Cell viability was calculated using the following formula.

Cell viability (%) = (amount of absorbance of treated cells / amount of absorbance of control cells) x 100

2.6. Determination of Antioxidant/Free Radical Scavenging Activity

The antioxidant activity of the extracts were measured by a DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) assay as described previously. In brief, 0.1 mL of final concentrations of 0.5 %, 1%, and 5% extracts was treated in 0.1 mL of 0.1 mM of DPPH (Sigma-Aldrich, Cat.#D9132) in the presence of 0.4 mL of ethanol. We used 5 μg/mL ascorbic acid (vitamin C) (Sigma-Aldrich) as a positive control. The samples were mixed well for 10 s and incubated at room temperature in dark conditions for 30 min. The wavelength absorbance was measured at 517 nm using a Thermo Scientific Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The free radical scavenging activity was calculated using the following formula.

$$\text{Scavenging activity (\%)} = [(Abs_{control} - Abs_{sample}) / Abs_{control}] \times 100\%$$

2.7. Real-Time (RT)-PCR

To examine the effect of Sweet Yellow and 18120-11 callus extract on anti-oxidants and skin barrier function, we carried out RT-PCR with known primers amplifying marker genes using QuantiTect Primer Assays (Qiagen, Hilden, Germany) according to the manufacturer 's instructions. HaCaT cells at a density of 5×10^4 cells per well were incubated in a 96-well plate for 24 h. After that, final concentrations of 0.5 %, 1 %, and 5% callus extraxts were treated for 24 h. cDNA was synthesized from the respective HaCaT cells treated with different callus extracts concentrations using a SuperPrep Cell Lysis & RT Kit for qPCR (Toyobo, Osaka, Japan) containing lysis reagents and RT reagents according to the manufacturer's instructions. For the evaluation of anti-oxidant through Sweet Yellow and 18120-11 callus extract treatment, the expression of the gene encoding superoxide dismutase 1 (SOD1), catalase (CAT) nuclear factor (erythroid-derived 1)-like 1 (NRF1) was quantified by real-time RT-PCR using a Thunderbird SYBR qPCR Mix kit (Toyobo) based on the manufacturer's instructions.

In the case of the skin barrier improvement, HaCaT cells at a density of 5×10^4 cells per well were incubated in a 96-well plate for 24 h. After that, final concentrations of 0.5 %, 1 %,

and 5 % callus extracts were treated for 24 h. We used 1 % Glyceryl glucoside (GeneChem, Cat.#GCBD0027) as a positive control. For the skin barrier improvement effect from callus extracts treatment, the expression of genes encoding FLG (fillaggrin), respectively, were quantified by real-time RT-PCR. The expression of individual genes was normalized to GAPDH gene expression.

2.8. Statistical Test

We conducted a one-way ANOVA test for the comparison between the control and test samples. The results were shown with mean and standard deviation (Mean ± SEM). The p-values $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ were considered statistically significant. All statistical tests were declared statistically significant at the 0.05 level. We used IBM SPSS Statistics version 21.0 (SPSS, Chicago, IL, USA) for the statistical analysis.

Results.

3.1. Production of rose plant callus using plant tissue culture technology

Sweet Yellow and 18R 120-11 were obtained as shown in Figure 1 (a-b). Sterilized leaves of Sweet Yellow and 18R 120-11 were cut into pieces and transferred to the gel media containing Plant Growth Regulator (PGR). The cut pieces were transferred to fresh gel media every 3 to 6 weeks. After 6 weeks, it was starting to be found yellowish plant callus. We selected in the media containing 3 $mg \cdot L^{-1}$ 2,4-D, 300 mgL^{-1} proline with 3 $mg \bullet L^{-1}$ 2,4-D and 3 % sucrose grown faster than other media conditions without browning (Fig. 1 (c-d)). Plant calluses were harvested and used for further experiments.

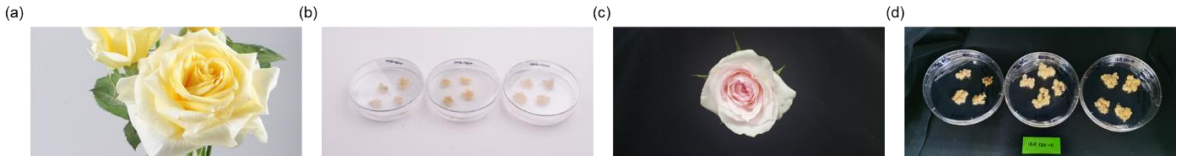

Fig. 1. Callus Induction of Sweet Yellow and 18R 120-11 callus

(a) Sweet Yellow rose (b) Sweet Yellow callus (c) 18R 120-11 rose (d) 18R 120-11 callus

3.2. HPLC results

Sweet Yellow and 18R 120-11 callus extracts were analyzed using HPLC with diode array detector, then a lot of peaks were shown. Checking the chromatogram at 255 nm, a

remarkable peak of ellagic acid at 33.6 min was observed in both rose callus extracts (Figure 2(a-b)). As a result of calculation through the ellagic acid standard coumpound, The contents of this substance in Sweet Yellow and 18R 120-11 callus extracts were 3.0 mg/L and 12.0 mg/L, respectively. Additionaly, many minor peaks appeared at 210 nm, and gallic acid at 15.4 min was conspicuous (Figure 2(c-d)). The gallic acid peak was found to be particularly larger in 18R 120-11 callus extract. Calculating the contents of gallic acid through standard compound, this substance was contained in Sweet Yellow and 18R 120-11 callus extracts at 1.0 mg/L and 5.1 mg/L, respectively.

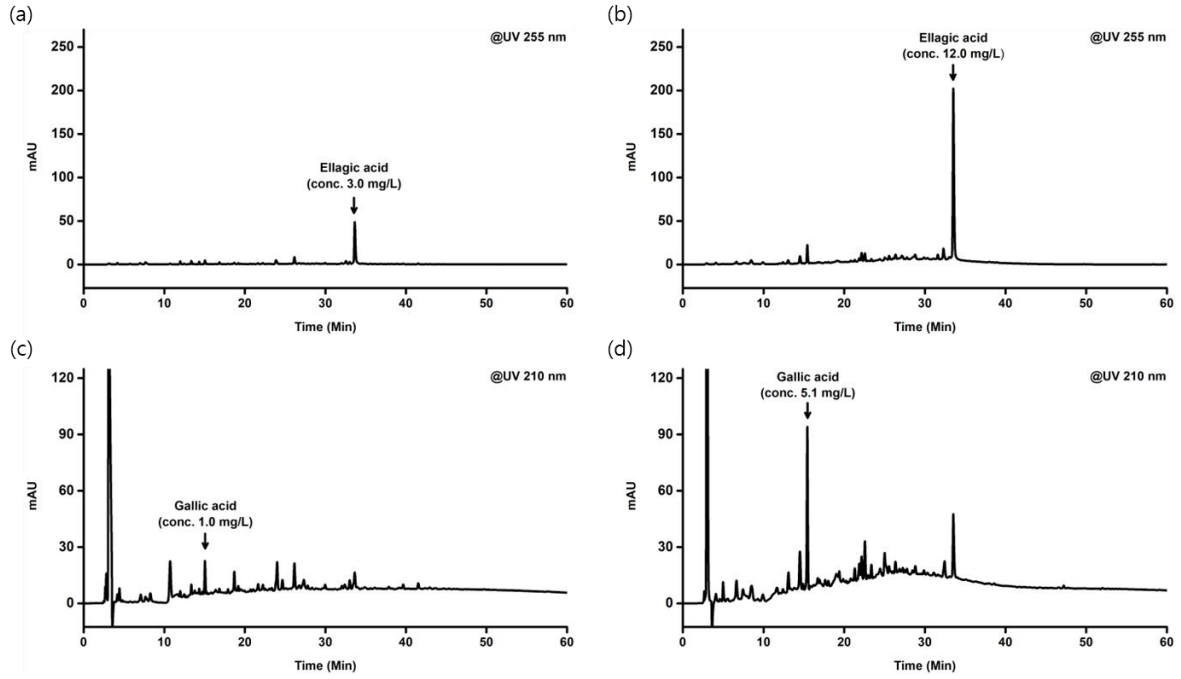

Fig. 2. HPLC chromatograms of Sweet Yellow and 18R 120-11 callus extracts. Among the peaks of each chromatogram, the identified peaks were ellagic acid (RT 33.5 min) and gallic acid (RT 15.4 min). These two substances were contained more in 18R 120-11 than in Sweet Yellow. (a) Sweet Yellow (detected at UV 255 nm) (b) 18R 120-11 (at UV 255 nm) (c) Sweet Yellow (at UV 210 nm) (d) 18R 120-11 (at UV 210 nm)

3.3. Evaluation of Cell Viability and Antioxidant Activity by Sweet Yellow and 18120-11 callus extract

To examine the cytotoxic effect of Sweet Yellow and 18120-11 callus extract, we performed MTT assay in HaCaT cells. As shown in Figure 3(a), the Sweet Yellow and 18120-11 callus

extract treatment at varying concentrations did not demonstrate the cytotoxicity in HaCaT cells. Further, to investigate the radical scavenging activity of Sweet Yellow and 18120-11 callus extract, the DPPH radical scavenging assay was used. Our data demonstrated that the Sweet Yellow and 18120-11 callus extract at the concentration of 5 % induced the most efficient antioxidant activity. Also, 18120-11 callus extract is an antioxidant effect more than Sweet Yellow callus extract (Figure 2(b)). In response to the accumulating ROS, cells require an efficient antioxidant activity achieved by the function of ROS-detoxifying enzymes and proteins.

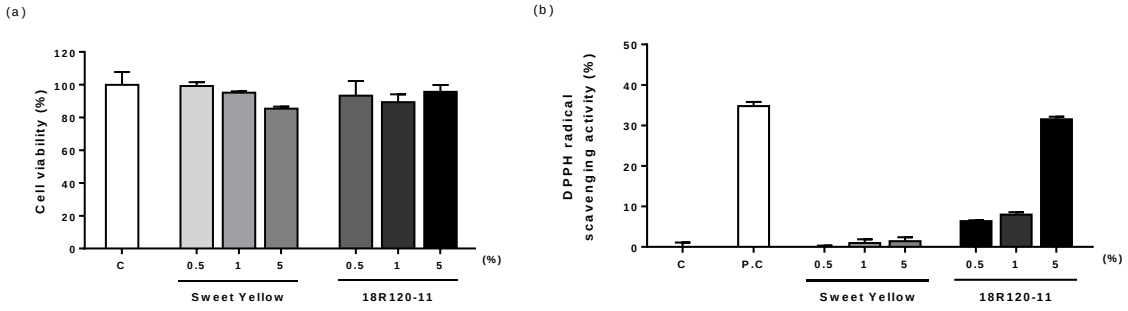

Fig. 3. The effect of Sweet Yellow and 18R 120-11 callus extract on HaCaT cell viability and Antioxidant Activity Cell viability for three different concentrations (0.5%, 1%, and 5%) of Sweet Yellow and 18R 120-11 callus Extract in HaCaT cells by MTT assay (a). DPPH Free Radical Scavenging Activity of Sweet Yellow and 18R 120-11 callus Extract (b). Data are expressed as mean± SD values (n=3) **P*<0.05,***P*<0.01, ****P*<0.001 vs non treated control

3.4. Antioxidant Effect of Sweet Yellow and 18120-11 callus extract by RT-PCR

To determine the antioxidant function, the expressions of the antioxidant genes were examined upon the Sweet Yellow and 18120-11 callus extract. As shown in Figure 4(c), the Sweet Yellow callus extract treatment increased the NRF1 expression. Next, we examined the downstream genes of NRF1, including superoxide dismutase 1 (SOD 1), Catalase (CAT) (Figure 4(a–b)). Interestingly, all the genes examined showed a significant increase, but we determined that the Sweet Yellow callus extract treatment don't increase the expression of genes NRF1(Figure 4(f)). On the other hand, the expression of superoxide dismutase 1 (SOD 1), and Catalase (CAT) genes which are the downstream genes of NRF1, examined increased (Figure 4(d–e)), the increase was more distinctive than that of the Sweet Yellow callus extract

(Figure 4(d–f)). These data indicate that 18R 120-11 callus extract has the potential to regulate oxidative stress through the activation of antioxidant genes.

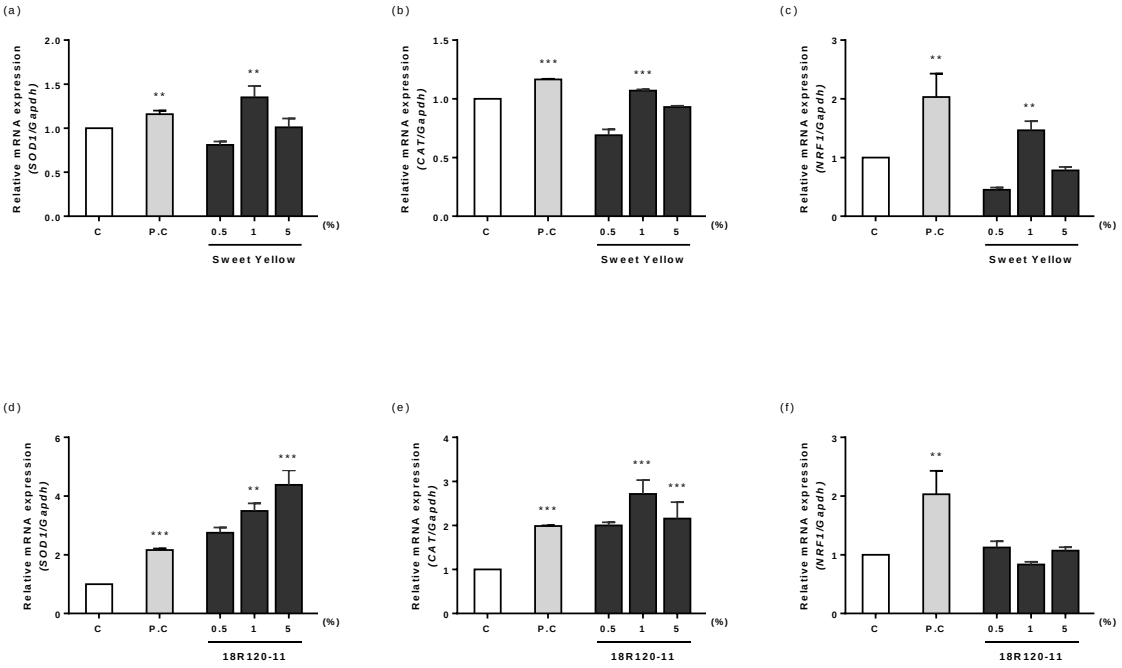

Fig. 5. Sweet Yellow and 18R 120-11 callus extract increases expression of antioxidant genes in HaCaT cells.

Relative expression of antioxidant genes encoding SOD1, CAT, NRF1 in response to treatment with three concentrations of Sweet Yellow(a~c) and 18R 120-11(d~f) and C, P.C (10mM N-acetyl-L-cysteine). Data are expressed as mean± SD values (n=3) **P*<0.05,***P*<0.01, ****P*<0.001 vs non treated control

3.5. Improvement skin barrier of Sweet Yellow and 18R 120-11 callus extract by RT-PCR

An attention for the role of filaggrin (FLG) in skin barrier function has been emphasized by a strong association between loss-of-function mutations in FLG and atopic dermatitis (AD), a common inflammatory skin disease (Schum A et al., 2003). Also, FLG may affect the skin barrier by the maintenance of skin hydration and acidic milieu, both crucial for the optimal activity of enzymes involved in skin inflammation, lipid synthesis and desquamation (Kezic S, Jakasa I et al., 2016). We found out that the skin barrier improvement effect of the callus extracts using a marker gene encoding Filaggrin (FLG) by RT PCR. Interestingly, as the concentration of the Sweet Yellow callus extract was increased, the expression of FLG was gradually decreased, but as the concentration of the 18R 120-11 callus extract was increased,

the expression of FLG was gradually increased. Comprehensively, both extracts confirmed the effects of skin barrier repair.

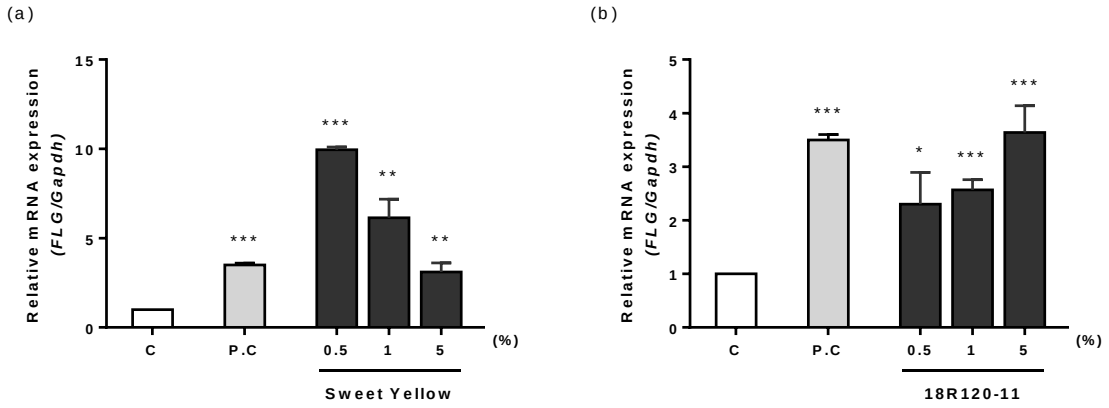

Fig. 6. In vitro assessment of Sweet Yellow and 18R 120-11 callus extract as skin barrier function by real-time RT-PCR. Relative expression of filaggrin genes encoding FLG in response to treatment with three concentrations and C, P.C (Glyceryl glucoside 1%). (a-b) Data are expressed as mean± SD values (n=3), **P*<0.05, ***P*<0.01, ****P*<0.001 vs non treated control

Discussion.

In study, callus was successfully induced in Sweet Yellow and 18R 120-11 which was cultivated and bred in National Institute of Horticultural & Herbal Science. Sweet Yellow and 18R 120-11 callus were extracted with distilled water at 121 ℃.

As a result of the analysis using HPLC, numerous peaks including ellagic acid and gallic acid were present, and it is thought that these substances may have affected the antioxidant activity. Especially, it has been reported that ellagic acid and gallic acid exhibit antioxidant effects such as high scavenging activity of reactive oxygen species (ROS) or reactive nitrogen species (RNS) and upregulating the NRF2 signaling pathway (Gao et al., 2019; Lin et al., 2020; Ríos et al., 2018). It was hypothesized that these two substances contributed in part to enhancing the antioxidant effects of these extracts.

Sweet Yellow and 18R 120-11 callus extract were investigated for inhibition of DPPH and ROS. Efficacy evaluation for ROS Scavenging activation and skin barrier function were conducted by treating the Sweet Yellow and 18R 120-11 callus extract at concentrations of

0.5 % and 5 %. Several in vitro assay results showed the strong antioxidant activity of Sweet Yellow and 18R 120-11 callus extract.

The expression of three antioxidant genes was increased by Sweet Yellow and 18R120-11 callus extract treatment, suggesting the possible role of Sweet Yellow and 18R 120-11 callus extract in DPPH scavenging activity, as shown previously. Interestingly, there was significant difference in the antioxidant effect among different callus extract concentrations, indicating that 5% concentrations of extracts might be sufficient for application as an anti-aging agent in cosmetics.

Also, Efficacy evaluation for skin barrier improvement were conducted by treating the callus extracts at concentrations of 0.5 % to 5 %. Taken together, the results of this experiment showed that the Sweet Yellow and 18R 120-11 callus extract restore the skin barrier function.

Conclusion. Sweet Yellow and 18R 120-11 callus extract derived from fragrant roses are expected to be used as a good material for various industries using skin barrier improvement as well as the supply of antioxidant products for the prevention of ROS as a nature-friendly and eco-friendly material.

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Conflict of Interest Statement. NONE.

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